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**Significance of Oxytocin and Oxytocin Receptors
in Bovine**

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Title

Significance of Oxytocin and Oxytocin Receptors in Bovine Pregnancy

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C. Abstract Oxytocin has multiple actions in bovine reproductive tract and it was our purpose to examine the nature of these actions and their significance for the physiology of bovine reproduction. The bovine oxytocin receptors (OTR) gene was cloned and its expression studied during the cycle and pregnancy. OTR mRNA changed in parallel with OTR with control occurring mainly at the transcriptional level. However, the endocrine regulation of OTR were found in endometrium and cervical mucosa at estrus and at parturition. In both tissues OTR were suppressed in the luteal phase and early pregnancy. Whereas cervical OTR remained suppressed throughout pregnancy, endometrial OTR began to increase soon after implantation and reached higher concentrations in midpregnancy than at estrus. OTR in caruncles did not increase until third trimester, and OTR in cervical mucosa, cotyledons and fetal membranes increased only at term. Myometrial OTR showed less variation and OTR were present throughout the cycle and pregnancy but increased significantly during mid- and late pregnancy. OTR were localized in endometrial epithelial cells and lumina epithelial cells of cervical mucosa as determined by immunohistochemistry. Endometrial OTR were functional throughout pregnancy and mediated $\text{PGF}_{2\alpha}$ release from day 50 onwards in a receptor density related manner. OTR in cervical mucosa mediated PGE release both in vivo and in vitro, as shown in cyclic cows. The ontogeny of uterine OTR was studied from third trimester fetal stage until puberty. OTR were present in endometrium and cervical mucosa in high concentrations throughout this period; myometrial OTR began to increase somewhat later but also reached adult values by 6-mo of age. In the prepuberal heifers OT injections failed to initiate $\text{PGF}_{2\alpha}$ release. The influence of steroids on the effect of OT was examined. Ovariectomy and E_2 were without effect, but P_4 with or without E_2 induced a massive $\text{PGF}_{2\alpha}$ release in response to OT in spite of reduced OTR. Bovine cyclooxygenases (COX-1 and COX-2) were cloned and their expression studied in the endometrium of prepuberal heifers and pregnant cows. Untreated and E_2 treated prepuberal heifers did not express COX-2 but P_4 treated heifers did express the mRNA for COX-2, albeit weakly. During the second half of pregnancy COX-2 mRNA was strongly expressed in cotyledons and somewhat less in caruncles, whereas endometrium, myometrium and cervical mucosa showed only weak, if any, COX-2 mRNA under basal conditions. However, 2 h after OT injection significant increases in COX-2 mRNA were found in endometrial RNA. Thus OT is capable of inducing the expression of the inducible COX-2 gene, and hence the conversion of arachidonic acid to prostanoids. The results indicate that the functions of OT are numerous and probably essential for successful pregnancy and parturition.

D. Objectives

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Significance of Oxytocin and Oxytocin Receptors in Bovine Pregnancy

The purpose of the proposed studies was to improve our understanding of the physiological significance of oxytocin during the second half of pregnancy and in the initiation and maintenance of labor in the cow. In particular:

- 1) To elucidate the significance of the high concentrations of oxytocin receptor found in all uterine tissues at term for bovine parturition, as well as the significance of the high density of receptors in the intercaruncular endometrium observed from mid-pregnancy to term.
- 2) To define the actions that are mediated by the oxytocin receptor present in the non-traditional target tissues of the bovine reproductive tract, with emphasis on a possible interaction with the prostaglandin generating enzymes.
- 3) To elucidate the factors that regulate concentrations of oxytocin receptor in the tissues of the bovine reproductive tract and the fetal-placental unit. Clear understanding of these factors will be helpful for the design of rational methods for the prevention of calving difficulties and retention of placentae- two conditions that cause significant losses to the cattle industry.

E. Description of Research Results

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Significance of Oxytocin and Oxytocin Receptors in Bovine Pregnancy

Oxytocin Induces PGF_{2α} Release in Pregnant Cows: Influence of Gestational Age and Oxytocin Receptor Concentrations: Brahman cows with known breeding dates were injected iv with either 10 or 100 IU oxytocin (OT) on Days 50, 150, 250 or 280 of gestation (n = 3 for each stage; Fuchs et al., 1996a). Concentrations of 13, 14-dihydro-15-keto-PGF_{2α} (PGFM) and OT were measured in samples of peripheral plasma collected at 15 min intervals for 1 h

FIG. 1. Least-square means of plasma concentrations of PGFM (SEM = 43 pg/ml) and OT (SEM = 16.8 pg/ml) in cows receiving i.v. injections of 100 IU OT on Days 50, 150, 250, and 280 of gestation.

before and 1 h after treatment followed by 30 min intervals for 3 h (Figure 1; Fuchs et al., 1996a). Plasma progesterone was measured daily for 14 days after OT injection on Days 50 and 250 of gestation. The increase in plasma OT after injection was dose dependent ($p = 0.001$), but was not affected by stage of gestation. Plasma PGFM increased after OT in a dose dependent manner and increased markedly with gestational age ($p = 0.001$). At Day 280, the increase in plasma PGFM after 100 IU OT was seven fold greater than at Day 50. Plasma progesterone declined

significantly over 7 days post-injection and returned to concentrations normally found during pregnancy except in two cows treated on Day 50 of gestation that later aborted. In a second experiment, the concentration of OT receptors was determined in endometrium collected from purebred Angus or Hereford cows slaughtered on Days 50, 150, 250 and 280 of gestation (n = 3 or 4 at each stage). Endometrial concentrations of OT receptor changed as a function of gestational age increasing six fold from Day 50 to Day 280 which was parallel to the ability of OT

TABLE 1. Intercaruncular endometrial concentrations of OT_R on Days 50, 150, 250 and 280 of gestation (experiment 2) and the integrated PGFM responses to injections of 10 and 100 IU OT at the same stages (experiment 1).^a

Day of gestation	Endometrial OT _R pmol/g wet wt.	PGFM response: ng/210 min ^b	
		10 IU OT	100 IU OT
50	4.4 ± 1.5 ^c	1.1 ± 1.1	5.4 ± 1.3 ^{cd}
150	14.9 ± 5.7	2.1 ± 1.4	26.8 ± 9.2 ^d
250	21.1 ± 4.8	4.1 ± 0.3 ^d	28.2 ± 11.7 ^d
280	26.9 ± 3.4	7.0 ± 6.7	40.5 ± 14.9 ^d

^aValues are mean ± SEM, n = 3 or 4 cows in each group. The OT-induced PGFM increased in a parallel manner to endometrial concentrations of OT_R (r = 0.97; p < 0.01).

to increase plasma PGFM

(Table 1; Fuchs et al.,

1996a). Thus,

endometrial OT receptors

are functionally coupled

to PGF_{2α} release during

pregnancy and their

concentration determines the magnitude of OT-induced PGF_{2α} release during gestation.

Consequently, endogenous OT is a factor in the regulation of PGF_{2α} release from the bovine uterus during pregnancy and parturition.

Effect of Oxytocin Antagonist on Oxytocin Induced PGF_{2α} Release In Late

Pregnant Cows In Vivo: We have reported (Fuchs et al., 1996c) that endometrial OT receptors are functionally coupled to PGF_{2α} release during pregnancy and their concentration determines the magnitude of OT-induced PGF_{2α} release during gestation. To ascertain whether endogenous OT is a factor in the regulation of PGF_{2α} release from the bovine uterus during pregnancy and parturition, an OT antagonist is required, which blocks endometrial as well as myometrial OT

receptors. This is not the case with all currently synthesized antagonists. We have established that $d(CH_2)_5$, Tyr(ME)², Thr⁴, Tyr-NH₂⁹)Ornithine Vasotocin (OTA, Bachem, Torrance, CA) binds

TABLE 2. Comparison of affinities of a number of pharmacologically defined OT analogues and antagonists to OT receptors in bovine endometrium and myometrium.

Rank ^a	Endometrium ^b		Myometrium ^c	
	Analogue ^d	K _d (nM)	Analogue ^d	K _d (nM)
1.	OTA	0.57 ± 0.1	OTA	0.45 ± 0.1
2.	OT	0.7 ± 0.05	AVT ^e *	0.8 ± 0.02
3.	AVP ^e *	0.9 ± 0.13	OT	1.0 ± 0.14
4.	PAVP ^e *	2.7 ± 0.35	IT	4.5 ± 1.08
5.	IT	5.4 ± 0.54	PAVP ^e *	4.7 ± 0.41
6.	AVT ^e *	8.7 ± 1.09	AVP ^e *	6.6 ± 2.3
7.	MC	16.9 ± 3.9	MC	13.3 ± 3.8
8.	AT ^e *	28.7 ± 4.4	AT ^e *	15.5 ± 0.3
9.	DDAVP	102.5 ± 23.9	DDAVP	62.2 ± 6.9

^a Compounds are ranked from highest to lowest affinity (K_d = 1/K_{1/2}).

with equally high affinity to OT receptors in both these tissues (Table 2; Fuchs et al., 1997a).

This was therefore chosen for experiments in late pregnant cows in which the antagonist was injected iv 5 min before an iv OT injection. Blood samples

were collected at 15 min intervals for 1 h before and 3 h after the injections (Figures 2 and 3; Fuchs et al., 1997a). A total of 24 cows on Day 260 of gestation were randomly assigned to the following treatments: 1] saline; 2] 50 IU OT; 3] 1200 ug OTA ; 4] 400 ug OTA and 50 IU OT; 5]

FIG. 2. Peripheral plasma concentrations of PGFM in three groups of Brahman cows (n = 4 each) on Day 259 or 260 of gestation (groups 1, 2, and 3). They were injected i.v. with saline (controls and OT group), or 1200 µg OT antagonist (OTA group) 5 min before Time 0 (arrow) and at Time 0 with saline (controls and OTA groups) or 50 IU OT (OT group).

FIG. 3. Peripheral plasma concentrations of PGFM in four groups of Brahman cows injected i.v. on Day 259 or 260 of pregnancy with 50 IU OT. Five minutes before the injection of OT, cows in each group received an i.v. injection of either saline or 400 µg, 1200 µg, or 4000 µg of OTA (groups 2, 4, 5, and 6, respectively).

1,200 ug OTA and 50 IU OT; 6] 4,000 ug OTA and 50 IU OT. Concentrations of PGFM increased as expected in cows treated with OT only. OTA alone did not increase plasma PGFM but caused a slow decline in basal concentrations of PGFM. OTA, 400 ug and 1,200 ug, did not have a significant effect on OT induced increase in plasma PGFM, but 4,000 ug suppressed the increase almost completely (Figures 2 and 3; Fuchs et al., 1997a). Two calves were born dead, both to cows that received the 4,000 ug dose of OTA.

In conclusion, OTA, an effective inhibitor of the uterotonic action of OT, is also a potent inhibitor of $\text{PGF}_{2\alpha}$ release, at least in cattle, and appears to effective at lower concentrations than atosiban, a well known antagonist of OT action. We conclude that the bovine uterus possesses separate OT and arginine vasopressin (AVP) receptors, that OT receptors are present in greater numbers in both endometrium and myometrium than AVP receptors, but that AVP competes with greater avidity for OT receptors than OT competes for AVP receptors. Myometrial OT receptors bind OT with two times higher affinity than endometrial OT receptors; therefore at low endogenous OT levels, as most of pregnancy, contractile responses may be elicited without concomitant release of endometrial $\text{PGF}_{2\alpha}$.

Oxytocin Receptor Gene Expression in the Bovine Cervix During the Estrous Cycle:

The present results confirm and expand our preliminary observations on the presence of high concentrations of OT receptor in the bovine cervix at estrus (Fuchs et al., 1996b). Oxytocin receptor concentrations were determined in cross-sectional segments of the cervix of nonpregnant cows on cycle Days 0, 3, 7-8, 17, and 19 (n = 3-4 cows each day); [^3H]OT was used as the labeled ligand. Mucosal and muscle layers of the cervix were also analyzed separately for both ligand binding and expression of the OT receptor gene using a newly developed RNase protection

assay. Cellular localization of OT receptor protein was determined by immunohistochemistry. All regions of cervix from cows at estrus had high concentrations of OT receptor. In the luteal phase the receptors were sharply down-regulated, whereas, receptor affinity was unaltered. At estrus

(Figure 5; Fuchs et al., 1996c). On Days 7-17, OT receptor mRNA signals in both mucosa and muscle were faint or nondetectable. The correlation between OT receptor density and mRNA expression was good suggesting that OT receptor concentrations are mainly regulated at the transcriptional level. Immunoreactive OT receptor protein was concentrated in the epithelial cells at the luminal surface of the mucosa; muscle cells showed significantly weaker signals. Histological analysis of the cervix at estrus, Day 7 post-estrus of pregnancy revealed that the three regions of the cervix appeared somewhat different in the relative amount and arrangement of connective tissue and smooth muscle (Figure 6; Fuchs et al., 1996c).

We conclude that cervical mucosa of nonpregnant cows is an important target organ for OT, whereas effects of OT on cervical muscle appear to be of minor significance, due to the sparse receptor population in the muscular tissue. The cervical OT receptor concentrations are modulated in a striking fashion during the estrous cycle, and the regulation appears to be mainly at the transcriptional level. Since OT stimulates PGE₂ output in cervical tissue in vitro (Fuchs et al., 1995; Shemesh et al., 1998a), we propose that the cellular response to systemic OT in cervical mucosa consists of PGE₂ release from the epithelial cells; the free PGE₂ may in turn act in a paracrine manner on the adjacent connective and muscle tissues to promote cervical dilatation and facilitate sperm transport at the time of ovulation.

Oxytocin Receptor Gene Expression in the Bovine Cervix During Pregnancy and Parturition: The expression of transcripts encoding the OT receptor protein, the cellular localization of immunoreactive OT receptor protein, and OT receptor concentrations were determined in bovine cervix during pregnancy and parturition (Fuchs et al., 1996b,d). Cervical tissues were obtained from 20 Angus and Hereford cows from the herd of the US Subtropical

FIG. 6. Light microscopic photographs from different regions of the bovine cervix at Day 7 of the estrous cycle. A,D) Cranial end. B,E) Mid-portion. C,F) Caudal end. A,B,C,E,F) Gomori's trichrome was used for staining the connective tissue. The connective tissue collagen stains blue-green and the smooth muscle and epithelial cells stain red. D) Orcein staining was used for elastin with hematoxylin counterstain in a cranial region of the cervix. The elastin fibers stain brown (small arrows) while the basal nuclei of mucosa epithelial cells stain bluish purple (large arrows). Note the clear apical cytoplasm of the luminal epithelial cells, which is distended with secretory material (arrows) into the lumen (L) of the cervix. A,B,C) The cervical mucosa consists of mucin-filled columnar epithelial cells (arrows) and underlying stromal cells. The underlying tissue layer consists of two distinct regions, mucosa (MC) and muscle (MS). The mucosa layer contains bundles of longitudinal smooth muscle dispersed in the connective tissue. The muscle layer contains abundant smooth muscle dispersed in connective tissue in a complex arrangement. The arrowhead in C represents the area of magnification shown in F. E,F) The muscle fibers are arranged in longitudinal (LF), circular (CF), and oblique transverse (OF) configurations dispersed throughout the different regions of the cervix and throughout the muscle layers. A-C, $\times 40$; D, $\times 400$; E and F, $\times 100$.

Agriculture Research Station, Florida on Days 90, 175, 250, 278, term, and day of delivery ($n = 2$ or 3 each day). Samples of mucosa and muscle were obtained from three regions of the cervix as previously described. Gene transcripts for OT receptor were detected by RNase protection assay and glyceraldehyde- 3-phosphate dehydrogenase (GAPDH), a general "housekeeping" gene, was used as control for the RNase protection assay. Immunohistochemistry for the OT receptor protein was performed on cryostat sections using the alkaline phosphatase technique (APAAP-complex, Dianova, Hamburg), the antibody was a gift from Dr. T Kimura, Osaka, Japan. Oxytocin receptor mRNA was readily detected in cervical tissues of estrous cows used as positive controls. As in nonpregnant cows OT receptor density and mRNA expression was significantly higher in mucosa than muscle. The concentration of OT receptor and its mRNA was very low in all regions of the cervix throughout pregnancy, whereas GAPDH mRNA was readily

Fig. 7. Ribonuclease protection assays specific for mRNA encoding for oxytocin receptor (OT_R) (length of protected fragment 302 bp) and GAPDH as control (length of protected fragment 198 bp). *Lanes 1 to 25*, Cervical mucosa (A) and muscle (B) from cows in order of advancing gestational age and during delivery as indicated; *lanes 27 and 29*, (C), two cRNA probes used; *lanes 26 and 28*, ribonuclease-digested probes; *lane 31*, (P), *HinfI*-digested PhiX174 deoxyribonucleic acid size marker, end-labeled with α - 32 P-labeled deoxycytidine triphosphate.

detectable in all tissues (Figure 7; Fuchs et al., 1996d). Cows in active labor and immediately post-partum had high concentrations of OT receptors and OT receptor mRNA in mucosa but weak signals only were present in the muscular layer of the cervix (Figure 8 and 9; Fuchs et al., 1996d). Immunoreactive OT receptor was localized in the epithelial cells of the mucosa and in

Fig. 8. Oxytocin receptor (OT_R) density in mucosal and muscular layers of pregnant and parturient bovine cervix among three regions of cervix ($n = 3$ to 4).

Fig. 9. Oxytocin receptor (OT_R) concentrations in mucosal layer of cervical midportion in relation to cervical dilatation. Tissues were obtained from cows at term not in labor and cows in advanced labor or immediately post partum.

some muscle cells.

In conclusion, the results indicate that OT cells of bovine cervical mucosa are targets for OT action. Mucosal epithelium is the primary location of OT receptors in the bovine cervix. Both the level of OT receptor mRNA and protein concentration is much lower in cervical muscle than mucosa, with rare exceptions. Oxytocin receptor gene expression in cervical epithelial cells is suppressed during gestation and dramatically up-regulated at parturition. Oxytocin receptor protein and mRNA concentrations show parallel variations during pregnancy, suggesting control at the transcriptional level. In cervical tissue from nonpregnant cows OT stimulates PGE_2 release in a receptor density-related manner (Fuchs et al., 1995; Shemesh et al., 1998a). Oxytocin

receptor therefore may also mediate release of PGE_2 from the cervical mucosa of pregnant cows. Endogenous OT secreted during delivery may therefore stimulate release of PGE_2 from cervical mucosa, resulting in softening of adjacent, tight collagenous tissues, thereby increasing cervical compliance. Thus OT has multiple functions during bovine parturition: stimulation of myometrial contractions, stimulation of $\text{PGF}_{2\alpha}$ release from the endometrium, resulting in luteolysis and potentiation of OT induced myometrial contractions, and also stimulation of PGE_2 from cervical mucosa, resulting in cervical ripening.

The Effect of Oxytocin on the Bovine Cervix In Vitro: Cross-sectional segments of the bovine cervical tissues (external os, mid-section and internal os) were minced and incubated for 6 h in 1.5 ml Minimum Essential Medium 199 (MEM) and production of PGE_2 and $\text{PGF}_{2\alpha}$ were measured by radioimmunoassay (Fuchs et al., 1995a,b; Shemesh et al., 1997a). The tissues were incubated in the presence or absence of OT (10 ng/ml); progesterone (0-10 ng/ml) and indomethacin (0-10 g/ml) alone or in combination. Oxytocin stimulated PGE_2 secretion ($P < 0.03$) in segments of external os from peri-estrous cows. The stimulatory effect of OT on tissues from the mid-section or internal os was not significant, probably because of admixture of the OT receptor rich mucosa with large amounts of receptor poor muscle tissues. Oxytocin did not stimulate PGE_2 secretion in any segment of cervix obtained from cows on Days 2-6 after ovulation. Additional experiments were therefore conducted using tissues from the region of the external os obtained from peri-estrous cows. In such tissues progesterone induced a dose dependent inhibition of basal PGE_2 secretion, maximally $50 \pm 3\%$ ($P < 0.03$; Figure 10; Shemesh et al., 1997a). Indomethacin (1 g/ml) completely inhibited both basal and OT stimulated PGE_2 secretion. $\text{PGF}_{2\alpha}$ production was minimal (< 1 ng/100 mg) compared to PGE_2 in all three

Fig. 10. Effect of progesterone (5 ng mL⁻¹ 6 h⁻¹) on oxytocin stimulation of prostaglandin E₂ (PGE₂) production. Tissues were obtained from 5 cows at pre-oestrus-oestrus and treatments were tested in quadruplicate.

Fig. 11. Effect of oxytocin (10 ng mL⁻¹) and/or progesterone (5 ng mL⁻¹) on the induction of cyclooxygenase by cervical tissues in pre-oestrus-oestrus cows. The immunoblot is representative of tissues from 7 individual cows. The lower panel represents the mean $\pm$ s.e.m. of samples from separate experiments expressed as the percentage stimulation or inhibition relative to controls ((control - treatment)/control). SV, bovine seminal-vesicle microsomes (40 μ g protein). Lane A, control; Lane B, oxytocin; Lane C, progesterone; Lane D, progesterone+oxytocin.

segments and OT had no effect on PGF_{2 α}

production at any time of the cycle.

Expression of

cyclooxygenase was determined by Western blots analysis in minces of external os segments (100 mg) after incubation in MEM for 1 to 3 h in the presence or absence OT (10-50 ng/ml) alone or in combination with progesterone (5 ng/ml; Figure 11; Shemesh et al., 1997a). The antibody used for identification cross-reacted with both forms of the cyclooxygenase enzyme, I and II. Oxytocin stimulated the induction of cyclooxygenase mRNA ($P < 0.05$) whereas progesterone inhibited basal cyclooxygenase mRNA production ($P < 0.05$). The OT induced stimulation of cervical PGE₂ production is therefore mediated through the induction or stabilization of cyclooxygenase mRNA. The suppression by progesterone of cervical PGE₂ production also appeared to be mediated through modulation of cyclooxygenase mRNA expression.

In summary, production of cyclooxygenase and of PGE₂ by cervical tissues of the cow is regulated by OT and progesterone, i.e, stimulation by OT and inhibition by the steroid compound. The increase in OT receptor (Fuchs et al., 1996c) and the fall in peripheral plasma progesterone at

estrus suggest that cervical PGE₂ release is regulated by more than one hormone.

Effects of Oxytocin and PGE₁ on the Bovine Cervix at Estrus: Cervical mucosa

possesses OT receptors that are tightly regulated by ovarian and placental hormones. The OT receptors are localized in luminal epithelial cells (Fuchs et al., 1995a,b; 1996b,c,d), which are the site of PGE₂ production. Oxytocin stimulates PGE₂ production in cervix from periestrous cows in vitro (Shemesh et al., 1997a) but the effects in vivo are not known. All cows were synchronized with SyncroMate B implants, 48 h after withdrawal the experiments were begun (Graddy et al., 1998a,b). PGE₂ release was determined by inserting into mid cervix a dialysis bag which was left in place for 2 h (control period). A fresh bag was then inserted. Oxytocin (100 IU) was injected im and the bag was removed 2 h later. PGE₂ was measured in the dialysates by EIA. PGE₂ concentrations were significantly greater after OT injection than in the control period ($P < 0.01$, $n=21$; Figure 12; Graddy et al., 1998a,b). Cervical resistance to stretching was measured by a

Fig. 12. Concentrations of plasma PGFM in estrous cows which a bag made of dialysate tubing was inserted inside the cervix for 2 h each time. At time zero the first bag was removed, a second bag was inserted, and an im injection of 100 IU oxytocin (OT, open circles) or saline (solid circles) was administered; $n = 4$ in each group.

balloon tipped Foley catheter, attached to a pressure strain gauge and a polygraph, and inserted into the mid cervix. The increase in resting tension after injection of a fixed volume of water into the balloon was used as an index of cervical resistance.

The resistance measurements were done 10 h and 24 h after

Fig. 13. Cervical resistance to sudden stretch induced by inflation of a Foley balloon, placed in midcervix, with 3.5 ml water. Cervical wall pressure was measured continuously with a strain gauge manometer connected to the foley catheter with a three-way valve. The cows were periestrous in the beginning of the experiments (48 h after removal of a progestin pessary). Control; group (open columns) had inert jelly (20 ml) applied within the cervical lumen at 8 am; OT group (hatched columns) received 100 IU im at 8 am; PGE₁ group s (cross hatched columns) had PGE₁ suspended in jelly (2.5 mg x 20 ml⁻¹) applied within the cervical lumen at 8 am. All treatments were administrated on three consecutive days. Measurements were made before the treatments were administered and 10 h later each day. The values shown are antilog of the log normal mean $\pm$ SE. Values with the same letter in each vertical column are different (ANOVA, followed by Waller-Duncan *k* ratio procedure). Asterisks indicate significant difference between am and pm values during a single day (paired *t* test).

each treatment. They were: (1) OT injection; (2) application of PGE₁ (2.5 mg in 20 ml K-Y jelly intracervically; and (3) application of placebo jelly (Figure 13; Graddy et al., 1998 a, b). Each treatment was performed on 3 consecutive days in the same cow, n = 4

cows each. In the placebo group cervical resistance was lowest at estrus and increased during the next 24 h to relatively steady level. PGE₁ treatment resulted in significant decrease in cervical resistance within 24 h, the decrease was similar during and after all three PGE₁ applications. The first OT injection had no effect on the cervical resistance, but 24 h after the second OT injection cervical resistance had declined to similar values as in PGE₁ treated cows. However, the third OT injection did not have any measureable effect on cervical resistance. In conclusion, OT causes release of PGE₂ from bovine endocervix in vivo which in sufficient amounts results in decreased cervical resistance to stretch. Intracervical PGE₁, in amounts much greater than those released by one injection of OT, significantly lowers cervical resistance to stretching.

In conclusion, OT receptors in cervical mucosa mediate the release of PGE₂ by the ligand, OT. PGE₂ has a softening effect on the surrounding collagenous cervix, making it more extensible. We postulate that cervical OT receptors play an important function in the mechanism of parturition, because the concentrations of mucosa OT receptors are very high at the onset of

parturition and large amounts of OT are released into circulation from the posterior pituitary gland. The role of OT receptors during estrus, when the mucosa OT receptor density is also high, may be to facilitate the act of copulation and the process of fertilization.

Oxytocin Induction of Prostaglandin H Synthase-2 Gene Expression in Pregnant

Cows: Two isoforms of prostaglandin H synthase (PGHS-1 and PGHS-2) generate the same prostanoids but are encoded by different genes, and are differently regulated. Tissues of the reproductive tract reportedly express transcripts of both isoforms. The isoform of the cyclooxygenase enzyme which is encoded by the PGHS-1 gene (also called COX-1) is rather ubiquitously present in low concentrations and presumably functions as a so called “house-keeping” enzyme, which is constitutively expressed. PGHS-2 enzyme can produce prostanoids rapidly from endogenous or exogenous precursors when need for prostanoids arises in the cells.

Ovarian and placental steroids markedly influence prostanoid production in tissues of the reproductive tract. In prepubertal heifers endometrial PGHS-2 mRNA expression and $\text{PGF}_{2\alpha}$ release in response to OT in vivo increased several fold after progesterone pretreatment whereas estrogen pretreatment had a suppressive effect, but given together with progesterone, estradiol enhanced the effect of progesterone (Fuchs et al., 1997c, 1998a,e). This occurred paradoxically in spite of suppression by progesterone of OT receptor concentrations. The increased estrogen and progesterone secretion during bovine pregnancy may therefore cause enhanced PGHS-2 mRNA expression and prostaglandin release. Because $\text{PGF}_{2\alpha}$ is luteolytic in cows, increased production of $\text{PGF}_{2\alpha}$ could be detrimental for the maintenance of pregnancy. The purpose of this study was to examine this apparent paradox and determine PGHS-2 gene expression in various bovine uterine tissues during the second half of gestation (Figures 14-17; Fuchs et al., 1998b,c).

Fig. 14. Ribonuclease protection assay for PGHS-2 mRNA in bovine caruncles and cotyledons at Days 150, 275, 280 (nonlabor), 280 (labor) of gestational age; post partum Day 1 is included for caruncles. Tissues from two animals are shown for each day. Length of protected fragment for PGHS-2 was 274b, and for GAPDH 198b. Caruncles (lanes 1 to 10); cotyledons (lanes 11 to 18); stage of gestation is indicated on the figure.

Fig. 15. Ribonuclease protection assay for PGHS-2 mRNA in intercaruncular endometrium, myometrium and cervical mucosa of the same cows as in Figure 12. Endometrium (lanes 1 to 12); myometrium (lanes 13 to 23); cervical mucosa (lanes 24 to 29).

Fig. 16. Relative intensities of PGHS-2 mRNA vs GAPDH mRNA, shown in Figure 12, in endometrium, myometrium and cervical mucosa of pregnant cows. Values for endometrium from postpartum cows and cervical mucosa of cows in labor were greater than the respective initial values (Day 150 and 275), differences among other samples from the second half of gestation and labor were not significant (ANOVA, $P < 0.05$).

Fig. 17 Plasma concentrations of PGFM in midpregnant cows injected with 299 IU oxytocin (OT, closed diamonds) or saline (open circles) at time 0. Blood samples were collected at times indicated. Plasma levels of PGFM were significantly greater in OT than saline injected cows from 60 to 90 min following the injection. Tissues were collected at 120 min.

additionally, the purpose was to determine whether OT affects the expression of PGHS-2 in the endometrium of pregnant cows. PGHS-1 and PGHS-2 mRNA were measured by ribonuclease protection assays (RPA) in intercaruncular and caruncular endometrium, myometrium, cotyledons, and cervical mucosa of pregnant cows. Samples, were obtained at gestational ages of Days 150, 275, 280 (at term not in labor), 280 (at term in labor), and 6 to 12 h post partum. Bovine GAPDH cRNA was used as a control for the RPAs. The effect of OT on PGHS-2 expression in intercaruncular endometrium was determined in pairs of third trimester cows (Days 230-270); one in each pair was injected with 200 IU of OT and the other with saline 2 h before tissues were removed for total RNA preparation and RPA. PGFM was measure in plasma samples taken at 15 min intervals before and after OT injection. PGHS-2 isoform was the dominant gene transcript in all tissues studied;

PGHS-1 mRNA was expressed weakly or not at all. Cotyledons had the highest expression of PGHS-2 mRNA followed by caruncles, which had about 50% fewer transcripts than cotyledons. PGHS-2 mRNA in cotyledons and caruncles was greatest at term with no difference between Days 280 and cows in or not in labor. PGHS-2 mRNA expression in endometrium and myometrium was low and variable during pregnancy with no increase at term. PGHS-1 mRNA was detected in low amounts in myometrium and caruncles only. During pregnancy cervical mucosa expressed PGHS-2 mRNA in only minute amounts but during parturition PGHS-2 transcripts were markedly increased (Figures 14-16; Fuchs et al., 1998b,c). The half life of endometrial PGHS-2 transcripts in the pregnant cow is apparently short and PGHS-2 expression has to be induced when the need for PGE_2 or $\text{PGF}_{2\alpha}$ production arises. This may be a protective mechanism to prevent preterm activation of the uterus and preterm birth. In the cow, OT receptors are localized in endometrial epithelial cells, which also are the main site of PGHS in the uterus. Because concentration of endometrial OT receptors increases dramatically before the onset of labor OT is likely to be of major importance for prostaglandin release during spontaneous labor. Because OT stimulates expression of PGHS-2 mRNA in intercaruncular endometrium, and OT secretion increases in the last two weeks of gestation and during parturition, OT may be the cause for the increased output of $\text{PGF}_{2\alpha}$ near term and during labor observed by many authors.

Oxytocin Receptors and Oxytocin-Induced Stimulation of Prostaglandin Synthesis in Prepubertal Heifers: The influence of the specific endocrine conditions prevailing before puberty on OT receptor concentrations in the reproductive tract has not been established in any species. Indirect evidence from the poor PGFM responses to OT injections in prepubertal heifers suggests that the endometrium may not possess OT at this stage. Elucidation of OT receptors

ontogeny from birth to puberty may provide new insights into endocrine regulation of OT receptors in bovine uterus. The purpose of this study was to investigate temporal aspects of OT receptor expression and localization of the OT receptors protein in prepubertal heifers from fetal age to adulthood. A second aim was to examine the functional capacity of the putative OT receptors to stimulate $\text{PGF}_{2\alpha}$ release from the endometrium in vivo as well as endometrial cell structures in vitro. Crossbred Angus-Brahman heifers, in which puberty occurs around 12 mo of age, were used to determine the concentrations of OT receptors from the late fetal stage to adulthood. $\text{PGF}_{2\alpha}$ release in response to OT was determined in 3, 6, and 9-mo-old heifers ($n = 4$ each). Myometrium, endometrium, and cervical mucosa were obtained from 3-week old, 3-mo old, 6-mo old, and 9-mo old heifers and from adult cows at estrus. Whole uterus and cervix were taken from third trimester fetuses at birth. [^3H]OT binding and specificity, localization of immunoreactive (ir) OT receptors, OT receptor messenger RNA, and OT induced release of $\text{PGF}_{2\alpha}$ were determined (Tables 3 and 4; Figures 18 and 19; Fuchs et al., 1997b,c; 1998a). The uterus from fetuses and the neonate expressed OT receptor concentrations per mg protein were very low, but at 3 mo of age they had increased markedly in all three tissues. At 6 and 9 mo of age, levels of OT receptors had risen further and were similar to those in adult cows at estrus. Prepubertal uterus also possessed separate vasopressin VP1 subtype receptors. The ir-OT

TABLE 3. Affinity of uterine OT receptors in prepubertal heifers at different stages of development, expressed as the dissociation constant, K_d (nanomoles per liter)

Age of heifers	n	Endometrium ^a	Myometrium	Cervical mucosa
Fetus, third trimester	3	0.45 ± 0.06^b		0.86 ± 0.18^c
Fetus, at birth	1	0.52^b		0.59^c
3 wk old	4	0.61 ± 0.14	0.74 ± 0.15	1.10 ± 0.39
3 months old	4	0.84 ± 0.06	0.93 ± 0.15	1.00 ± 0.11
6 months old	3	1.19 ± 0.21	0.96 ± 0.10	0.58 ± 0.96
9 months old	4	1.00 ± 0.07	0.70 ± 0.15	0.74 ± 0.05
Adult, in estrus	4	1.00 ± 0.07	0.94 ± 0.12	1.31 ± 0.18

^a Linear regression for all K_d values against age was significant: $r = -0.535$; $P < 0.05$.

^b Endometrium and myometrium were not separated.

^c Cervical mucosal and muscle layers were not separated.

TABLE 4. The affinities of various oxytocin and arginine vasopressin agonists and antagonists to [³H]OT-binding sites in pooled endometrial membranes from prepubertal heifers, here ranked in the order of decreasing affinity ($K_i = 1/K_d$)

Compound	Rank (1-site model)	K_d (nmol/liter; 1-site model)	B_{max} (%; 1-site model)	K_{d1} (2-site model)	K_{d2} (2-site model)
OTA	I	0.54	2530 ± 5	0.05	Not possible
AVT ^a	II	0.58	2578 ± 14		1.04
MT ^b	III	0.73	2529 ± 8	0.33	1.41
OT	IV	1.09	2475 ± 3	0.41	Not possible
AVP ^a	V	2.74	3004 ± 5		6.49
IT ^a	VI	6.20	2425 ± 26	0.91	24.3
PAVP ^a	VII	6.94	2423 ± 7	0.30	11.8
MC	VIII	14.9	2440 ± 1.5	Not possible	
DDAVP	IX	>1000	No displacement		

OTA, [1-D(CH₂)₅,Tyr(Me)²,Thr⁴,Tyr-NH₂⁵]ornithine vasotocin, a specific OT antagonist; AVP, arginine vasopressin; IT, isotocin; PAVP, [Phe²,Ala⁴]ornithine vasopressin, a highly specific vasopressor (VP1) agonist; MC, Manning compound, a specific VP1 receptor antagonist; DDAVP, a highly specific VP2 receptor agonist (antidiuretic).

^a Binding data fitted two-site model significantly better than one-site model.

^b Binding data fitted two-site model, but not significantly better than one-site model, $P = 0.22$.

^a Whole uterus and cervix were used.

FIG. 18. Developmental changes in bovine uterine OTR concentrations from late third trimester fetuses to adulthood. Concentrations are expressed as femtomoles per mg protein in a crude microsomal pellet; values are the mean ± SE for four heifers in each group, except for fetuses in late pregnancy ($n = 3$) and one at birth. The increase in OTR concentrations with age was highly significant for each of the tissues examined ($P < 0.001$ for all three tissues, by ANOVA).

FIG. 19. Effect of 50 IU OT, iv, on plasma concentrations of PGFM in heifers of different ages. Values are the mean ± SE ($n = 4$ in each group). No significant increase in plasma PGFM was observed in response to OT in 3- and 6-month-old heifers. In 9-month-old heifers, OT induced a slight increase in plasma PGFM, and in adult cows, OT induced a 4-fold increase, which lasted over 2 h ($P < 0.0025$ vs. 3- and 6-month-old animals; $P < 0.01$ vs. 9-month-old animals). Basal levels of PGFM were significantly higher in adult cows than in prepubertal heifers ($P < 0.01$).

receptor was localized in luminal epithelial cells of endometrium and cervical mucosa, most of which were ir positive, whereas in myometrium, clusters of ir-OT receptor-positive cells were found among large numbers of ir-OT receptor negative cells. The PGF_{2α} response to OT was insignificant in heifers of all age groups, in contrast to that in cows of estrus. Endometrial cells from 4- to 5- mo-old heifers did not respond to OT with prostaglandin release in the absence or presence of added arachidonic acid. Tumor promoters, lipopolysaccharide and interleukin-2 also failed to elicit prostaglandin release in vitro, although they induced prostaglandin release in similar

cell cultures from cyclic cows. In summary, uterine tissues of prepubertal heifers have high levels of OT receptors, which appear to be developmentally regulated.

The endometrial OT receptors are not functionally coupled to prostaglandin synthase in prepubertal heifers, or alternatively, prostaglandin synthase is not expressed in the uterine tissues of prepubertal heifers. The *in vitro* experiments indicated that endometrial cells derived from the prepubertal heifer are capable of prostaglandin synthesis, albeit at much lower rates than cells from adult bovine endometrium. The paucity of phospholipase A₂ was not responsible for the failure of OT to induce prostaglandin release, because addition of arachidonic acid did not reverse this failure. Other agents known to elicit prostaglandin release from by induction of PGHS-2 also failed to elicit prostaglandin release from endometrial cells from prepubertal heifers in the presence and absence of added arachidonic acid; therefore, the alternative explanation, lack of expression of the PGHS-2 gene, seems closer to the truth. Indeed in pregnant cows, OT injections caused within 2 h a significant increase in endometrial PGHS-2 mRNA expression (Fuchs et al., 1998b,c). We, therefore, infer that OT mediates prostaglandin release from endometrial epithelial cells through induction of PGHS-2 transcription. The gene for the constitutively expressed PGSH-1 is probably expressed in prepubertal bovine uterus at low concentrations, but the gene for the inducible PGHS-2 is not. Progesterone is required for the expression of PGHS-2 in bovine uterine tissues in prepubertal heifers (Fuchs et al., 1998e).

In summary, the OT receptor gene is expressed in the fetal bovine uterus in the late third trimester if not earlier. Postnatally, the expression of OT receptors increases rapidly, and high levels of receptor protein are maintained in the luminal epithelium of endometrium and cervical mucosa throughout puberty. The changes in OT receptor concentrations with age are probably developmentally determined rather than induced by circulating estrogen levels present in heifers

before puberty (~2 pg/ml) probably enhance OT receptor expression indirectly by promoting growth and differentiation of the uterus and cervix. Oxytocin does not elicit prostaglandin release from the endometrium of prepubertal heifers, apparently because the uterus does not express the inducible PGHS-2 gene before puberty.

Influence of Ovarian Steroids on Endometrial Oxytocin Receptor, and Induced Prostaglandin $F_{2\alpha}$ Secretion and Prostaglandin H Synthase-2 Gene Expression in Prepubertal Heifers: Oxytocin does not elicit $PGF_{2\alpha}$ release in prepubertal heifers in spite of high endometrial concentrations of OT receptors suggesting absence of PGHS-2 before puberty. We have examined expression of PGHS-1 and PGHS-2 gene transcripts in endometrium of prepubertal heifers and the influence of estradiol and progesterone on this expression (Figures 20

Fig. 20. Experimental design

Fig. 21. Concentrations of plasma PGFM in 9-mo old ovariectomized heifers before and after injection of 50 IU OT. Injections of OT were given on Day 0, 13, 20, and 23 after the experiment began.

Fig. 22. Concentrations of plasma PGFM in 9-mo old heifers implanted with estradiol-17 β releasing pellets before and after injection of 50 IU OT on Day 0, 13, 20, and 23 after the experiment began.

Fig. 23. Concentrations of plasma PGFM in 9-mo old heifers, that were equipped with progesterone releasing vaginal device on Day 0, before and after i.v. injection of 50 IU OT on Day 0, 13, 20, and 23 after the experiment began.

Fig. 24. Concentrations of plasma PGFM in 9-mo old heifers that were equipped with both estradiol-17 β pellets and a progesterone releasing vaginal device inserted on Day 0. Blood samples were taken every 15 min before and after i.v. injection of 50 IU OY at time 0; injections were given on Days 0, 13, 20, and 23 after the experiment began.

Relative Intensity of Cox-2 vs GAPDH (%)

Fig. 25. Relative density of PGHS-2 mRNA protected fragments in comparison to the corresponding density of GAPDH mRNA protected fragments (controls) in Rnase protection assay. Values are expressed as percent of each control.

to 25; Fuchs et al., 1998e).

Additionally, the effect of ovarian steroids on uterine OT receptor concentrations and the OT induced prostaglandin release in vivo was investigated. Four study groups of 9-mo old heifers were studied: 1) ovariectomized; 2) with estradiol implants; 3) with progesterone releasing vaginal device (CIDR);

4) with estradiol implants and a CIDR. Steroids were withdrawn 21 days later. Oxytocin (50 IU) was injected iv 13, 20, and 23 days after treatments began. PGFM was measured in plasma after each OT injection. Endometrial biopsies were taken on Days 14, 21, and 24 for determination of OT receptor concentration by ligand binding, and PGHS-1 mRNA and PGHS-2 mRNA by RNase protection assay. PGHS-1 mRNA was not detected in any of the heifers. Weak signals for PGHS-2 mRNA were found in intact and ovariectomized heifers; after progesterone or estradiol treatment concentration of the transcripts increased. Oxytocin had no effect on plasma PGFM levels in intact, ovariectomized, or estradiol treated heifers but elicited massive increases in progesterone or estradiol treated heifers in spite of substantial decrease in OT receptor density (Figures 21 to 24; Fuchs et al., 1998e). Hence action of progesterone is necessary for PGHS-2 mRNA expression in bovine endometrium. Prepubertal heifers have a super-abundance of uterine OT receptor, because a fraction of the initial OT receptors sufficed for induction of $\text{PGF}_{2\alpha}$ release in progesterone treated heifers. The expression of OT receptor gene in prepubertal heifers seems

to be constitutively expressed and is suppressed by progesterone and to a lesser extent by estrogen.

We conclude that in the absence of ovarian steroids, OT receptor expression in the uterus is developmentally regulated and constitutively up-regulated. Progesterone has a marked inhibitory effect on OT receptor gene transcript expression, whereas estradiol in low concentrations has no effect and in high concentrations estradiol has a minor suppressive effect. Estradiol potently augments the effects of progesterone on both OT receptor and PGHS-2 mRNA expression when given together with progesterone. Ovarian steroids also exert important, not yet completely understood, modulating effects on components of the signaling pathway for OT receptor. Our results indicate that the action of progesterone on endometrium is a necessary prerequisite for the induction of PGHS-2 expression by OT and other activators of protein kinase C. We postulate that PGHS-2 expression in the endometrium may be essential for the onset of puberty in the bovine.

Dexamethasone Treatment Induces Oxytocin Gene Expression in Corpora Lutea and Increases Oxytocin Receptor Gene Expression in Late Pregnant Cows: Twelve pregnant Angus and Hereford cows were grouped in pairs of equal gestational length; one of each pair received an im injection of 20 mg dexamethasone and the other the vehicle (inactive oil; Fuchs et al., 1995b). The cows were slaughtered 24 or 48 h after the injection. Ovaries and uteri were dissected as quickly as possible and samples of CL, endometrium, and myometrium were frozen in liquid N₂ and kept at -80° C until processed for total RNA extraction by guanidine isothiocyanate and for preparation of microsomal pellets by sequential centrifugation of homogenates for [³H]OT binding measurements by saturation analysis. RNase protection assays were performed for the detection of bovine OT mRNA and bovine OT receptor mRNA. Bovine

GAPDH mRNA was also measured as a control for equal loading of total RNA on the gels. cRNA probes were prepared using amplified cDNA fragments for bovine OT, OT receptor, and GAPDH, subcloned into pBluescript transcription vectors. OT mRNA was not detected in the uterine tissues of any of the cows, but was present in the CL of all dexamethasone treated cows. Only one of the CL of the six control cows showed a weak signal for OT mRNA, whereas the signals for GAPDH mRNA were equivalent in all CL. OT receptor mRNA signals were present in the uterine tissues of both groups of cows, but they were stronger in dexamethasone treated than control cows. B_{max} values for OT binding were also significantly greater for the dexamethasone cows (Fuchs et al., 1995b). Thus, dexamethasone induces within 24 to 48 h after injection similar changes in OT and OT receptor gene expression in the bovine ovary and uterus as those observed at the onset of spontaneous labor.

Oxytocin Gene Expression in Extra Hypothalamic Tissues of the Pregnant Cow:

Rescue of Luteal Oxytocin Production at Term: The peptide hormone OT is produced both in the hypothalamus and in certain peripheral organs. The extent of this extra-hypothalamic hormone synthesis in the pregnant cow has not previously been examined. In this study different tissues were analyzed from the pregnant bovine reproductive tract and CL for the presence of mRNA encoding the OT peptide as well as the OT receptor (Ivell et al., 1995a,b). In uterine tissues oxytocin mRNA could only be detected sporadically with the help of a reverse transcription-polymerase chain reaction method, implying only very low levels of expression (Figure 26; Ivell et al., 1995a,b). The caruncles showed a consistent, albeit, low level of OT gene expression, which appeared up-regulated at term. In the CL of mid- and late-pregnant cows mRNA was not detected, however, at term, particularly following the onset of labor a significant level of OT gene expression was detected. The transcript levels were sufficiently high to be

measurable by both RNase protection assay and by Northern hybridization; these levels imply a rescue at term of OT gene expression which is present in the CL of cyclic and early pregnant

cows but then disappears in midgestation. Immunohistochemistry of the OT peptide confirmed that the gene was functionally active. A sensitive RNase protection assay was developed to detect transcripts encoding the bovine OT receptor. Transcripts were detected in most uterine tissues, including the caruncles, with highest levels in the endometrium and myometrium at term. In cotyledons and amnion transcripts were detected only at term and during labor. No transcripts could be detected in the CL at any stage of pregnancy (Ivell et al., 1995a,b). The results suggest that the bulk of circulating OT in pregnant and parturient cows is of post pituitary origin, however, there is a possibility of paracrine effects of OT within the uterus of the pregnant cow at term. The rescue of luteal OT production at term could act to supplement the circulating hormone of pituitary origin.

The factor causing the rescue of OT gene expression in bovine CL at term are presently unknown. It seems likely that this rescue of luteal OT-generating function reflects the changes in ovarian and placental steroid secretion, e.g., high estrogen, low progesterone, at parturition. Interestingly, an increase in LH secretion, which is closely associated with the up-regulation of OT gene expression in cyclic cows, does not appear to occur at term. The changes in the systemic steroid concentrations may be responsible, among other stimuli, for the up-regulation of OT synthesis wherever it is produced, whether in the hypothalamus or in the CL.

LH Receptors Its mRNA and the Prostanoid System in the Bovine Endometrium:

Other factors beside OT probably play a role in regulating the prostanoid system in the bovine uterus. One such candidate is LH. LH/hCG binding sites were characterized in bovine endometrium with a radioreceptor assay performed with membrane homogenate of endometrial tissues and analyzed for binding site specificity and capacity (Shemesh et al., 1996). There was little competition for receptor occupancy between hCG and ovine FSH (5%) or oPRL (<0.1%)

but there was a 20% cross-reaction with eCG. There was no affinity for LH/hCG in crude membrane preparations of kidney or skeletal muscle. Concentrations of endometrial LH/hCG binding sites were found in cell preparations of Days 15-17 of the cycle, but not in preparations from the other stages of the cycle tested (Days 8-12, pre- and post-estrus, and ovulation). The concentration of uterine LH/hCG receptor varied during the estrus cycle with higher values at Days 15-17 (3.1 fmol/mg protein). However, the binding capacity of hCG by luteal cells (9.7 fmol/mg protein) was three fold higher ($P < 0.0.1$) than endometrial tissue on any day studied. No significant differences in affinity constant for endometrial LH/hCG receptor were seen from Days 2-4 ($0.65 \times 10^{11} \text{ M}^{-1}$) or Days 15-17 ($0.86 \times 10^{11} \text{ M}^{-1}$), and mid-cycle luteal cells ($0.60 \times 10^{11} \text{ M}^{-1}$). Using RT-PCR in a limited number of tissues, the presence of the mRNA for the LH receptor was clearly indicated in endometrium on Day 16 and to a lesser extent on Day 18 of the cycle. Using Western blot analysis, we determined the expression of cyclooxygenase during the estrous cycle of the cow. It was found that the signal for cyclooxygenase was strongest at Days 15-17. In the presence of LH, a three fold increase in cyclooxygenase expression was observed on Days 15-17. Incubation of endometrial cells with LH resulted in an increase in $\text{PGF}_{2\alpha}$ production from 0.2 ng to 0.8 ng $\text{PGF}_{2\alpha} / 3 \times 10^5 \text{ cells/6 h}$. These data suggests that LH receptors are of physiological significance as LH induces endometrial cyclooxygenase and increases $\text{PGF}_{2\alpha}$ release.

Expression of LH Receptor, Its mRNA, Induction of Cyclooxygenase, and

Augmentation of Prostaglandin Production in Uterine Veins: We have previously reported that bovine endometrium contains LH/hCG binding receptors and that LH increases cyclooxygenase and prostaglandin production in the bovine endometrium. The present study (Shemesh et al., 1997a) investigated 1) whether bovine uterine vein and artery contain LH receptor mRNA and receptor protein and 2) whether LH can regulate the formation of vasoactive

eicosanoids by the uterine vein. The uterine vein endothelium, but not the uterine artery, contained LH receptor mRNA transcript essentially identical to that found in the bovine CL. The uterine vein endothelium also contained a 95-kDa immunoreactive receptor protein that bound to rat anti-LH receptor antibody in Western blots. The LH receptor mRNA and LH receptor protein were greater in the uterine vein from cows in proestrus/estrus compared with cows in luteal or postovulatory phases. Incubation of endothelial minces of uterine vein with LH resulted in a two-fold increase in cyclooxygenase concentration as determined by Western blot by using an antibody to ram seminal vesicle cyclooxygenase. The increase in cyclooxygenase was maximal in cows in proestrus/estrus compared with postovulatory and luteal phase cows. Incubation of proestrus/estrus uterine vein or artery minces with LH or mellitin (a phospholipase A₂ simulator) caused increased production of eicosanoids. In the uterine vein, LH caused a significant increase in both PGF_{2α} (basal 5.7 ± 0.3 ng/100 mg·6 h, $P < 0.01$; N = 6 cows) but had no effect on prostaglandin production by the artery. Mellitin increased PGF_{2α} production by both uterine vein and artery minces but had no effect on PGE₂ production in either tissue. Addition of steroids (progesterone, estradiol) or cytokines (tumor necrosis factor- α , IL-6) to the incubation medium of uterine vascular tissue had no effect on prostanoid production (Shemesh et al., 1997a). Expression of the LH receptor in uterine veins may have physiological significance as LH increases the concentration of cyclooxygenase and increases prostaglandin release in this tissue. The increase in the concentration of the LH receptor and its mRNA at proestrus/estrus may serve to increase the amounts of prostanoids reaching the regressing CL either by increasing prostanoid production or by increasing the blood flow of the ovary. The role of LH on prostanoid production by the uterus of pregnancy is unknown.

Secretory Proteins of the Bovine Corpus Luteum During the Estrous Cycle and

Pregnancy: The CL is a major source of OT during the estrous cycle of the cow (Fields and Fields, 1996). The observation that large luteal cells contain secretory granules which undergo exocytosis indicated that these cells are involved in protein secretion. The granule population is sparse during early pregnancy (Day 40), increases to maximum levels in mid-pregnancy, and declines during late pregnancy (Fields and Fields, 1996). The contents of luteal secretory granules of the estrous cycle are partially known, but those of pregnancy have not been elucidated. This study was carried out to test the hypothesis that protein synthesis and secretion by the bovine CL of pregnancy differs qualitatively and quantitatively from that by luteal phase CL, and that temporal variations exist within each condition (Ndikum-Moffor et al., 1995, 1996, 1997; Fields et al., 1996).

Corpora lutea from Days 3, 7, 11, 14, 17, and 19 of the estrous cycle, and from Day 17 of pregnancy as well as from first, second and third trimester of pregnancy were sectioned and incubated in MEM (500 mg/15 ml) containing 50 μ Ci [3 H]-leucine for 24 h at 37° C in at atmosphere of N₂:O₂:CO₂ 50:45:5 (v/v/v). Luteal-conditioned medium was dialyzed against Tris buffer (pH 8.2), and then against water. Proteins in the retentate were separated by two dimensional gel electrophoresis, gels were stained with Coomassie blue, dried and exposed to x-ray film. Molecular weights of the proteins present in the retentates dialyzed LCM ranged from 12,000 to >200,000. Separated proteins were transferred onto polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA) and subjected to N-terminal amino acid sequencing.

Amino acid sequences for the 20, 22, 27, 30 and 35 kDa proteins matched that of bovine tissue inhibitor of metalloproteinases-2 (TIMP-2), human manganese superoxide dismutase (Mn SOD), bovine apolipoprotein A-I (Apo A-I), bovine TIMP-1, and bovine apolipoprotein E (Apo E), respectively (Ndikum-Moffor et al., 1996, 1997; Fields et al., 1996). Except for Apo E,

which was secreted only on Day 3 of the estrous cycle and Apo A-I which was not secreted on Days 3 and 7 the other proteins were secreted throughout pregnancy and on all days of the estrous cycle except on Day 3. Densitometric analysis of fluorographs revealed quantitative differences in protein secretion across days of the cycle, and between cycle and pregnancy. During the estrous cycle Apo A-I secretion was first detected on Day 11 and increased to highest levels ($p < 0.06$) on Day 19. Secretion of the unidentified 58 kDa protein was greatest ($p < 0.01$) on Day 17, while Apo A-I, TIMP-1, and the unidentified 44 kDa protein were secreted in greatest amounts on Day 19 of the cycle. The secretion of TIMP-2 and Mn SOD proteins across the cycle were highest ($p < 0.08$) on Day 17 and declined on Day 19. During pregnancy the secretion of the 58 kDa protein was significantly ($p < 0.03$) greater than during the cycle, whereas Apo A-I and TIMP-1 were secreted at four and two times greater rates, respectively, during the estrous cycle than during pregnancy.

These data indicate that the bovine CL synthesizes and secretes apolipoproteins, in addition to TIMP-1, TIMP-2, and Mn SOD. Except for TIMP-1 and TIMP-2, this is the first demonstration of secretion of these proteins by the CL. Apo A-I is the major protein constituent of high density lipoprotein (HDL), which is the major source of circulating cholesterol in the cow, it is also a potent activator of lecithin: cholesterol acetyltransferase (LCAT). Secretion of this protein may therefore regulate availability of cholesterol to steroidogenic cells within the CL and placenta. According to our results secretion of the Apo A-I increases as the cycle progressed. Luteal MnSOD may be involved in protecting the CL from damage by converting reactive superoxide anions to hydrogen peroxide. The H_2O_2 generated may also drive the peroxidase-ascorbate pathway responsible for production of progesterone. Our results showed an increase in MnSOD secretion from Day 7 to Day 17, and a decline on Day 19 of the cycle. Secretion of Apo

E (a ligand for the LDL receptor) was observed only on Day 3, thus Apo E may regulate movement of cholesterol required for membrane synthesis during early luteal development. Apo E may also play a role in steroidogenesis. TIMP-1 and TIMP-2 may be involved in the remodeling of luteal and uterine tissues. In summary, the bovine CL synthesizes and secretes proteins which may act in an autocrine, paracrine, and/or endocrine manner to regulate luteal, uterine and placental development, tissue remodeling and steroidogenesis.

Circulating Levels of Apolipoproteins A-I and B in Cows During Estrous Cycle and Peri-partum Period: We have reported earlier that the bovine CL secretes Apolipoproteins (Apo) E and A-I and expresses their mRNAs during the estrous cycle and pregnancy (Ndikum-Moffor et al., 1995, 1996, 1997; Fields et al., 1996). Luteal synthesis of Apo A-I increased in late luteal phase and during the second half of pregnancy from levels observed during the first half (Ndikum-Moffor et al., 1996, 1997). The role of luteal Apolipoproteins is not well understood. They may be involved in membrane synthesis and steroidogenesis. The objectives of the present study were two fold: to determine the circulating levels of Apo A-I and Apo B during the estrous cycle and during the peri-partum period, and to compare these profiles with the pattern of their luteal secretion and mRNA expression reported earlier. Daily blood samples were collected during estrous cycle and twice a week during 7 weeks before the expected delivery date and 7 weeks after calving. Serum concentrations of Apo A-I and Apo B were measured by enzyme immunoassay and progesterone by radioimmunoassay. Circulating levels of Apo A-I were similar before and after parturition whereas Apo B concentrations, which were lower during pregnancy than in cyclic cows, increased postpartum.. Multivariate analysis showed that during the estrous cycle serum concentrations of progesterone and Apo-I were correlated (partial correlation coefficient $r = .36$) but those of progesterone and Apo B were not. Apo A-I, Apo B or

progesterone in any combination were not correlated in the peri-partum period. Luteal activity and luteal synthesis of Apo A-I in particular has only a minimal effect on circulating levels of these Apolipoproteins. Hormonal changes during the estrous cycle and pregnancy appear to participate in the regulation of circulating levels of Apolipoproteins A-I and B (Ndikum-Moffor et al., 1996, 1997). Future studies are required to resolve the question of the relative importance of systemic and local contribution of these Apolipoproteins for luteal steroidogenesis.

Differential Gene Expression in Bovine Corpus Lutea During Pregnancy: In addition to progesterone, the bovine CL secretes proteins, including, apolipoproteins E and A-I, and tissue inhibitor of metalloproteinases-1 and -2. The CL undergoes a series of important changes during gestation, one of which is the appearance of secretory granules on Day 45 of pregnancy and reaches its maximum formation on Day 160 to Day 200. The objective of this experiment to isolate and identify the genes which are differently expressed between the CL of Day 40 and Day 160 of pregnancy (Kowalski et al., 1998). Total luteal RNA from Day 40 and Day 160 of pregnancy were isolated using the Trizol procedure, DNase treated, and stored at -80°C. The total RNAs were reverse transcribed and subjected to polymerase chain reaction using a combination of anchor primers (T7dT₁₂AP6 and AP7) with 5' upstream primers (M13r-APR13) and [α P³³]dATP. Products were separated in a 4.5% polyacrylamide denaturing gel. Two candidate cDNA bands C-1 (800 bp) and C-2 (820bp) appeared to be differentially expressed. These bands were excised from the gel, reamplified, and used as probes in Northern blots to confirm differential expression of these RNAs. The C-2 was up regulated on Day 40 and the C-1 gene was upregulated on Day 160. Cloning and subsequent DNA sequencing of these cDNAs revealed 747 nucleotides for C-1 and 744 nucleotides for C-2. These cDNAs appear to be novel since no homologies with nucleotide sequences reported in the GenBank were found. Translation

of the determined nucleotide sequence of C-2 cDNA indicated an open reading frame of 74 amino acids that contained six cysteine and 35 hydrophobic amino acids which may suggest that the encoded protein is secreted. These results indicate that morphological and physiological changes by the CL across pregnancy may be reflected in changes in specific gene expression.

Secretory Proteins of the Ovine Corpus Luteum During the Estrous Cycle,

Pregnancy, and Hysterectomy: In the ewe, the CL is a source of OT and is essential for the maintenance of pregnancy up to Day 50, after which placental progesterone suffices to maintain pregnancy. Although the CL is not necessary for the synthesis of progesterone in the later stages of pregnancy, it does not atrophy, and remains active throughout gestation. The purpose of this investigation was to test the hypothesis that the synthesis and release of luteal proteins differ among cyclic, pregnant, and hysterectomized ewes. Corpora lutea were obtained from cyclic (Day 7, 10, and 14; $n = 3$ CL each day), pregnant (Day 14, 80, 120, and 129; $n = 3$ or 4 CL each day) or hysterectomized (Day 80 or 120; $n = 3$ CL each day) ewes. Luteal slices (500 mg) were incubated in 15 ml leucine-deficient Eagle's MEM in the presence of 50 μ Ci [3 H]-leucine. Cultures were maintained at 37° C in an atmosphere of $N_2:O_2:CO_2$ 50:45:5 (v/v/v) for 24 h. The LCM was dialyzed (MW cutoff 3500) first against Tris buffer and then against deionized water. The percentage of [3 H]-leucine incorporated into the proteins present in the retentates of the LCM was determined. Retentates were lyophilized and submitted to isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Coomassie blue, dried, and exposed to x-ray film. The intensity of each radioactive signal on the fluorographs was determined by densitometry. Fluorographs showed qualitative and quantitative differences in de novo synthesis and release of luteal proteins among the CL of cyclic, pregnant, and hysterectomized ewes. Differences were also detected within each group. Four of the

proteins were synthesized and released by all CL regardless of their status: 24 kDa (pI 6.5), 32 (pI 7.6), 32 (pI 7.8) and 60 (pI 7.0). Proteins of a higher MW, >100,000, were observed on Day 7 of the estrous cycle and on Days 14, 80, 120, and 129 of pregnancy as well as on Day 80 but not on Day 120 after hysterectomy. The percentage of radiolabel incorporated by the CL of pregnant ewes was greater than that of the cyclic ($p = 0.07$) or hysterectomized ($p = 0.001$) ewes. Our results indicate that qualitative and quantitative differences exist among cyclic, pregnant, and hysterectomized ewes in regard to the proteins secreted by their CL in vitro.

F. Description of Cooperation

BARD Project No. US-2333-93

Significance of Oxytocin and Oxytocin Receptors in Bovine Pregnancy

Collaboration between the three laboratories pursuing the objectives of the BARD grant resulted in the publication of 26 refereed journal articles and 17 abstracts. Samples were provided from experiments conducted at the University of Florida to Kimron Veterinary Institute and Cornell University Medical College. Samples were also provided to the Institute of Hormone and Fertility Research, Hamburg, Germany (Dr. Richard Ivell) and the University of South Alabama (Dr. Phillip A. Fields). Dr. Ron Randel at Texas A&M University also participated in some of the experiments by providing samples. The annual meetings of the Society for the Study of Reproduction in the US provided the investigators an opportunity to meet and discuss the results, present results and plan for future experiments.

Dr. Anna-Riitta Fuchs was awarded a Fogarty International Senior Fellowship to work in the laboratory of Dr. Richard Ivell in Hamburg, Germany where she studied expression of the oxytocin receptor gene. Dr. Fuchs was the recipient of the Interdisciplinary Reproductive Biology Group Donald Henry Barron Lecturer Award at the University of Florida for her contribution to reproductive sciences. While at Florida in 1995 and again in 1997 research results were reviewed.

Dr. Mordechai Shemesh visited the University of Florida in 1996 and again in 1998 to conduct experiments that took advantage of the availability of cows for study. While at the University of Florida in 1998 Dr. Shemesh was recognized as a Donald Henry Barron Lecturer awarded to those who have made contributions to the reproductive sciences.

G. Conclusions and Evaluation of Research Achievement

BARD Project No. US-2333-93

Significance of Oxytocin and Oxytocin Receptors in Bovine Pregnancy

Our earlier studies had shown that oxytocin receptors were present in all uterine tissues, including maternal and fetal placenta and fetal membranes. Oxytocin and vasopressin receptors were particularly abundant in the endometrium and myometrium during estrus and decreased abruptly in early pregnancy. During later stages of pregnancy oxytocin receptors in the endometrium, myometrium, and fetal membranes increased with a steep rise during labor. The concentrations of the oxytocin receptors in the different tissues were differently regulated during estrous cycle and pregnancy. The distribution and concentration of oxytocin receptors suggested that oxytocin may have important, novel physiological functions in bovine pregnancy and parturition. We therefore proposed to investigate the actions mediated by oxytocin receptor in the different tissues as well as the factors involved in the regulation of their concentrations.

In collaboration with Dr. Richard Ivell the bovine oxytocin receptor gene was cloned and a ribonuclease protection assay was developed for the bovine oxytocin receptor mRNA. The expression of oxytocin receptor transcripts was determined in tissues from pregnant cows in which oxytocin receptor concentrations had previously been measured. The results indicated that the oxytocin receptor concentrations were controlled mainly at the transcriptional level. Dr. Ivell and his colleagues have further explored the sequence of the bovine oxytocin receptor gene upstream promoter, and found no estrogen or progesterone response elements up to 3,500 bp from the transcription site. This is puzzling, since the concentrations of the OT binding sites are clearly influenced by ovarian steroids, which therefore must exert their influence indirectly,

through steroid induced transcription factors or growth factors.

The effects of oxytocin on myometrium and endometrium have been known for some time; oxytocin initiates and augments myometrial contractions and causes release of $\text{PGF}_{2\alpha}$ from the endometrium which results in augmentation of myometrial contractions. The mechanism of action of oxytocin in both types of cells has also been elucidated. In myometrial cells the primary action of oxytocin is initiation of action potentials carried by inward calcium currents, brought about by opening of receptor operated calcium channels, and secondarily by initiation of phospholipase C and G-protein coupled intracellular inositol 3 phosphate formation and subsequent increase in calcium by liberation from calcium sequestering intracellular vesicles. In endometrium oxytocin is coupled to phospholipase C and phosphokinase C which initiate the phosphoinositol reaction and arachidonic acid mobilization, resulting in prostaglandin synthesis. We had originally proposed to determine whether this was the mechanism of action of oxytocin in bovine endometrium, but other groups had already been doing similar work and published their results ahead of us, so that aspect of our proposal was not pursued.

Further insight into the mechanism of oxytocin action on the endometrium was gained by the demonstration that oxytocin acts as a ligand for the ligand-induced cyclooxygenase-2 isoform, and initiates the expression of cyclooxygenase-2 mRNA in bovine endometrium.

Oxytocin was shown to also increase the cyclooxygenase protein in bovine cervical minces in vitro. On the other hand bovine endometrium expresses very little cyclooxygenase-1 isoform, which is the constitutively expressed isoform of the enzyme. The expression of COX-2 mRNA under basal conditions was also very low. The finding that oxytocin is capable of inducing the inducible isoform, cyclooxygenase-2, is therefore of great significance. These findings are quite

novel and of great importance for understanding the series of events that culminate in parturition in the bovine.

We had shown earlier that endometrial oxytocin receptor concentrations in pregnant cows are suppressed during implantation but begin to rise from Day 50 onwards reaching very high levels in mid-pregnancy. Suppression of endometrial oxytocin receptor and $\text{PGF}_{2\alpha}$ release are considered essential for maintenance of early pregnancy in ruminants and the role of oxytocin in luteolysis in ruminants is well established. Our findings of high concentrations of endometrial OTR therefore presented a puzzle, and prompted us to investigate whether the endometrial oxytocin receptors of pregnancy are fully functional before onset of labor. The results obtained in collaboration with Dr. Ronald Randel indicated that oxytocin did in fact induce $\text{PGF}_{2\alpha}$ release from Day 50 onwards in proportion to the oxytocin receptor concentrations. Because many other endogenous factors can induce $\text{PGF}_{2\alpha}$ release, it was important to verify that the release observed in vivo was indeed oxytocin receptor mediated. In a second study a specific oxytocin antagonist was administered to pregnant cows before oxytocin injection, and showed that the antagonist prevented the release of $\text{PGF}_{2\alpha}$ in a dose dependent manner.

It had been our intention to use oxytocin antagonists in late pregnant cows to determine whether blocking the action of endogenous oxytocin interferes with the onset and course of parturition. Unfortunately, the accurate timing of parturition in beef cattle is difficult, therefore it would have been necessary to administer prolonged infusions of the antagonist. Because the cows are very big animals and the oxytocin antagonist is very expensive, we have not been able to carry out these experiments. Plans were to circumvent this problem by using dexamethasone

induced parturition as a model for spontaneous parturition, but in the preliminary experiments carried out with dexamethasone injections in near term cows, the timing of parturition has been almost as unpredictable as in spontaneously calving cows. This aspect of the project therefore remains unresolved.

The finding of high concentration of oxytocin receptor in the endocervix of both estrous and parturient cows demonstrated that oxytocin has other target organs besides endometrium and myometrium. We have subsequently shown that the oxytocin receptors are localized in the luminal epithelial cells of the endocervix in both nonpregnant and pregnant cows. The concentration of oxytocin receptor in the muscular wall of the cervix was always lower than in the endocervix. Moreover, the oxytocin receptor concentrations were very tightly regulated both during the estrous cycle and pregnancy. Oxytocin receptor and the oxytocin receptor gene transcripts were very rare in bovine cervix except at estrus and at parturition. Oxytocin was shown to stimulate PGE_2 release from bovine cervical tissue in vitro and cause increases in the concentration of cyclooxygenase-2 enzyme in cervical tissue minces. We also demonstrated that oxytocin injections result in the accumulation of PGE_2 in the cervical lumen of estrous cows in vivo and cause transient cervical softening. Additionally, we demonstrated that PGE_1 , a closely related analog of PGE_2 , causes persistent cervical softening when relatively large doses were inserted into the cervical lumen. We postulate that the action of oxytocin in the parturient cervix is the same, namely, release of PGE_2 and subsequent softening of the rigid collagenous framework of the pregnant cervix, but this is not yet proved.

As already mentioned the relationship between OT and the two isoforms of the cyclooxygenase enzyme were examined in bovine uterine tissues. In cooperation with Werner

Rust at the Institute for Hormone and Fertility Research in Hamburg complementary DNA's for bovine cyclooxygenase-1 and bovine cyclooxygenase-2 were cloned and the expression of messenger RNA for both isoforms was examined in tissues of pregnant cows using ribonuclease protection assay. The expression of cyclooxygenase-1 mRNA, which is the constitutively expressed isoform and believed to be involved in the "housekeeping" functions of cells, was very low in bovine uterine tissues, and could only be demonstrated in myometrial and caruncular RNA and in fetal uterus. The expression of cyclooxygenase-2 mRNA was abundant in the cotyledons and maternal caruncles during the third trimester, it was also expressed in fetal membranes and myometrium, albeit in much lower concentrations. Surprisingly, cyclooxygenase-2 mRNA was very weak or absent in intercaruncular endometrium except in the early post partum period. However, injections of oxytocin induced the expression of cyclooxygenase-2 mRNA. This is a very exciting and important finding. Until now only mitogens and inflammatory cytokines were thought to be able to induce the expression of the cyclooxygenase-2 isoform. Oxytocin presumably acts through protein kinase C to elicit the expression of cyclooxygenase-2 transcription, like the tumor promoters.

Another important outcome from these studies was the discovery that oxytocin receptor gene transcripts are expressed in uteri from third trimester fetuses. This led us to investigate the ontogeny of oxytocin receptor in bovine uterus. High concentrations of oxytocin receptor were found in endometrium, myometrium and endocervix of 3-wk, 3-mo, 6-mo, and 9-mo old prepubertal heifers. Six months and 9-mo old heifers had equally high concentrations as adults at estrus. The immunohistochemical localization of these oxytocin receptors performed in collaboration with M. Balvers at the Institute for Hormone and Fertility Research, Hamburg,

indicated that practically all luminal epithelial cells of the endometrium and endocervix expressed the receptor protein, whereas epithelium of glandular endometrial was less uniformly stained and only limited numbers of immunoreactively positive myometrial cells were scattered among numerous immunoreactive negative cells. In prepuberal heifers endometrial oxytocin receptors were apparently constitutively expressed but they were not functionally coupled to cyclooxygenase and $\text{PGF}_{2\alpha}$ production. Injections of oxytocin failed to increase plasma concentrations of the metabolite of $\text{PGF}_{2\alpha}$ and neither COX-1 nor COX-2 mRNA was detected in endometrial RNA. The influence of ovariectomy, estradiol-17 β , progesterone and estradiol-17 β and progesterone in combination was examined on concentrations of oxytocin receptors and oxytocin induced $\text{PGF}_{2\alpha}$ release in prepuberal heifers. We found that progesterone is essential for cyclooxygenase-2 gene expression and oxytocin induced $\text{PGF}_{2\alpha}$ release in prepubertal heifers, estradiol-17 β and ovariectomy were without effect. Certain differences were observed among the effects of these steroids in prepubertal heifers and cyclic and ovariectomized cyclic cows. We concluded that prepubertal heifers provide an interesting alternative model for studies of hormone effects on the reproductive tract.

There was a total of 26 refereed papers and 17 abstracts resulting from the research supported by this grant. In addition to the study of oxytocin and its effects during several important studies on the secretory products of the corpus luteum were performed. It was also shown that the uterus contains receptors for LH/hCG and that LH stimulates prostanoid secretion in the uterus of cycling cows. Results from our studies indicate that during estrous cycle and pregnancy oxytocin has multiple actions on the bovine uterus and cervix which may be essential for a successful pregnancy and outcome at parturition.

H. Published Journal Articles and Abstracts

BARD Project No. US-2333-93

Significance of Oxytocin and Oxytocin Receptors in Bovine Pregnancy

1. Altszuler N, Fuchs A-R. 1994. Oxytocin secretion is stimulated by changes in glucose metabolism. *Proc Soc Exp Bio. Med.* 207:38-42.
2. Behrens O, Mascher HJ, Kupsch E, Fuchs. 1995. Oxytocin receptors in human ovaries during the menstrual cycle. In: Ivell R, Russell J (eds.) *Oxytocin. Cellular and Molecular Approches in Medicine and Research.* *Adv Exp Med Biol* 395:485-486.
3. Dibbs KI, Sadovsky Y, Li X-J, Koide SS, Adler S, Fuchs A-R. 1995. Estrogenic activity of RU486 (mifepristone) in rat uterus and cultured uterine myocytes. *Am J Obstet Gynecol.* 173:134-140.
4. Fields MJ, Fields PA. 1996. Morphological characteristics of the bovine corpus luteum during the estrous cycle and pregnancy. *Theriogenology.* 45:1295-1325.
5. Fields MJ, Ndikum-Moffor FM, Simmen RCM, Buhi WC, Rollyson MK, Kowalski AA, Chang S-MT, Fields PA. 1996. Bovine luteal secretory proteins of the oestrous cycle and pregnancy. *Reprod Dom Anim.* 31:407-425.
6. Fuchs A-R, Drolet P, Fortier MA, Balvers M, Fields MJ. 1998a. Ontogeny of oxytocin receptors and oxytocin induced stimulation of prostaglandin synthesis in prepubertal heifers. *Endocrinology.* 139:2755-2764.
7. Fuchs A-R., Rust W, Fields MJ. 1998b. Prostaglandin endoperoxide H synthase-2 gene expression in pregnant cows: stimulation of transcription by oxytocin. (Accepted;

8. Fuchs A-R, Rust W, Fields MJ. 1998c. Cyclooxygenase-1 and -2 transcript accumulation in bovine uterine tissues during the second half of pregnancy and parturition. *Biol Reprod.* 58(Suppl 1):122.
9. Fuchs A-R., Fields MJ. 1998d. Parturition, nonhuman mammals. In: *Encyclopedia of Reproduction*. Editors E. Knobil, J.D. Neill. Academic Press, San Diego, CA. 3:69-82.
10. Fuchs A-R, Rust W, Drost M, Chang S-M, Fields MJ. 1998e. Influence of ovarian steroids on endometrial oxytocin receptor concentrations and oxytocin induced prostaglandin F_{2α} secretion in prepubertal heifers: relation to prostaglandin H synthase-2 gene expression. (In Review).
11. Fuchs A-R, Behrens O, Einspanier A, Mascher HJ, Kupsch E. 1998f. Oxytocin and vasopressin receptors in nonpregnant endometrium, myometrium and leiomyomas: Characterization, cellular localization, and relation to cycle stage. *Human Reprod* (In Press).
12. Fuchs A-R, Fields MJ, Chang S-M, Thatcher WW, Willard CC, Randel RD. 1997a. Oxytocin antagonist [d(CH₂)₅,Tyr(ME)²,Thr⁴,Tyr-NH₂⁹]ornithine vasotocin inhibits oxytocin induced PGF_{2α} release in late pregnant cows. *Biol Reprod.* 57:436-441.
13. Fuchs, A-R, Fields MJ, Blocker M, Balvers M. 1997b. Ontogeny of uterine and cervical oxytocin receptors. *Ann Meet Soc Gynecol Invest.* San Diego, CA, March 20-22.
14. Fuchs AR, Drost M, Morissey P, Chang SM, Fields MJ. 1997c. Steroid effects on

oxytocin and PGF_{2α} interaction in endometrium of prepubertal heifers. *Biol Reprod.* 56 (Suppl 1):187.

15. Fuchs A-R, Fuchs F. 1997d. Physiology of parturition. In: Gabbe S, Niebyl J, Simpson JL, (eds.): *Obstetrics, Normal and Problem Pregnancies, Third Edition*, Churchill Livingstone, New York.
16. Fuchs A-R. 1997. Physiology and endocrinology of lactation. In: Gabbe S, Niebyl J, Simpson JL, (eds.) *Obstetrics: Normal and Problem Pregnancies, Third Edition*, Churchill Livingstone, New York.
17. Fuchs AR, Rollyson MK, Meyer M, Fields MJ, Minix JM, Randel RD. 1996a. Oxytocin induces PGF_{2α} release in pregnant cows: influence of gestational age and oxytocin receptor concentrations. *Biol Reprod.* 54:647-653.
18. Fuchs A-R, Ivell R, Balvers M, Chang S-M, Fields MJ. 1996b. Oxytocin receptor (OTR) gene expression and cellular localization in pregnant and parturient bovine cervix. *Biol Reprod.* 54(Suppl 1):125.
19. Fuchs A-R, Ivell R, Fields PA, Chang S-M, Fields MJ. 1996c. Oxytocin receptors in bovine cervix: distribution and gene expression during the estrous cycle. *Biol Reprod.* 54:700-708.
20. Fuchs A-R, Ivell R, Balvers M, Chang S-M, Fields MJ. 1996d. Oxytocin receptors in bovine cervix during pregnancy and parturition: gene expression and cellular localization. *Am J Obstet Gynecol.* 175:1654-1660.
21. Fuchs A-R. 1995. Receptors regulating myometrial contractility and their hormonal modulation. *Sem Perinatol.* 19:15-30.

22. Fuchs A-R, Fields MJ, Freidman S, Shemesh M, Ivell R. 1995a. Oxytocin and the timing of parturition: influence of oxytocin receptor gene expression, oxytocin secretion, and oxytocin induced prostaglandin $F_{2\alpha}$ and E_2 release. Ivell R, Russell J. *Oxytocin: Cellular and Molecular Approaches in Medicine and Research. Adv Exp Med Biol* 395:405-420.
23. Fuchs AR, Fields MJ, Chang S-M, Ivell R. 1995b. Dexamethasone treatment induces oxytocin gene expression in corpora lutea (CL) and increases oxytocin receptor gene expression in late pregnant cows. *Biol Reprod.* 52 (Suppl 1):187.
24. Graddy LG, Fields MJ, Kowalski A, Chang S-M, Fuchs A-R. 1998a. Effects of oxytocin and PGE_1 on bovine cervix at estrus. *Biol Reprod.* 58(Suppl 1):174.
25. Graddy LG, Kowalski A, Fields MJ, Fuchs A-R. 1998b. Evidence for oxytocin induced PGE_2 release from the bovine cervix in vivo. *J Reprod Fertil. (Submitted)*.
26. Ivell R, Rust W, Fuchs A-R, Einspainer A. 1995a. Oxytocin and oxytocin receptor gene expression in the reproductive system of the pregnant cow: rescue of luteal oxytocin production at term. *Biol Reprod.* 50(Suppl 1):187.
27. Ivell R, Rust W, Einspanier A, Hartung, S, Fields MJ, Fuchs A-R. 1995b. Oxytocin and oxytocin receptor gene expression in the reproductive tract of the pregnant cow: rescue of luteal oxytocin production at term. *Biol Reprod.* 53:553-560.
28. Kowalski AA, Simmen RCM, Green ML, Chang S-M, Fields MJ. 1998. Differential gene expression in bovine corpora lutea during pregnancy. *Biol Reprod.* 58(Suppl 1): 125.
29. Ndikum-Moffor F, Simmen RCM, Fields PA, Katoh N, Oikawa S, Buhi WC, Rollyson

- MK, Chang S-MT, Fields MJ. 1997. Synthesis and messenger ribonucleic acid expression of apolipoproteins E and A-I by the bovine corpus luteum during the estrous cycle and pregnancy. *Biol Reprod.* 56:745-756.
30. Ndikum-Moffor F, Katoh N, Oikawa S, Kowalski A, Graddy L, Chang S-M, Fields MJ. 1996. Circulating levels of apolipoproteins A-1 and B in the cow during the estrous cycle and the pre- and post-partum period. *Biol Reprod.* 54 (Suppl 1):67.
 31. Ndikum-Moffor F, Simmen, RCM., Chang S-M, Fields MJ. 1995. Secretory proteins of the bovine corpus luteum: the estrous cycle and pregnancy. *Biol Reprod.* 52 (Suppl 1):110.
 32. Rollyson MK, Ndikum-Moffor FM, Chang S-M, Fields MJ. 1995. Differential release of ovine luteal proteins in vitro: effects of estrous cycle, pregnancy and hysterectomy. *Biol Reprod.* 52 (Suppl 1):110.
 33. Sharp DC, Thatcher MJ-L, Salute M, Fuchs A-R. 1996. Relationship between endometrial oxytocin receptors and oxytocin-induced prostaglandin $F_{2\alpha}$ release during the estrous cycle and early pregnancy in pony mares. *J Reprod Fertil.* 109:137-144.
 34. Shemesh M, Dombrovski L, Gurevich M, Shore LS, Fuchs A-R, Fields MJ. 1997a. Regulation of bovine cervical secretion of prostaglandins and synthesis of cyclooxygenase by oxytocin. *Reprod Fertil Develop.* 9:525-530.
 35. Shemesh M, Dombrovski L, Gurevich M, Stram Y, Fields MJ, Shore LS. 1997b. Expression of functional luteinizing hormone (LH) receptor and its messenger ribonucleic acid in bovine uterine veins; LH induction of cyclooxygenase and

augmentation of prostaglandin production in bovine uterine veins. *Endocrinology* 138:4844-4851.

36. Shemesh M, Freidman S, Gurevich M, Stram Y, Fields MJ, Shore LS. 1996a. Luteinizing hormone receptors and its mRNA in the bovine endometrium. *J Physiol Pharm.* 47(Suppl 1):15-27.
37. Shemesh M, Gurevich M, Shore LS, Davidson I, Freidman S, Dombrovski L. 1996b. Regulation of cyclooxygenase and products of the cyclooxygenase pathway in bovine uterus. *Reprod Dom Anim.* 31:431-436