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Investigations into the mechanisms controlling parturition in cattle

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Abstract

A pronounced increase in fetal cortisol concentrations stimulating an increase in estrogen production at the expense of progesterone precursors in the placenta, luteolysis, and progesterone withdrawal is considered as a key event during the complex signal cascade leading to the initiation of parturition in cattle. However, there are many questions concerning the exact functional and/or temporal relationships between these individual processes which finally result in the expulsion of the calf and the timely release of the placenta. Thus, parturition was induced in 270-day pregnant cows using the progesterone receptor blocker aglepristone (group AG, $n=3$), the prostaglandin $F_{2\alpha}$ analog cloprostenol (group PG, $n=4$), and the glucocorticoid dexamethasone (group GC, $n=4$) to characterize the effect on maternal steroid and prostaglandin levels and to identify immediate subsequent changes in placental morphology and gene expression as compared with untreated controls sampled on day 272 (group D272, $n=3$) and cows during normal parturition (group NT, $n=4$). All calves of the treatment groups were born on days 271–272, whereas gestational length in NT cows was 280.5 ± 1.3 days. However, none of the treatments significantly induced the prepartal remodeling of placentomes characterized by a decline in trophoblast giant cells and reduction of the caruncular epithelium. Data on placental CYP17 and COX2 expression confirm that these key enzymes are upregulated by GC, whereas placental aromatase expression was not affected by any treatment. Maternal progesterone and prostaglandin profiles suggest differential effects of the treatments on luteal function and placental or uterine prostaglandin production. The results provide new information on the initiation of parturition in cattle but raise many new questions.

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Introduction

For many mammalian species, the signal cascade leading to the initiation of parturition is still poorly understood with the information available pointing toward distinct species-specific differences (Thorburn & Challis 1979, López Bernal 2001, Zakar & Hertelendy 2007, Mitchell & Taggart 2009). So far the sheep has served as an important animal model and detailed information on the regulatory mechanisms leading to birth is available for this species (for review see Whittle *et al.* (2001)); in the ovine fetus at the end of gestation, maturation of the hypothalamo–pituitary–adrenal (HPA) axis leads to a substantial increase in fetal cortisol, triggering in uninucleated trophoblast cells (UTC) an upregulation of cyclooxygenase 2 (COX2) and of the steroidogenic key enzyme 17α-hydroxlyase-C17, 20-lyase (CYP17). The latter channels placental metabolism of pregnenolone into the Δ5-pathway resulting in a dramatic decrease in progesterone and an enhanced synthesis of estrogens. This switch in placental steroid production leads to an upregulation of contraction-associated proteins in the myometrium and the release of uterotonic prostaglandins in the uterus which eventually stimulates the onset of myometrial activity and expulsion of the fetus (Challis 1971, Tsang 1974, Mason *et al.* 1989, Gyomorey *et al.* 2000, Whittle *et al.* 2000). Important steps of this signal cascade have been confirmed as occurring in late bovine gestation like the increase of fetal cortisol levels (Comline *et al.* 1974, Hoffmann *et al.* 1977, Hunter *et al.* 1977) and the significant prepartal upregulation of CYP17 and COX2 in UTC (Schuler *et al.* 2006a, 2006b). Moreover, the collapse of bovine placental progesterone production and an enhanced production of estrogens as a result of an increase in placental CYP17 activity was shown in *in vitro* studies (Schuler *et al.* 1994). However, the signal

cascade controlling the initiation of parturition in the cow must clearly be more complex than in the late pregnant sheep where the placenta is the sole significant source of progesterone (Denamur & Martinet 1955, Bassett *et al.* 1969, Al-Gubory *et al.* 1999), while in the cow it is the corpus luteum (Estergreen *et al.* 1967, Day *et al.* 1977*a*, 1977*b*, Chew *et al.* 1979) with the prepartal decline in maternal progesterone levels clearly resulting from luteolysis (Hoffmann *et al.* 1979). The bovine placenta contributes – if at all – only insignificantly to systemic maternal progesterone levels during late gestation (Comline *et al.* 1974, Conley & Ford 1987) but is still capable of producing high local progesterone concentrations at the feto-maternal interface (Tsumagari *et al.* 1994). In contrast to luteolysis in cyclic cows with prostaglandin $F_{2\alpha}$ of endometrial origin being the crucial factor (for review see Okuda *et al.* (2002) and Skarzynski *et al.* (2008)), the mechanisms leading to luteolysis in prepartal cows are virtually unknown. During prepartal luteolysis, i.e. 36–48 h prior to birth, the increase in $PGF_{2\alpha}$ or of its major metabolite 15-keto-13,14-dihydro $PGF_{2\alpha}$ (PGFM) over basal levels is still minimal (Königsson *et al.* 2001), and PGFM profiles in maternal peripheral blood are distinctly different from luteolysis in cyclic cows, where pronounced prostaglandin spikes of endometrial origin are observed preceding or concomitant with luteolysis (Peterson *et al.* 1975, Kindahl *et al.* 1976, Vighio & Liptrap 1986). Thus, the only clear role that can be attributed to the substantial increase in $PGF_{2\alpha}$ or PGFM during the immediate peripartal period (Fairclough *et al.* 1975, Edqvist *et al.* 1978, Bosu *et al.* 1984, Meyer *et al.* 1989) seems to be related to myometrial activity. However, largely due to the fact that luteolysis and consequently parturition is readily induced in late pregnant cows by the application of $PGF_{2\alpha}$ or its analogs (Zerobin *et al.* 1973, 1975, Henricks

et al. 1977*a*, 1977*b*), it is widely accepted that $PGF_{2\alpha}$ is also in prepartal cows the endogenous luteolytic agent.

Though in the cow cessation of placental progesterone production does not significantly contribute to the prepartal progesterone withdrawal, the prepartal changes in placental endocrine functions induced by the increase in fetal cortisol must play an essential role in the signal cascade controlling bovine parturition as is indicated by the significant prolongation of pregnancy in cases of disturbed fetal HPA axis function (Kennedy *et al.* 1957, Holm & Short 1962, Buczinski *et al.* 2007, Cornillie *et al.* 2007). From these observations it may be concluded that the prepartal changes in bovine placental endocrine function induced by the increase in fetal cortisol cause the generation of luteolytic prostaglandins of hitherto unknown origin. Based on these observations and assumptions a hypothetical model of the signal cascade leading to bovine parturition was developed (Fig. 1). In order to test the validity of this model and to further assess the mechanisms and the sequence of events leading to onset and completion – i.e. release of the placenta – of bovine parturition, we have compared at a clinical and molecular level three treatments inducing parturition in 270-day pregnant cows: i) administration of the glucocorticoid dexamethasone (Bosc 1971, Welch *et al.* 1973, Wagner *et al.* 1974) to possibly mimic the increase in fetal cortisol (group GC, $n=4$); ii) application of the $PGF_{2\alpha}$ analog on cloprostenol to induce luteolysis (Day 1977*a*, 1977*b*) and consequently parturition (group PG, $n=4$); and iii) administration of the competitive progesterone receptor (PR) blocker aglepristone (group AG, $n=3$) to abolish PR-mediated effects irrespective of the source of the ligand. The effects of treatments were assessed by clinical observations, investigations into placental histomorphology and leucocyte infiltration,
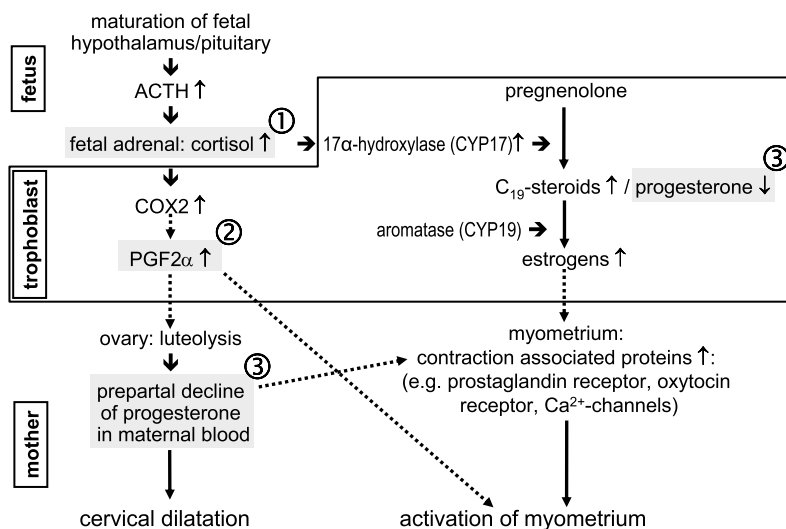
Figure 1 Hypothetical model of the initiation of parturition in cows based on our own previous observations and data from other investigators (see text for detailed information). The encircled numbers indicate the sites where the treatments applied intervene with the underlying signal cascade. Treatments were (1) the application of dexamethasone to mimic the late gestational rise in fetal cortisol; (2) the application of the prostaglandin $F_{2\alpha}$ analog cloprostenol to mimic prepartal luteolysis; and (3) the blockage of progesterone receptors by the antigestagen aglepristone to withdraw the effects of progesterone of both luteal and placental origin. Solid lines, established effects; dotted lines, hypothetical effects; ↑, increase, and ↓, decrease.

determination of maternal progesterone, estrogen, and PGFM concentrations, and assessment of the expression of genes in placentomes putatively involved in the control of parturition. Controls were untreated cows calving spontaneously at normal term (group NT, $n=4$) and cows on which elective cesarean sections were performed on day 272 for the collection of placental tissue in order to characterize placental morphology and gene expression prior to the occurrence of the final signals inducing parturition (group D272, $n=3$). Parts of this study with data from D272, AG, and NT cows have been published previously (Shenavai *et al*. 2010). In order to facilitate a comparison, clinical observations and data on maternal steroid profiles and placental morphology from these three groups are also reviewed in this paper.

Results

Clinical observations

Gestational length in the AG (272.4±0.3 days), GC (271.7±0.3 days), and PG treatment groups (271.7 ±0.2 days) was not different but significantly shorter ($P<0.001$) compared with spontaneously calving cows (280.5±1.3 days). Other clinical observations are summarized in Table 1. All cows of the GC, PG, and NT groups calved spontaneously, whereas in all cows of the AG group, parturition – despite a completely open cervix – had to be finished by extraction of the calves due to an apparently insufficient myometrial activity and an impaired softening of the caudal birth canal as previously reported (Shenavai *et al*. 2010). Calves from D272 cows and AG cows exhibited a significant delay in standing and suckling (impaired neonatal vitality), which was not observed with the newborns from the other groups. All cows from the D272 and AG groups retained their placenta for more than 1 week *postpartum* (Shenavai *et al*. 2010), the incidence of retained fetal membrane (RFM) was 1/4 in GC and 3/4 in PG cows; only cows with normal shedding of the placenta were assigned to group NT.

Histomorphological observations on prepartal tissue remodeling in placentomes

The mean percentage of trophoblast giant cells (TGC) related to the total number of trophoblast cells (Table 1) was virtually identical in the three treatment groups (AG, GC, PG; 20.1–20.4%) and was not statistically different from the late pregnant control animals (D272, 22.1%). However, NT animals showed the characteristic prepartal decrease in TGC numbers (9.8%; $P<0.05$). The condition of the caruncular crypt epithelium – measured as the ratio (area) of the caruncular epithelium related to the total area encircled by its basal membrane (Table 1) – was not statistically different in D272 (31.5±1.4%), AG (30.5 ±3.3%), and GC animals (29.6±2.7%). Again, NT animals showed the characteristic prepartal reduction of the caruncular epithelium to 21.1±6.1% ($P<0.05$). The intermediate ratio in PG animals of 24.3±3.3% was not statistically different from D272, AG, and GC animals on the one hand and from NT animals on the other.

### Effect of PR blockage on hormone profiles (group AG)

Following two aglepristone applications 24 h apart on days 270 and 271, a substantial decline in maternal progesterone levels occurred immediately prior to or at the time when the calves were extracted, which was 48.5±7.3 h after the start of treatment (Fig. 2 and Table 1). In two animals a slight increase in PGFM levels was observed from the basal level (<0.5 ng/ml) to values of 1.7–2.7 ng/ml between the onset of the treatment and the time when signs of impending parturition became obvious (Fig. 2). During extraction of the calf a short transient PGFM peak occurred, followed by a massive increase in PGFM levels starting 8–12 h later and reaching peak levels of 12–17 ng/ml between 15 and 25 h after birth. A similar profile was observed in the third animal, albeit on a lower level. The prepartal levels of free and sulfonated estrone were virtually maintained for another 36–48 h after birth (Fig. 3), which was clearly longer as compared with GC and PG animals (Fig. 3).

Table 1 Calving traits and placental morphology in cows with induced parturition on days 271–272 (group AG: aglepristone; group GC: dexamethasone; group PG: cloprostenol) and in cows calving spontaneously at normal term (d280.5±1.3; group NT). To characterize the situation immediately prior to spontaneous initiation of parturition, tissue samples were collected from day 272 pregnant animals during elective cesarean section (group D272).

Parameter	D272[a] ($n=3$)	AG[a] ($n=3$)	GC ($n=4$)	PG ($n=4$)	NT[a] ($n=4$)	p_G
	Experimental group					
Time between onset of treatment – parturition (h)	–	48.5±7.3	40.6±6.8	40.7±5.9	–	0.2687
Dystocia due to insufficient myometrial activity	–	3/3	0/4	0/4	0/4	–
Impaired neonatal vitality	3/3	3/3	0/4	0/4	0/4	–
Incidence of retained fetal membrane (≥12 h)	3/3	3/3	1/4	3/4	0/4	–
%TGC related to the total number of trophoblast cells	22.1±4.8*	20.1±1.4*	20.3±3.3*	20.4±2.2*	9.8±3.9[†]	0.0012
Ratio (area) of the caruncular epithelium/caruncular crypt (in %)	31.5±1.4*	30.5±3.3*	29.6±2.7*	24.3±3.3*,[†]	21.1±6.1[†]	0.0114

p_G, error probability for an effect of the experimental group (one-factorial ANOVA). Groups with different superscripts differ with $P<0.05$.
[a]Data are included from a previous study (Shenavai *et al*. 2010) for statistical evaluation and discussion.
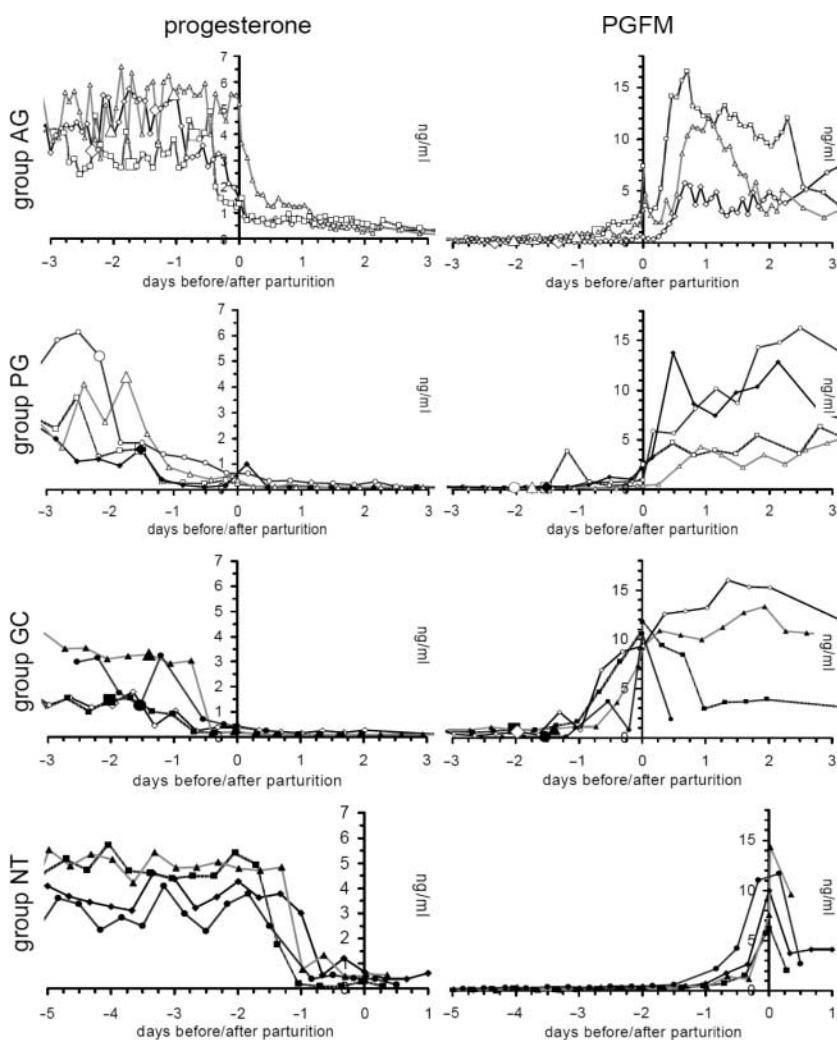
Figure 2 Concentrations of progesterone and 15-keto-13,14-dihydro $PGF_{2\alpha}$ (PGFM) in cows before and after induced parturition on days 271–272 (group AG: aglepristone, group PG: cloprostenol, group GC: dexamethasone) and in spontaneously calving cows at normal term (day 280.5 ± 1.3, group NT). Large symbols indicate the time of the treatment, open symbols are used for animals that retained the placenta for more than 12 h, closed symbols denote animals with timely release of the placenta. Progesterone concentrations in AG and NT animals were previously published (Shenavai *et al.* 2010) and are included for comparison.

Effect of prostaglandin treatment on hormone profiles (group PG)

Application of cloprostenol resulted in an immediate and substantial decrease of progesterone, indicating a rapid induction of luteolysis (Fig. 2), followed by a lag phase of 30–40 h until parturition in which basal or slightly suprabasal progesterone concentrations were measured. Initially, PGFM concentrations remained on a basal level (<0.5 ng/ml) in three animals; in one animal a transient increase up to around 4 ng/ml was observed. During the last 24 h preceding parturition, a weak increase in PGFM concentrations occurred from 0.54 ± 0.31 to 1.51 ± 1.01 ng/ml at the time of parturition. Values started to increase substantially immediately after expulsion of the calves and remained elevated until the end of the observational period (Fig. 2). Estrone concentrations increased slightly between the time of treatment and the immediate peripartal period from 1.11 ± 0.31 to 1.63 ± 0.54 ng/ml ($P = 0.0410$). Estrone

sulfate concentrations showed no obvious changes between treatment and parturition (Fig. 3).

Effect of glucocorticoid treatment on hormone profiles (group GC)

Following a lag phase of at least 8 h, progesterone concentrations declined from 1.84 ± 0.96 ng/ml at the time of treatment to 0.31 ± 0.12 ng/ml at parturition (Fig. 2). PGFM levels increased substantially from 0.64 ± 0.38 ng/ml at the time of treatment to 10.24 ± 1.22 ng/ml at parturition; onset of this increase was about 8–16 h after dexamethasone application (Fig. 2). Estrone concentrations increased only slightly between treatment and parturition from 1.14 ± 0.24 to 1.71 ± 0.20 ng/ml and declined to values of <0.2 ng/ml within 24 h after parturition. Estrone sulfate concentrations increased or were still high between treatment and parturition in three animals, in the fourth animal a decrease of about 50% was observed. After a rapid initial
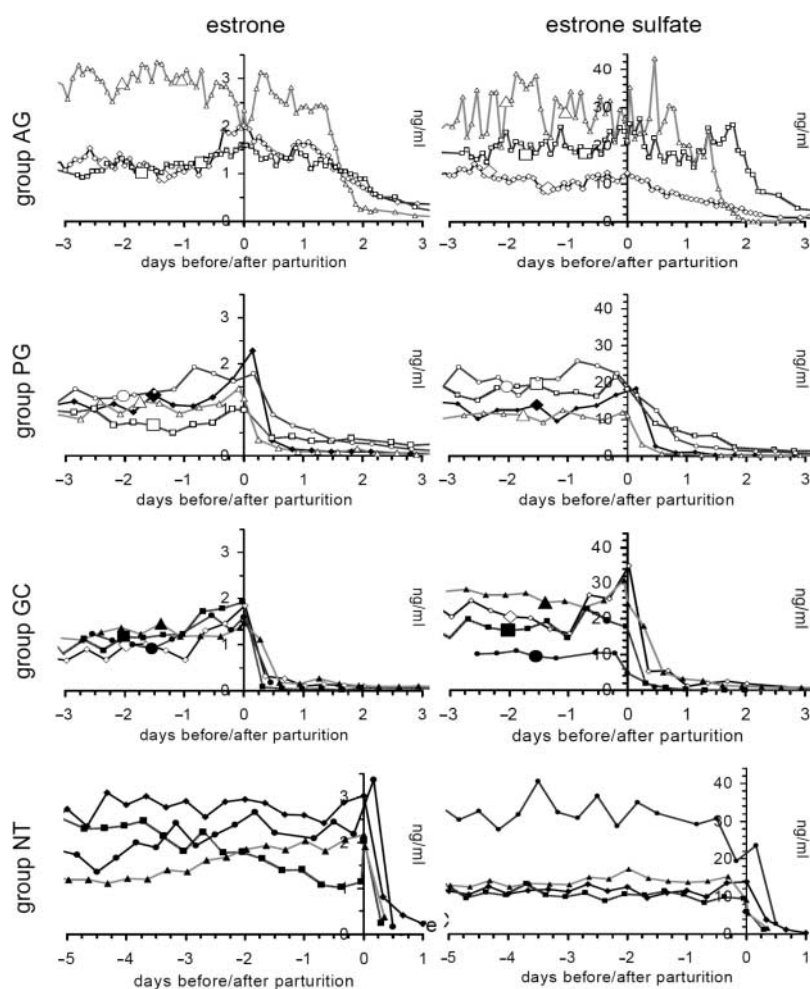
Figure 3 Concentrations of estrone and estrone sulfate in cows before and after induced parturition on days 271–272 (group AG: aglepristone, group PG: cloprostenol, group GC: dexamethasone) and in spontaneously calving cows at normal term (day 280.5 ± 1.3, group NT). Large symbols indicate the time of the treatment, open symbols are used for animals that retained the placenta for more than 12 h, closed symbols denote animals with timely release of the placenta. Estrogen concentrations in AG and NT animals were previously published (Shenavai *et al.* 2010) and are included for comparison.

decline during the first 12 h after parturition, a more gradual decrease to basal level (<0.2 ng/ml) occurred during the following 1–3 days (Fig. 3).

Effect of treatments on the placental expression of mRNA specific for genes related to steroidogenesis, for COX2 and for PR and glucocorticoid receptor

Results from the measurements of relative expression of target gene-specific mRNA in cotyledons or caruncles by real-time RT-PCR are presented in Table 2. One-factorial ANOVA for an influence of the experimental group yielded significant error probabilities for the expression of STAR ($P=0.0069$), 3β-hydroxysteroid dehydrogenase (HSD3B1; $P=0.0374$), CYP17 ($P=0.003$), and COX2 ($P=0.0344$) in cotyledons and for PR and glucocorticoid receptor (GR) expression in caruncles ($P=0.0165$ and 0.0490 respectively). For the expression of CYP19 and GR in cotyledons, no statistically significant influence of the experimental group could be detected.

The pair-wise comparison of experimental groups showed that relative gene expression (RGE) levels for *STAR* mRNA were higher ($P<0.05$) in D272 compared with AG or GC animals. Expression in groups PG and NT

was intermediate and not statistically different from the above-mentioned groups. Expression of *HSD3B1* mRNA was lowest in group AG and significantly different from group D272. *CYP17A1* mRNA expression was significantly higher in GC and NT animals compared with the other experimental groups. For *PTGS2* mean expression levels in GC and NT animals were also higher compared with D272, AG, and PG animals. However, in pair-wise comparisons of groups no significant *P* values were obtained.

Caruncular PR expression was significantly higher in group NT compared with PG animals. Expression in groups D272, AG, and GC was intermediate and not significantly different from PG animals on the one hand and NT animals on the other. Caruncular *NR3C1* mRNA expression was highest in group PG but no significant *P* values were obtained from pair-wise comparisons.

Observations on the expression of PTGS2, CYP17A1, PGR, and NR3C1 in bovine placentomes on the protein level

Immunostaining pattern for COX2 in placentomes was as previously described in detail (Schuler *et al.* 2006b) with UTC being the only positive cell type apart from

Table 2 Expression of genes related to steroidogenesis (STAR, HSD3B1, CYP17, CYP19), COX2, and the progesterone receptor (PR) and glucocorticoid receptor (GR) in placentomes from late pregnant cows (group: D272) and from cows after induced (group AG: antigestagen, group GC: dexamethasone, group PG: cloprostenol) or spontaneous parturition (group NT).

Gene	Parameter	Tissue/cell type	Experimental group					p_G
			D272 ($n=3$)	AG ($n=3$)	GC ($n=4$)	PG ($n=4$)	NT ($n=4$)	
STAR	RGE	Cotyledon	8.5 (1.6)*	1.7 (1.6)[†]	2.7 (1.4)[†]	5.4 (2.2)*,[†]	3.0 (1.7)*,[†]	0.0069
HSD3B1	RGE	Cotyledon	5.1 (1.9)*	1.5 (1.6)[†]	5.0 (1.9)*,[†]	3.8 (1.8)*,[†]	6.0 (1.4)*,[†]	0.0374
CYP17A1	RGE	Cotyledon	3.5 (3.7)*	2.6 (2.4)*	42.6 (2.0)[†]	4.6 (1.7)*	37.4 (1.3)[†]	0.0003
CYP19A1	RGE	Cotyledon	10.0 (2.7)	2.5 (2.6)	6.4 (2.1)	7.6 (1.8)	8.1 (1.2)	0.1742
PTGS2	RGE	Cotyledon	1.9 (2.5)	1.7 (1.7)	6.7 (3.0)	4.0 (2.0)	9.5 (1.2)	0.0344
PGR	RGE	Caruncle	1.2 (2.1)*,[†]	1.0 (1.5)*,[†]	1.4 (1.4)*,[†]	0.7 (1.9)*	2.9 (1.3)[†]	0.0165
	% Positive cells	Caruncular stroma	70.4±8.5*	64.4±1.7*,[†]	62.5±5.6*,[†]	50.8±10.4[†]	67.7±8.6*,[†]	0.0376
NR3C1	RGE	Cotyledon	4.1 (1.2)	2.3 (2.0)	7.3 (2.8)	4.1 (2.1)	5.1 (1.1)	0.3026
	RGE	Caruncle	7.2 (1.8)	8.0 (1.2)	15.4 (1.4)	23.8 (2.2)	4.9 (3.0)	0.0490
	IRS	Trophoblast giant cells	3.1±1.3	2.3±0.4	2.3±0.8	1.7±0.3	ND	0.1933
	IRS	Caruncular epithelium	3.3±1.7	4.1±0.6	2.1±1.1	2.5±0.7	ND	0.1395

p_G, error probability for an effect of the experimental group (one-factorial ANOVA). RGE, relative gene expression assessed by real-time RT-PCR (geometric mean and deviation factor); IRS, immunoreactive score from evaluation of immunostained tissue sections (mean±S.D.). ND, not determined, as the mode of quantification applied does not yield meaningful results in placentomes of term cows (see text). Groups with different superscripts differ with $P<0.05$.

sporadic signals in the vascular system. In animals of the NT group, a ubiquitous strong to intense signal was found in UTC of the chorionic plate and the chorionic villous tree (Fig. 4A). In D272 animals, immunostaining was generally weaker with a gradient from the chorionic plate to the tips of the villous trees. Staining patterns in placentomes from AG and PG animals were similar to D272 animals. Mean staining intensity in placentomes from GC animals was higher compared with D272, AG, and PG but clearly lower than in term animals, with a gradient in staining intensity from the chorionic plate to the tips of the chorionic villi. Two-factorial ANOVA of immunoreactive score (IRS) values yielded a significant effect for the group ($P=0.0014$) and the localization within the placentome ($P<0.0001$). Moreover, a significant interaction group×localization was found ($P=0.0207$).

Immunostaining for CYP17 was restricted to UTC of chorionic villi as previously described (Schuler *et al.* 2006*a*). Results from semiquantitative evaluation of immunostaining yielded a similar pattern as was observed for COX2 with an intermediary CYP17 expression in placentomes of GC animals (Fig. 4B). IRS values showed a significant effect for the group ($P=0.0001$) and localization within the placentome ($P=0.0025$), whereas the interaction group×localization was not significant.

As previously described (Schuler *et al.* 1999), PR were immunolocalized solely in cells of the caruncular stroma. There was a significant effect for the group ($P=0.0376$) and subsequent pair-wise comparisons showed that the percentage of PR-positive cells in caruncles was significantly ($P=0.05$) lower in the PG group compared with D272 controls (Table 2).

Immunostaining for GR was predominantly found in the nuclei of caruncular epithelial cells and of mature and invasive TGC confirming earlier observations by Boos *et al.* (2000). Consistent with observations by these authors, immunostaining of weaker intensity was also found in a proportion of stromal cells of chorionic villi and in the cotyledonary and caruncular vascular systems. Statistical evaluation did not consider NT animals as in term placentomes the few remaining TGC are inhomogeneously distributed and the identification of caruncular epithelial cells is dubious due to significant prepartal tissue remodelling; there was no group effect (Table 2).

Observations on pre- and intrapartum leucocyte invasion in placentomes

Leucocytes in bovine lymphatic tissue used as a positive control showed a distinct staining of cytoplasm for the pan-leucocyte marker CD45, mostly concomitant with a pronounced staining of the cellular membrane (Fig. 5A). No staining was found in negative controls, in which the primary antibody was replaced with an isotype-specific control antibody (Fig. 5B). In placentomal tissue, distinctly CD45-positive cells were regularly observed intralumenally in blood vessels (Fig. 5C and D), especially of major caruncular septa and of the caruncular stalk. However, irrespective of the experimental group, in the extravascular compartment of placentomes they only occurred very sporadically. Thus, a quantitative assessment of leucocyte invasion was considered inappropriate.
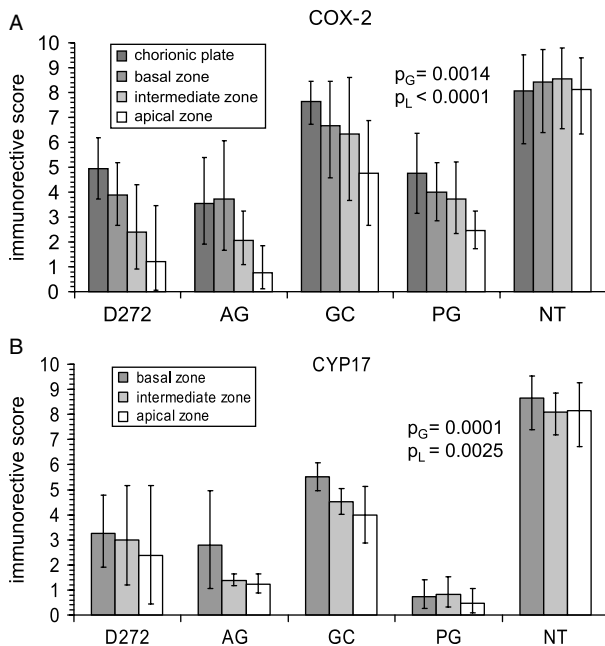
Figure 4 Expression of (A) cyclooxygenase 2 (*PTGS2*) and (B) 17α-hydroxylase-C17,20-lyase (*CYP17A1*) in placentomes from cows at induced parturition on days 271–272 (group AG: aglepristone, group PG: cloprostenol, group GC: dexamethasone), from day 272 pregnant animals and from cows at spontaneous parturition (group NT). IRS as a median±deviation computed after preceding arcsine transformation; p_G, p_L: error probabilities for an influence of the experimental group and of the localization within the placentome.

Discussion

The effects observed after induction of parturition with glucocorticoids provide clear evidence that the prepartal rise in fetal cortisol upregulates the expression of *CYP17A1* and *PTGS2* in bovine UTC. The substantial upregulation of *PTGS2* in UTC supports the concept that these cells are the primary source of prostaglandins in prepartal cows including $PGF_{2\alpha}$. This conclusion is substantiated by earlier observations that have shown that UTC also express aldoketoreductase 1B5 (AKR1B5), an enzyme with a significant PGF synthase activity (Madore *et al.* 2003, Schuler *et al.* 2006*b*) and that *PTGS2* expression in the bovine uterus around the time of spontaneous prepartal luteolysis is only low in comparison with the expression in the cotyledons (Fuchs *et al.* 1999, Wehbrink *et al.* 2008). The contribution of the placenta to systemic maternal PGFM concentrations is further supported by observations in D272 cows, in which – albeit on a low level – a decrease in maternal PGFM concentrations was observed after disruption of the fetoplacental circulation following the surgical removal of the fetus (Fig. 6).

Among the treatments applied, only glucocorticoid application stimulated a detectable upregulation of *PTGS2* in placentomes, and 8–16 h after treatment a

rapid increase in PGFM levels was observed, reaching similar values around the expulsion of the fetus as found in NT animals (Fig. 2). Consistent with earlier observations (Fairclough *et al.* 1975, Edqvist *et al.* 1978, Bosu *et al.* 1984, Meyer *et al.* 1989, Königsson *et al.* 2001) maternal PGFM levels were still basal or only slightly elevated around the time of onset of prepartal luteolysis. However, this observation does not necessarily contravene a role of $PGF_{2\alpha}$ of cotyledonary origin as the luteolytic signal in prepartal cows, as maternal PGFM levels in peripheral blood might not closely reflect the local availability in the ovaries as has also been discussed for luteolysis in cyclic animals (Krzymowski & Stefańczyk-Krzymowska 2008).

The considerable lag phase between glucocorticoid treatment and the time when progesterone started to decline suggests that the stimulatory effect of dexamethasone on placental *PTGS2* expression and consequently $PGF_{2\alpha}$ secretion was mediated by genomic mechanisms via GR. However, as GR immunostaining was virtually absent in UTC, the prepartal upregulation of *PTGS2* as well as the upregulation of *CYP17A1* in these cells is probably mediated by paracrine mechanisms via receptors expressed in the neighboring GR-positive cells, which are the TGC, the cotyledonary stroma, or even the caruncular epithelial cells (Boos *et al.* 2000).

PGFM profiles in AG animals in connection with the absence of a detectable upregulation of placental *PTGS2* mRNA or protein expression provide convincing evidence that functional progesterone withdrawal subsequent to PR blockage allows the release of $PGF_{2\alpha}$ from an extraplacental source. The concept that progesterone withdrawal leads to extraplacental prostaglandin synthesis in periparturient cows is further corroborated by observations in the PG group and particularly in D272 control cows, in which after surgical removal of the offspring an increase in PGFM levels was only observed in conjunction with the final stages of spontaneous or cloprostenol-induced luteolysis (Fig. 6). However, different from PG animals, in which a distinct lag phase was observed between progesterone withdrawal and the massive increase in maternal PGFM levels, luteolysis in D272 cows was immediately followed by a pronounced increase in PGFM levels. These observations may indicate that the extraplacental prostaglandin source may initially have been attenuated by the presence of suppressive factors provided by the intact fetoplacental circulation or the absence of additional stimulatory factors. There is strong evidence that the uterus is the source of this *postpartum* increase, as *PTGS2* expression in the uterus of late pregnant cows is only low until the expulsion of the calf, after which a rapid and significant upregulation has been shown to occur specifically in the intercaruncular endometrium (Fuchs *et al.* 1999, Wehbrink *et al.* 2008). Our study suggests that withdrawal of progesterone is a key event
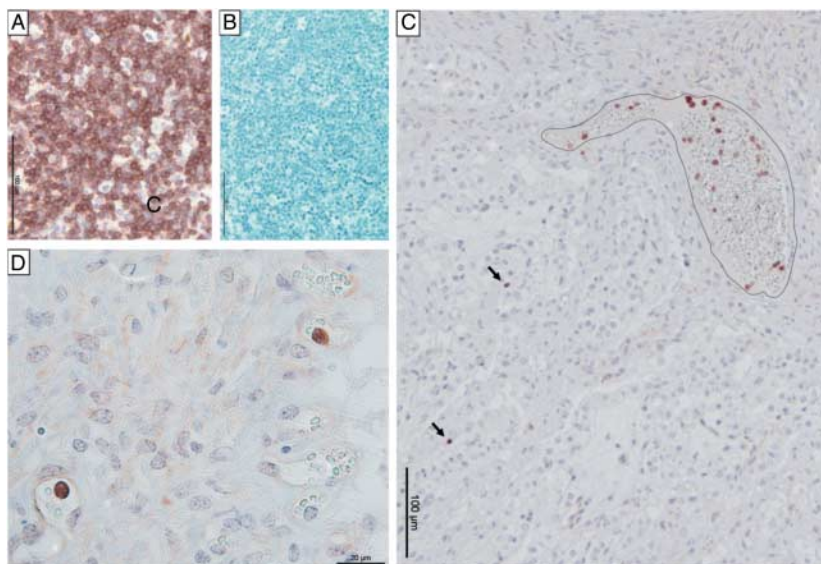
Figure 5 Identification of leucocytes in placentomes by immunostaining for the pan-leucocyte marker CD45. (A and B) Results from control experiments using bovine lymphatic tissue. In (B), the specific primary antibody was replaced by an irrelevant isotypic murine mAb. (C) Placentome of a parturient cow. Positive staining in leucocytes situated in a blood vessel of a major caruncular septum (surrounded by dotted line). Positive reactions in the adjacent tissue formed by interdigitating tertiary chorionic villi and maternal crypts only occur sporadically (arrows). (D) Leucocytes in caruncular capillaries.

allowing this endometrial prostaglandin production. However, as previously assumed, the release of oxytocin and the trauma set to the endometrium during expulsion of the fetus may contribute to the upregulation of endometrial *PTGS2* expression in the immediate post-partal period (Fuchs *et al*. 1999, Wehbrink *et al*. 2008). High postpartal PGFM levels have been previously described in cases of RFM after glucocorticoid-induced parturition, whereas in non-RFM animals a rapid decline occurred after fetal expulsion (Königsson *et al*. 2001). In our study continuously high postpartal PGFM levels around 10 ng/ml were also found in individual PG – or GC – non-RFM cows, suggesting that not the retained placenta *per se* but the associated inflammatory processes stimulate endometrial PGFM production. Thus, our study has shown that UTC are the major source of prostaglandins in prepartal cows, their production rapidly is ceasing after breakdown of the fetoplacental circulation and with the prostaglandin of endometrial origin increasing gradually after pro-gesterone withdrawal. In the case of undisturbed parturition they may soon return to a relatively low level or may be further stimulated and remain at high concentrations in the case of severe intrauterine inflammation.

Many studies have shown that, except for the occurrence of RFM, prostaglandin-induced parturition can generally be considered as normal despite the low PGFM levels at the time of expulsion observed in this study and by previous investigators (Kornmatitsuk *et al*. 2000, Königsson *et al*. 2001). In contrast cows of the AG group with similarly low PGFM levels showed sub-stantial dystocia. This observation is difficult to assess as progesterone concentrations were at 2.67 ± 1.77 ng/ml in AG cows but significantly lower (0.47 ± 0.17 ng/ml) in PG cows. It is unclear, whether in spite of blocking the PR, the still circulating progesterone in AG cows might have exerted some suppressive activity on myometrial excitability, possibly via nongenomic actions (Stormshak & Bishop 2008). At present, a clear explanation for the underlying mechanisms also cannot be given as to why calves from AG cows needed significant care to adapt to extrauterine life similar to calves obtained by elective cesarean section on day 272 (Shenavai *et al*. 2010), while calves born after induction of parturition with cloprostenol were vital after birth, consistent with earlier observations (Zerobin *et al*. 1973).

One major aim of this study was to gain further information on the role of placental progesterone. As no method was available to selectively block the synthesis or effects of placental progesterone in late pregnant cows, it was hypothesized that the comparison of effects after PG and AG treatment – selective withdrawal of luteal progesterone vs blockage of PR – could yield relevant information. However, no significant difference was found between the two groups with regard to placental histomorphology, placental gene expression, leucocyte infiltration, and the incidence of RFM. These observations are inconsistent with our previous concept that a withdrawal of the high local progesterone concentrations predominantly of placental origin trig-gers important mechanisms leading to placental release.

Despite a clear upregulation of *CYP17A1* in GC and in NT animals, no substantial difference concerning prepartal estrogen profiles could be detected when comparing these two groups with the AG- and PG-treated animals exhibiting only basal *CYP17A1* expression similar to the untreated D272 control group. All cows with spontaneous or induced parturition exhibited – if at all – only moderate increases of estrogens during the last week of gestation. This is clearly different from sheep, where an increased expression of CYP17 results in a distinct increase of free and sulfonated estrogens during the last few hours
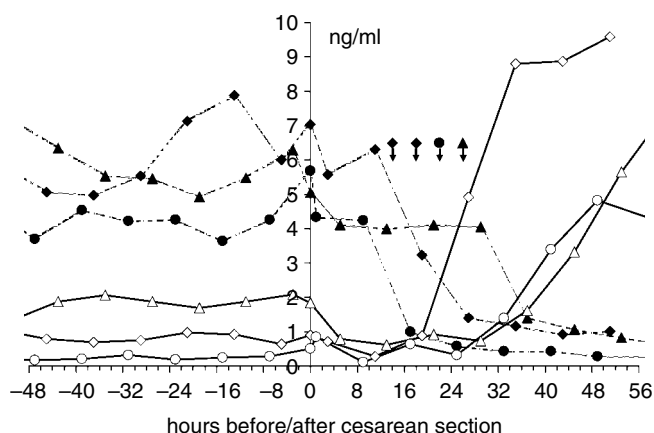
Figure 6 Concentrations of 15-keto-13,14-dihydro PGF$_{2\alpha}$ (PGFM; open symbols) in three late gestational control cows (group D272) around surgical removal of the fetus by cesarean section on day 272 of gestation. Arrows indicate the time when cloprostenol was applied to the individual animals intended to facilitate luteolysis after surgery. Concentrations of progesterone (closed symbols) published in a previous paper (Shenavai *et al*. 2010) were included for discussion. All three animals exhibited placental retention until day 9 *postpartum*.

prior to parturition (Challis 1971, Tsang 1974). We cannot explain our observation but hypothesize that in the prepartal cow an increase in placental estrogen production following *CYP17A1* upregulation is either masked by metabolism or excretion or – alternatively – that the prepartal upregulation of placental *CYP17A1* may predominantly lead to the increased conversion of placental progesterone to its inactive 17α-hydroxylated metabolite, of which a further conversion on the Δ4-pathway to C19-steroids and estrogens in ruminants is very inefficient (Schuler *et al*. 1994, Conley & Bird 1997). Thus, in the cow the prepartal upregulation of *CYP17A1* may serve the metabolization of placental progesterone rather than the production of estrogens. This assumption needs further confirmation but is not consistent with our previous *in vitro* results on placental steroid metabolism, suggesting that also in the bovine placenta a pronounced prepartal switch of placental steroidogenesis from progesterone to estrogens occurs (Schuler *et al*. 1994). However, different from placental homogenates, under *in vivo* conditions in the bovine trophoblast, steroidogenic enzymes are clearly compart-mentalized at a cellular level, possibly effecting a different substrate flow under *in vivo* conditions (Schuler *et al*. 2008).

The application of glucocorticoids induced – as far as investigated – at least qualitatively all the endocrine changes observed around normal parturition. However, in this group the prepartal remodeling of placentomal histomorphology, so far considered as a prerequisite for the timely release of the placenta, was also not detectable to any significant extent. In particular and in contrast to sheep (Ward *et al*. 2002, Braun *et al*. 2007), treatment with dexamethasone had no effect on

the number of TGC. Yet, three of four GC animals subsequently timely released the placenta, indicating that the processes leading to placental release had been sufficiently induced in these animals.

Another concept forwarded is that placental release may be an immune-mediated event, and the hypothesis was developed that at the end of gestation the placenta becomes a foreign body due to the cessation of blood supply (Gunnink 1984*a*, 1984*b*) or the expression of paternal antigens (Joosten *et al*. 1991, Joosten & Hensen 1992, Davies *et al*. 2000, 2004). Correspondingly, it has been reported that MHC class I compatibility between dam and calf increases the risk of RFM (Joosten *et al*. 1991). Moreover, chemotactic activity in placentomes was significantly higher in cows with timely release of the placenta compared with cows with RFM (Gunnink 1984*a*, Heuwieser *et al*. 1986, Heuwieser & Grunert 1987, Benedictus *et al*. 2011) and cows developing RFM exhibited impaired peripartal neutrophil functions (Gunnink 1984*b*, Kimura *et al*. 2002). Consistently, Heuwieser *et al*. (1986) found a significantly higher leucocyte infiltration immediately after expulsion of the calf in placentomal tissue of cows with timely release of the placenta compared with cows with RFM. In contrast, in our study using immunohistochemical detection of the pan-leucocyte marker CD45, irrespective of the experi-mental group and the presence or absence of RFM, no noteworthy leucocyte infiltration was observed in any of the placentomes investigated, challenging the concept of feto-maternal separation as an immune cell mediated event. However, a role of 'inflammatory cytokines' in the control of placental release cannot be excluded from this observation particularly since a transcriptome study of bovine placentomes ante- and intrapartum revealed an upregulation of the genes related to innate immune response at parturition (Streyl *et al*. 2012).

In conclusion, the results of this study are broadly in line with our concept of the initiation of parturition in cows. However, many questions still remain open on parturition-related processes in the cow, especially concerning the functions of bovine placental steroids and the mechanisms immediately controlling placental release.

Materials and Methods

Animals, treatments, and sample collection

All cows were of the Holstein-Friesian breed with registered dates of artificial insemination and exhibited an undisturbed singleton pregnancy. Cows of the aglepristone treatment group (group AG; *n* = 3) received 3 g antiprogestin (∼ 5 mg/kg of body weight s.c.; kindly provided by Virbac Tierarzneimittel GmbH, Bad Oldesloe, Germany) on days 270 and 271 as previously described (Shenavai *et al*. 2010). Animals of the glucocorticoid (group GC; *n* = 4) and prostaglandin (group PG; *n* = 4) treatment groups were given 25 mg dexamethasone-21-undecanoate (Devamed; Topkim, Istanbul, Turkey) or

150 µg D-cloprostenol (Dalmazin; Selectavet Dr Otto Fischer GmbH, Weyarn-Holzolling, Germany) i.m. on day 270. For comparison with the situation at normal term, a group of four spontaneously calving cows on day d280.5±1.3 (NT) with a timely release of the placenta within 12 h after expulsion of a healthy calf was also included in the present study. Immediately after the birth of the calves three to five placentomes were removed per vaginam. In order to characterize the situation immediately prior to the occurrence of the final parturition inducing signals, placental tissue was collected from three untreated cows during elective cesarean sections on day 272 (group D272), when induced parturition was expected to occur in animals of the three treatment groups.

Blood samples were taken from the jugular vein. Sampling in AG animals was at 2 h intervals between days 269 and 274 and at 8 h intervals on days 268 and 275. In groups D272, PG, and GC animals, blood sampling was at 8 h intervals from day 268 until day 275, with an incomplete set of samples in one GC animal. Animals of the term group were sampled at 8 h intervals from day 271 after insemination until the day after parturition.

The animal experiments were approved by the local ethical committees on the use of animals for research purposes (Regierungspraesidium Giessen, No. V54-19c-20-15(I) Gi 18/14-No. 41/2007; LAVES, 33.9-42502-04-09/1634; Ethical Committee of Uludag University of Veterinary Medicine, No. 401/1510-26.03.2007).

Hormone measurements

Measurements of progesterone, estrone, and estrone sulfate were performed as previously described (Shenavai *et al*. 2010) by well-established in-house RIA. For PGFM measurements the PGFM EIA kit (No. 516671; Cayman Chemical Company, Ann Arbor, MI, USA) was used basically following the instructions of the supplier. However, as the extraction procedure recommended by the supplier for the use in plasma samples in our case yielded erroneous results, direct measurements were performed and a reduction of the sample volume to 10 µl was applied to overcome the potential matrix effects. In order to evaluate the assay for the use in bovine plasma, a dilution series was prepared from the plasma of a parturient cow (high PGFM content) in bull plasma. The concentrations measured paralleled the standard curve exactly. Interassay coefficients of variation (CV) were 22.4, 8.9, and 17.4% at a relative binding (B/B_0) of 80, 35, and 10% ($n=9$) respectively. Intraassay CV values were 19.6% ($n=10$) at 80% B/B_0 and 7.3% at 10% B/B_0.

Investigations into placentomal histomorphology

The prepartal decrease in TGC numbers and the reduction of the caruncular epithelium were used as markers of morphological placental maturation. In order to identify TGC in advanced stages of differentiation, the ability of *Phaseolus vulgaris* leucoagglutinin (PHA-L) to specifically bind to sugar moieties of glycoproteins produced by differentiating and mature TGC was used. Lectin histochemistry was performed on sections from formalin-fixed, paraffin-embedded placentomal tissue as previously described (Shenavai *et al*. 2010).

In order to assess the percentage of mature TGC, the total number of trophoblast cells and the number of distinctly PHA-L-positive trophoblast cells were registered in one section from each animal by viewing 20 chorionic villi randomly distributed over the complete height of the interdigitation zone.

Evaluation of caruncular epithelium reduction was performed as previously described (Shenavai *et al*. 2010) using sections prepared from formalin-fixed tissue stained with hematoxylin–eosin. Maternal crypts surrounding nine cross-sectioned chorionic villi of a predefined size range randomly distributed over the complete height of the interdigitation zone were evaluated in one section from each animal. The area corresponding to the caruncular epithelium surrounding each of the villi was measured using the IM1000 software (Leica, Bensheim, Germany) and expressed as a percentage of the area encircled by the basal membrane of the caruncular epithelium. Finally, for each animal the mean percentage was calculated.

Immunohistochemical detection of COX2, CYP17, PR, GR, and the pan-leucocyte marker CD45 and evaluation of immunostaining

Sections prepared from formalin-fixed paraffin-embedded placentomal tissue samples were immunostained following an indirect immunoperoxidase standard procedure including microwave irradiation for unmasking epitopes and the streptavidin–biotin technique for signal enhancement as previously described (Schuler *et al*. 1999, 2006*a*, 2006*b*). Primary antibodies used were the following: a rabbit mAb against a synthetic peptide from the C-terminus of rat COX2 (Thermo Fisher Scientific, Fremont, CA, USA), a polyclonal rabbit antiserum against recombinant bovine CYP17 (gift from Prof. A Conley, UC Davis; reference: Peterson *et al*. 2001), a monoclonal murine antibody against the highly conserved C-terminus of PR (clone 10A9; Immunotech, Hamburg, Germany), a polyclonal rabbit IgG antibody against amino acids 346–367 of human glucocorticoid receptor (PA1-511A; Affinity BioReagents, Golden, CO, USA), and a murine mAb against bovine CD45 (MCA832S; AbD Serotec, Düsseldorf, Germany). In negative controls, the primary antibodies were replaced with isotypic nonspecific immunoglobulin or non-immunized rabbit serum respectively.

For semiquantitative assessment of immunostaining for each animal, one section from one placentome was evaluated using different procedures dependent on the respective staining pattern. For quantification of PR expression, the percentage of PR-positive cells in the caruncular stroma was determined as previously described (Schuler *et al*. 1999) based on the evaluation of 500 cells. For COX2 and CYP17, cytoplasmic staining in UTC was evaluated using an IRS taking into account the proportions of negatively, weakly, moderately, intensely staining cells as previously described (Khatri *et al*. 2011), which may range from 0 (all cells under investigation negative) to 10 (all cells under investigation intensely positive). To test for an influence of the localization, the interdigitation zone of the placentomes formed by chorionic villi situated in maternal crypts was subdivided into three zones of equal width (basal, i.e. close to the chorionic plate; intermediary; and apical, i.e.

close to the caruncular stalk), which were evaluated separately. Additionally, for COX2 immunostaining in the chorionic plate was analyzed. Based on the evaluation of nuclear signals in five cross sections of chorionic villi surrounded by the corresponding caruncular crypt, the IRS was also used for the quantification of *NR3C1* expression in TGC and caruncular epithelial cells respectively without consideration of the localization within the interdigitation zone.

Measurement of relative target gene mRNA levels by real-time RT-PCR (SYBR Green method)

Coarse pieces of deep-frozen ($-80\,°C$) cotyledons or caruncles were broken up using a clean hammer. The tissue fragments were quickly placed in a prechilled mortar and pulverized using a pestle under liquid nitrogen. Total RNA was isolated from 100 mg of tissue powder using TRIzol reagent (Invitrogen) and treated with DNase (Roche Diagnostics GmbH) for the elimination of genomic DNA. Seventy-four nanograms of total RNA were then reverse transcribed with a random primer, dNTP mixture, $MgCl_2$, RNase inhibitor, and reverse transcriptase (GeneAmp RNA PCR Kit; Perkin Elmer, Foster City, CA, USA). Real-time PCR was performed in a CFX 96 C1000 thermocycler (Bio-Rad Laboratories, Inc.) using the SYBR Green method (Absolute Blue QPCR SYBR; Thermo Fisher Scientific, Epsom, UK). Each sample was measured in triplicate. Primer pairs for the target genes *PTGS2*, *CYP17A1*, *CYP19A1*, *PGR*, *NR3C1*, and the reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were adopted from previous publications (see Table 3) or designed using Oligo Explorer (Version 1.1) and Oligo Analyzer (Version 1.1) software (Teemu Kuulasmaa, University of Kuopio, Finland; freeware from the Internet) and purchased from Eurogentec (Cologne, Germany). Amplification conditions were the same for the target and the reference genes: initial denaturation for 3 min at $95\,°C$ followed by 40 cycles at $95\,°C$ for 10 s and $60\,°C$ for 60 s. After completion of the amplification a melting curve of the PCR product was generated to test for the occurrence of nonspecific products and primer dimers. Further evidence for the specificity of the products was obtained from conventional RT-PCR and subsequent analysis of the amplicons using agarose gel electrophoresis, in which only one band respectively of the expected size was obtained. In order to further validate the real-time RT-PCR method, a standard curve was generated by serial dilutions of the cDNA according to the standard protocol provided by the supplier of thermocycler (Bio-Rad Laboratories, Inc.). The slope of the standard curve was used to calculate the efficiencies of the PCRs, which were between 94 and 99% for the target genes and the two housekeeping genes respectively.

RGE were calculated using the comparative C_T method ($\Delta\Delta C_T$ method) and reported as *n*-fold differences in comparison with the sample with the lowest amount of the respective target gene transcripts (calibrator) after normalizing the samples referring to the housekeeping genes *GAPDH* and β-actin.

Statistical analysis

Data obtained from the evaluation of prepartal tissue remodeling in placentomes and from the quantification of immunostaining for PR and GR were tested for an influence of the experimental group using one-factorial ANOVAs and in the case of significant error probabilities ($P < 0.05$) followed by pair-wise comparison of experimental groups by Tukey–Kramer multiple comparison test (GraphPad Software, Inc., San Diego, CA, USA). The results are presented as an arithmetic mean $\pm$s.d. ($\bar{X}\pm$s.d.).

Data from RT-PCR were analyzed for an influence of the experimental group in the same manner. However, as data from real-time RT-PCR are commonly right skewed, they were transformed logarithmically prior to statistical evaluation to obtain nearly normal distributions, and the results are presented as a geometric mean (X_g) and dispersion factor.

Data from the semiquantitative evaluation of immunostaining for COX2 and CYP17 were tested for an influence of the experimental group and the localization within the placentome

Table 3 Sequences of primer pairs used in real-time RT-PCR (SYBR green method), expected length of amplicons (bp) and sequence information used for primer design or reference.

Gene	Primer sequence ($5' \rightarrow 3'$)	Amplicon size (bp)	Accession no. or reference
GAPDH	Forward: GCGATACTCACTCTTCTACCTTCGA	82	U85042
	Reverse: TCGTACCAGGAAATGAGCTTGAC		
β-Actin	Forward: TATTGCTGCGCTCGTGGTCG	218	NM_173979
	Reverse: TGACGATGCCGTGCTCAATGG		
NR3C1	Forward: GGAATGAGACCAGACGTAAGTTCTC	83	AY238475
	Reverse: GAGCAGACTAGGCAGAGTTTGGA		
PGR	Forward: GAGAGCTCATCAAGGCAATTGG	227	Pfaffl *et al*. (2002)
	Reverse: CACCATCCCTGCCAATATCTTG		
PTGS2	Forward: GCACAAATCTGATGTTTGCATTC	77	Schuler *et al*. (2006*b*)
	Reverse: GGTCCTCGTTCAAAATCTGTCT		
STAR	Forward: GAGAGGCTTTATGAAGAGCTTGTG	115	NM_174189.2
	Reverse: CGTGAGTGATGACCGTGTCTTT		
HSD3B1	Forward: TGTTGGTGGAGGAGAAGGATCTG	208	Nimz *et al*. (2009)
	Reverse: GCATTCCTGACGTCAATGACAGAG		
CYP17A1	Forward: TGATGATTGGACACCACCAGTTG	296	Vanselow *et al*. (2008)
	Reverse: AGAGAGAGAGGCTCGGACAGATC		
CYP19A1	Forward: GGATCGGCAGTGCCTGCAATTACTA	175	Nimz *et al*. (2009)
	Reverse: ATGCCGATGAACTGCAACCCAAGTT		

using two-factorial ANOVAs with repeated measurements for the factor localization (statistical software BMDP2V, Dixon 1993). The IRS values are inherently limited by the borders 0 and 10, and the datasets contained values close to these borders to a substantial extent. Thus, these data were transformed by the arcsine function in order to obtain nearly normal distributions (Sachs 1982) prior to statistical evaluation. According to the mode of data transformation, these results are expressed as re-transformed values of $\bar{X} \pm$ S.D. of the arcsine transformed values (median $\pm$ deviation).

Declaration of interest

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