Quantitative characterization of prostaglandins in the uterus of early pregnant cattle

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Abstract

Prostaglandins (PGs) are important regulators of reproductive processes including early embryonic development. We analyzed the most relevant PG in bovine uteri at different preimplantation pregnancy stages when compared with non-pregnant controls. Additionally, endometrium and trophoblast tissues were examined regarding specific enzymes and receptors involved in PG generation and function. Simmental heifers were artificially inseminated or received seminal plasma only. At days 12, 15, or 18, post-estrus uteri were flushed for PG determination by liquid chromatography–tandem mass spectrometry. Endometrium and trophoblast tissues were sampled for RNA extraction and quantitative real-time PCR analysis. At all days and points of time examined, the concentration of 6-keto PGF_{1α} (stable metabolite of PGI₂) was predominant followed by PGF_{2α} > PGE₂ > PGD₂ ≈ TXB₂ (stable metabolite of TXA₂). At days 15 and 18, PG increased from overall low levels at day 12, with a much more pronounced increase during pregnancy. The PGF_{2α}/PGE₂ ratio was not influenced by status. The highest PG concentration was measured at day 15 with 6-keto PGF_{1α} (6.4 ng/ml) followed by PGF_{2α} (1.1 ng/ml) and PGE₂ (0.3 ng/ml). Minor changes in endometrial PG biosynthesis enzymes occurred due to pregnancy. Trophoblasts revealed high transcript abundance of general and specific PG synthases contributing to uterine PG. As PGI₂ and PGF_{2α} receptors were abundantly expressed by the trophoblast, abundant amounts of PGI₂ and PGF_{2α} in the uterine lumen point towards an essential role of PG for the developing embryo. High amounts of PG other than PGE₂ in the preimplantation uterus may be essential rather than detrimental for successful reproduction.

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Introduction

The uterine histotroph contains signaling molecules that critically account for an intact embryo–maternal communication. Most importantly during preimplantation development, a precise orchestration of all participants of this dialog is essential. Prostaglandins (PG) are local paracrine mediators important for a vast variety of functions. During normal reproductive development, PG are not only involved in ovulation, cycle determination through induction of luteolysis in ruminants, and fertilization, but also they take part in early embryonic development and implantation (Lim *et al.* 1997) thus contributing to the uterine milieu.

PG are generated from arachidonic acid (AA). The complex regulation involves synthesis, transport, as well as catabolism and action through specific receptors (Fortier *et al.* 2008). AA is liberated from membrane phospholipids by the action of phospholipase A₂ (PLA₂) isoenzymes, namely PLA₂G4A and

PLA₂G6. Cyclooxygenases (PTGS1/2) convert AA to PGH₂, the common precursor of all further downstream functional PG comprising prostacyclin (PGI₂), PGs (PGD₂, PGE₂, and PGF_{2α}), and thromboxane A₂ (TXA₂). The particular conversion of each of the substrates is mediated directly by specific enzymes (PTGIS, PTGDS, PTGES, AKR1B1 (PTGFS), and TBXAS1), whereby the progesterone (P₄) inactivating enzyme 20α-hydroxysteroid dehydrogenase has been ascribed the most prominent PGF_{2α} synthesis activity (Madore *et al.* 2003). Next to biosynthesis, local metabolism may take place due to the expression of 15-hydroxyprostaglandin dehydrogenase (HPGD), the first enzyme of inactivation forming 15-ketoprostaglandin metabolites in peripheral tissues such as endometrium (Parent *et al.* 2006). The primary inactivating step comprises the oxidation of the 15-hydroxyl group to a 15-keto group by HPGD. Further inactivation is performed by the reduction at Δ13 double bond by a Δ13-15-keto PG reductase to

the stable metabolites 13,14-dihydro-15-keto PG referred to as PGFM (Tai *et al.* 2002). PGI_2 is chemically unstable, the stable breakdown product 6-keto $\text{PGF}_{1\alpha}$, however, reflects PGI_2 concentrations directly. Following synthesis, PG are transported through and released from the cells predominantly by active transport. Only recently a specific transporter (SLCO2A1 (PGT)) has been described (Banu *et al.* 2003), which is not limited to a specific PG substrate (Arosh *et al.* 2003). PG act through G-protein-coupled membrane receptors, namely PTGIR (PGI_2), PTGDR (PGD_2), PTGER (comprising four subtypes PTGER1–4, whereby PTGE2 is the most prominent form in the bovine uterus (Arosh *et al.* 2003); PGE_2), and AKR1B1 (PTGFR) ($\text{PGF}_{2\alpha}$; Coleman *et al.* 1994). PG can also act via nuclear receptors (Forman *et al.* 1997), namely PGI_2 binds heterodimers of peroxisome proliferator-activated receptors (PPAR) and retinoic X receptors (RXR; Lim *et al.* 1999, Tarrade *et al.* 2001, Cammas *et al.* 2006).

The pulsatile $\text{PGF}_{2\alpha}$ release from bovine endometrium eventually triggering luteolysis and commencing estrus is well described (Kindahl *et al.* 1976, Schams 1987, Wathes & Lamming 1995). During early pregnancy, trophoblast-derived IFNT appears in significant amounts in the ruminant uterus (Roberts *et al.* 2003) increasing dramatically due to embryo elongation (Robinson *et al.* 2006). IFNT is thought to inhibit oxytocin (OXT) mediated stimulation of PG indirectly (Spencer *et al.* 1996) through suppression of estrogen receptor α (ESR1) transcription (Fleming *et al.* 2006), which thus impedes estrogen-induced OXT receptor (OXTR) upregulation (Bazer *et al.* 2009). Finding ESR1 present in the bovine endometrial luminal epithelium at cycle days 12–14 (Robinson *et al.* 2001) might resemble a species-specific peculiarity, which could require the direct binding of interferon-regulatory factors on OXTR interferon response elements for preventing estrogen-induced OXTR upregulation in case of pregnancy (Mann *et al.* 1999, Telgmann *et al.* 2003). Only recently, Krishnaswamy *et al.* (2009) have reported that *in vitro* IFNT was able to reduce OXT-induced $\text{PGF}_{2\alpha}$ without downregulating either OXTR or PTGS2, implying a direct regulatory mechanism of IFNT on OXT has been yet to be unraveled. Clearly, in the presence of IFNT, $\text{PGF}_{2\alpha}$ pulses impairing luteal function are absent.

The lack of pulsatile luteolytic $\text{PGF}_{2\alpha}$ during pregnancy is accompanied by a steady-state low level of PGE_2 in the bovine caudal vena cava (Schallenberger *et al.* 1989), although the rise of basal levels and occasional pulses in $\text{PGF}_{2\alpha}$ have been reported in sheep (Zarco *et al.* 1988). The general PG synthase PTGS2 is not inhibited due to the presence of an embryo, instead there is an inconsistent evidence of the effect of IFNT on the expression of the general PG synthase PTGS2, showing either stimulative or no effects (Kim *et al.* 2003, Arosh *et al.* 2004b, Emond *et al.* 2004). While $\text{PGF}_{2\alpha}$ has primarily been ascribed to conduct luteolytic

effects, elevated PGE_2 is associated with beneficial actions on uterine receptivity during the establishment of pregnancy (Arosh *et al.* 2004b). During the last decade, a great effort has therefore been laid on the description of the relative contribution of each of the specific synthases and/or receptors of general and specific PG synthesis, catabolism, as well as transport contributing to the PG turnover (Arosh *et al.* 2003, Banu *et al.* 2003, Kim *et al.* 2003, Madore *et al.* 2003, Emond *et al.* 2004, Parent *et al.* 2006). Major changes occur in the endometrium predominantly between the estrus and diestrus state, which accentuate the endometrial synthesis of PG during the preimplantation period (Arosh *et al.* 2004b, Parent *et al.* 2006). As numerous *in vitro* studies demonstrated a shift from $\text{PGF}_{2\alpha}$ to PGE_2 production due to IFNT, an optimal PGE_2 to $\text{PGF}_{2\alpha}$ ratio during pregnancy favoring PGE_2 is generally accepted as an essential prerequisite of maternal contribution to successful establishment of pregnancy (Arosh *et al.* 2004b). Earlier studies showing the presence of high levels of $\text{PGF}_{2\alpha}$ in the uterus lumen during pregnancy when compared with the cycle might be part of a general PG increase (Ellinwood *et al.* 1979, Bartol *et al.* 1981), but there is a lack of *in vivo* evidence for the concomitant outranging increase in PGE_2 . Comprehensive data on the presence of the different PG hormones in the uterine lumen of ruminants *in vivo* are missing.

In this study, we simultaneously quantified absolute amounts of all PG present in bovine uterine flushings using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Maternal and embryonic PG contributions of the generating enzymes for these most prominent substrates were quantified using quantitative real-time PCR (RT–qPCR). The sensitivity of the endometrium and the embryo for the PG identified were assessed by quantifying the expression of specific receptors. Together the results comprehensively depict the principal PG actors as well as the temporal sequential role and the direction of action of the different PG in the bovine uterine lumen during the preimplantation period.

Results

Concentrations of PG in uterine flushings

Grading of different PG occurring in uterine flushings

The concentration of PG in uterus flushings varied over time in both cyclic and pregnant animals. The results obtained by ELISA for PGFM and PGE_2 were confirmed with LC–MS/MS (Table 1) and showed a highly significant correlation ($r=0.92$, $P<0.0001$ and $r=0.91$, $P<0.0001$ respectively).

The quantitative dynamics of the five predominant PG as determined by LC–MS/MS are shown in Fig. 1. At all days and time points examined, the concentration of 6-keto $\text{PGF}_{1\alpha}$ was predominant followed by $\text{PGF}_{2\alpha}$ > PGE_2 > $\text{PGD}_2 \approx \text{TXB}_2$. In general, the concentrations

Table 1 The concentrations of 13,14-dihydro-15-keto PGF_{2α} (PGFM) and PGE₂ as determined by ELISA vary with respect to day and status in uterine flushings and were verified by liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis.

Days after estrus	13,14-Dihydro-15-keto PGF _{2α} (pg/ml)		PGE ₂ (pg/ml)	
	ELISA	LC–MS/MS	ELISA	LC–MS/MS
12				
Control	139 ± 28 ^a	181 ± 35 ^a	15 ± 0 ^a	7 ± 2 ^a
Pregnant	192 ± 52 ^a	239 ± 71 ^a	15 ± 0 ^a	12 ± 2 ^a
15				
Control	395 ± 63 ^b	404 ± 62 ^b	42 ± 12 ^b	56 ± 15 ^b
Pregnant	532 ± 98 ^b	503 ± 101 ^b	244 ± 70 ^c	294 ± 94 ^c
18				
Control	422 ± 97 ^b	449 ± 88 ^b	60 ± 10 ^b	59 ± 7 ^b
Pregnant	245 ± 58 ^b	310 ± 115 ^{a,b}	377 ± 88 ^c	426 ± 244 ^c

Data are presented as means ± S.E.M. in 100 ml flushing/uterus. Different superscript letters indicate significant differences between groups per column ($P < 0.05$).

were much higher during pregnancy when compared with the cyclic controls. The highest PG concentration was measured for 6-keto PGF_{1α} at day 15 of pregnancy (6355 ± 2610 pg/ml in 100 ml flushing), followed by PGF_{2α} (1134 ± 416 pg/ml), PGE₂ (295 ± 94 pg/ml), PGD₂ (95 ± 47 pg/ml), and TXB₂ (95 ± 48 pg/ml). At day 12, the concentrations measured were at the lowest level for both pregnant and non-pregnant groups for all hormones under investigation. Correlation coefficients were highly significant ($P < 0.0001$) for all PG analyzed (Table 2).

The ratio of PGF_{2α}/PGE₂ was not significantly influenced by either day ($P = 0.4$) or status ($P = 0.2$; Fig. 2). On average, PGF_{2α} was fourfold more abundant than PGE₂. The ratio of 6-keto PGF_{1α}/PGE₂ was higher on day 15 than days 12 and 18 ($P < 0.0001$), but not significantly influenced by status. At day 15, 6-keto PGF_{1α} was 23-fold more abundant than PGE₂.

PGF_{2α} and PGE₂

The variation of hormone concentration of PGF_{2α} and PGE₂ over time appeared quite congruent. The increase in PGF_{2α} from day 12 to 15 was fourfold during the cycle

and 34-fold during pregnancy (Fig. 3A). PGE₂ increased sevenfold during the cycle and 24-fold during pregnancy (Fig. 3D). Between days 15 and 18, PGF_{2α} and PGE₂ remained constant in both control and pregnant groups.

The 15-keto metabolites of PGF_{2α} and PGE₂ (Fig. 3B and E) were only affected by day ($P < 0.0001$) but not by status. The upregulation between days 12 and 15 was concomitant to the respective active hormone, but 15-keto PGF_{2α} and 15-keto PGE₂ were significantly lower in pregnant animals at day 18 compared with day 15 and did not differ from controls at day 18. Highest coefficients of correlation were found between metabolites 15-keto PGF_{2α} and 15-keto PGE₂ ($r = 0.93$), as well as hormones PGF_{2α} and PGE₂ ($r = 0.89$; Table 2). While 13,14-dihydro-15-keto PGF_{2α}, significantly affected by the day of the cycle ($P = 0.005$), displayed higher concentrations than its 15-keto precursor (Fig. 3C), the concentration of 13,14-dihydro-15-keto PGE₂ was close to the detection limit of 4 pg/ml in all groups examined (Fig. 3F).

PGI₂ (6-keto PGF_{1α})

The short half-life of 2–3 min in buffer solution due to chemical instability requires the measurement of the more stable 6-keto PGF_{1α}. We do not underestimate PGI₂, as the concentration of the following catabolic metabolite 2,3-dinor-6-keto PGF_{1α} was below the detection limit of 150 pg/ml (data not shown). In controls, 6-keto PGF_{1α} was 13-fold higher abundant comparing days 12 and 15, while a 142-fold increase was detected in pregnant animals (Fig. 3G). Between days 15 and 18, 6-keto PGF_{1α} dropped twofold in pregnant and fourfold in control animals.

PGD₂ and TXB₂

Concentrations of PGD₂ and TXB₂ were highly correlated ($r = 0.88$, $P < 0.0001$; Table 2). In cyclic animals, the hormone concentration of PGD₂ (Fig. 3H) and TXB₂ (Fig. 3I) did not differ with respect to day.

Figure 1 Predominant PG (6-keto PGF_{1α}, PGI₂, PGF_{2α}, PGE₂, PGD₂, and TXB₂) present in control (left) and pregnant (right) bovine uterine flushings. Data are presented as means ± S.E.M. in 100 ml flushing/uterus quantified by LC–MS/MS.

Table 2 Pearson's correlation coefficients for hormone and transcript abundances.

	6-Keto PGF _{1α}	PGF _{2α}	15-Keto PGF _{2α}	PGE ₂	15-Keto PGE ₂	PGD ₂	TXB ₂
6-Keto PGF _{1α}							
PGF _{2α}	0.82 (<0.0001)		0.80 (<0.0001)	0.83 (<0.0001)	0.82 (<0.0001)	0.81 (<0.0001)	0.80 (<0.0001)
15-Keto PGF _{2α}	0.79514 (<0.0001)	0.70 (<0.0001)		0.89 (<0.0001)	0.74 (<0.0001)	0.80 (<0.0001)	0.78 (<0.0001)
PGE ₂	0.83 (<0.0001)	0.8925 (<0.0001)	0.79 (<0.0001)	0.79 (<0.0001)	0.93 (<0.0001)	0.70 (<0.0001)	0.65 (<0.0001)
15-Keto PGE ₂	0.82 (<0.0001)	0.74 (<0.0001)	0.93 (<0.0001)	0.84 (<0.0001)		0.79 (<0.0001)	0.79 (<0.0001)
PGD ₂	0.81 (<0.0001)	0.80 (<0.0001)	0.70 (<0.0001)	0.79 (<0.0001)	0.70 (<0.0001)		0.64 (<0.0001)
TXB ₂	0.80 (<0.0001)	0.78 (<0.0001)	0.65 (<0.0001)	0.79 (<0.0001)	0.64 (<0.0001)	0.88 (<0.0001)	

P values are given in brackets. Significant correlations ($P < 0.05$) are highlighted in bold.

However, during pregnancy, PGD₂ and TXB₂ displayed 17- and 19-fold higher concentrations at day 15 compared with day 12 (Fig. 3H and I) followed by a decrease towards day 18.

Endometrial and embryonic mRNA transcript abundance of PG enzymes, receptors, and transporter

Phospholipases PLA2G4A and PLA2G6 for AA substrate release

Neither the day of the cycle nor the pregnancy status affected the endometrial mRNA transcript abundance of PLA2G4A and PLA2G6 (Fig. 4A and B). PLA2G4A was 185- and 22-fold lower expressed in trophoblasts at days 15 and 18 respectively when compared to endometrium (Fig. 4A).

Enzymes for PGH₂ generation (PTGS2)

The day of the cycle had a significant effect on the endometrial transcript abundance of PTGS2 ($P = 0.0002$) increasing significantly between days 12 and 15 (Fig. 4C). The trophoblasts resembled 43-fold higher mRNA transcript abundance per µg RNA of PTGS2 than the endometrium at day 18 (Fig. 4C).

PG transporter PGT

Neither day nor status had an effect on endometrial expression of PGT. Trophoblasts displayed a fourfold higher transcript number than endometrium (Fig. 4D).

PGF_{2α} synthase PTGFS and receptor PTGFR

The endometrial expression of PTGFS dropped gradually ($P = 0.0002$) over time (Fig. 4E), while the trophoblasts displayed an increasing concentration comparing day 15 with day 18 (fourfold, $P = 0.0004$). At day 18, the transcript abundance was 43-fold higher than in the endometrium. At day 15, control animals had a significant higher endometrial PTGFR expression than the respective pregnant and all other groups examined (Fig. 4F). Trophoblast PTGFR increased significantly at

Figure 2 Ratios of PGF_{2α}/PGE₂ (A) and 6-keto PGF_{1α}/PGE₂ (B) during the preimplantation period. Data are expressed as means ± S.E.M. of the quotients of absolute PG in uterine flushings quantified by LC-MS/MS.

Figure 3 The concentrations of the prostaglandins and metabolites vary with respect to day and status in uterine flushings. Data are presented as means $\pm$ S.E.M. in 100 ml flushing/uterus quantified by LC-MS/MS. Different superscript letters indicate significant differences between groups ($P < 0.05$).

day 18 compared with day 15 ($P = 0.0007$), the latter displaying a 125-fold higher expression than the endometrium at day 18.

PGE₂ synthase PTGES and receptors PTGER2 and PTGER4

The endometrial *mPGES-1* (*PTGES*; $P = 0.0008$) rose steadily over the time of examination (Fig. 4G). The status had a significant effect on gene expression ($P = 0.003$), which at day 18 was 1.9-fold lower in pregnant than in control animals. *PTGER2* ($P < 0.0001$) transcript numbers increased (Fig. 4H), while *PTGER4* ($P = 0.0001$) decreased between days 12 and 15 (Fig. 4I). Both *PTGER2* and *PTGER4* displayed much lower transcript abundance in the trophoblasts compared to endometrium (23- and 1000-fold respectively; Fig. 4H and J).

HPGD metabolizing PGF_{2α} and PGE₂

Neither day nor status had an effect on endometrial expression of *HPGD* (Fig. 4I). The expression in the endometrium at day 18 was more than 600-fold higher than in trophoblasts at the respective day.

PGI₂ synthase PTGIS and receptors PTGIR and PPARG

While neither day nor status displayed an effect on transcript abundance of *PTGIS*, trophoblast expression was fourfold higher than endometrium at day 18 (Fig. 4K). Transcripts of *PTGIR* were twofold higher in

pregnant than in non-pregnant animals at day 18 (effect of day*status, $P = 0.03$; Fig. 4L). In trophoblasts, *PTGIR* displayed much lower transcript abundance compared to endometrium. *PPARG* expression was significantly lower at day 18 than days 12 and 15 (effect of day $P = 0.0001$; Fig. 4M). Trophoblasts at day 18 had a 22-fold higher mRNA abundance of *PPARG* than endometrium.

Discussion

This is the first comprehensive study simultaneously evidencing the most relevant PG in the bovine uterine lumen during the preimplantation period. Because PG metabolizing enzymes are NAD⁺-dependent, PG in the uterine flushings are not rapidly targeted for degradation as in tissue or serum. This allows the reliable assessment of the actual concentrations present *in vivo*. The ELISAs performed for PGFM and PGE₂ were comparable to the specific and highly sensitive method of LC-MS/MS.

During the estrous cycle, luteolytic PGF_{2α} peaks appear in the effluent vascularity of the uterus (McCracken *et al.* 1972) as early as day 14 in the ewe and day 18 in the cow depending on the individual cycle length. PG released from the endometrium not only reach blood vessels, but also appear in the uterine lumen. Early studies have demonstrated PGF_{2α} and PGE₂ in *in vivo* flushings (Ellinwood *et al.* 1979, Bartol *et al.* 1981, Shelton *et al.* 1990). Additionally, the conversion

Figure 4 mRNA transcript abundance of PG synthases (PLA2G4A (A), PLA2G6 (B), PTGS2 (C), PTGFS (E), PTGES (G), and PTGIS (K)), the metabolic enzyme HPGD (I), the prostaglandin transporter PGT (D), and specific receptors (FP (F), PTGER2 (H) and PTGER4 (J), PTGIR (L), and PPARG (M)) in endometrium and trophoblast tissue. For both endometrium (left) and trophoblast tissue (right), equal amounts of total RNA was used for reverse transcription and subsequent qPCR. Results are depicted as means of dCT (log₂) mRNA expression $\pm$ S.E.M. Different superscript letters indicate significant differences between groups ($P<0.05$).

of AA into the different PG by uterine cells in short-term culture supernatants was studied (Marcus 1981, Lewis *et al.* 1982, Charpigny *et al.* 1999). These studies differ with respect to the relative amounts of PG production and are

not consistent with the present data. Low levels of PGF_{2 α} , rising not earlier than day 19, have been described either with or without accompanied change of even lower levels of PGE₂ (Ellinwood *et al.* 1979,

Bartol *et al.* 1981, Lewis *et al.* 1982, Shelton *et al.* 1990). Highest levels of PGF_{2α} and PGE₂ opposed to low levels of PGD₂, TXB₂, and 6-keto PGF_{1α} were found in ovine luminal cell supernatants treated with [³H]AA, whereby the latter authors specifically point out that exogenous [³H]AA may not reflect endogenous synthesis capabilities (Charpigny *et al.* 1999). At present, it is believed that PGF_{2α} and PGE₂ are the primary PG produced in the endometrium during the cycle (Fortier *et al.* 2008). The present results clearly evidence that PGI₂ is the principle PG dominating the bovine uterus lumen followed by lower concentrations of PGF_{2α} and PGE₂, and subordinately PGD₂ and TXB₂.

The *PTGS2* increase observed was the most pronounced change that occurred concomitantly with rising intrauterine PG as reported elsewhere (Charpigny *et al.* 1999). This coincides with the accepted mechanism by which the blocking impact of P4 on PR is lost, and OXTR and ESR1 located in luminal and glandular epithelia of the endometrium rise during the estrous cycle (discussed in (Robinson *et al.* 2008)). The pronounced upregulation of *PTGS2* at day 15 might be of specific importance enabling the massive burst of PGF_{2α} peaks. The observed cyclic upregulation of *PTGS2* is linked to the upregulation of endometrial LHR (Fields & Shemesh 2004) as well as to the upregulation of ESR1/OXTR after day 12 (Asselin *et al.* 1997b, Kim *et al.* 2003), leading to high amounts of PGH₂ provided by the rate-limiting enzyme *PTGS2*. The different concentrations of the distinct PG may arise from variety in number of synthases as well as the different inherent frequencies. *PTGS2* is activated via allosteric feedback and this might be likewise true for the specific synthases.

Albeit peripheral PGF_{2α} pulses are not present (Schallenberger *et al.* 1989), the concentration of luminal PG was much higher during early pregnancy than during the cycle. The present data indicate that the pregnant day 15 and day 18 uterine flushings had much higher concentrations of PG than their cyclic counterparts. Our findings are consistent with the observed rise in PGF_{2α} and PGE₂ as well as 6-keto PGF_{1α} observed in the ewe (Ellinwood *et al.* 1979, Bartol *et al.* 1981, Marcus 1981, Lewis *et al.* 1982, Shelton *et al.* 1990). These studies are consistently showing that during pregnancy the luminal concentrations of PGF_{2α} by far exceed PGE₂. We clearly evidenced the most pronounced abundance of PGI₂ followed by PGF_{2α} and PGE₂, and low levels of PGD₂ and TXB₂ in the pregnant bovine uterus.

If in the presence of the embryo, endometrial OXTR and ESR1 are downregulated, how then can the major increase of PG in the uterus during pregnancy be explained?

A possible scenario has been proposed that a redirection of PG takes place, switching from an endocrine direction towards the uterine vasculature to a lumen-directed exocrine-driven synthesis (Bazer & Thatcher 1977), although there are convincing doubts

favoring a retrograde transfer in the area of the mesometrium (Krzyszowski & Stefanczyk-Krzyszowska 2008). Major assumptions are that the epithelial endometrium can specifically transport PG in either apical or basal direction as well as the conceptus-derived anti-luteolytic signal acts as a principal director (Ziecik *et al.* 2006). As the newly described PGT accounts for up to 80% of the PG transport (Banu *et al.* 2008), the former is quite likely. PGT has been demonstrated to facilitate OXT-induced PGF_{2α} secretion, and a possible tight regulation due to IFNT or another embryo-derived factor seems feasible.

The transcript abundance of neither endometrial PLA₂ nor PG enzymes downstream of *PTGS2* exerted prominent differences between pregnancy and controls great enough to explain vast differences in uterine luminal PG abundance. This is in contrast to observed IFNT effects described *in vitro* (Asselin *et al.* 1997a, 1997c, Parent *et al.* 2002, Emond *et al.* 2004, Tithof *et al.* 2007, Godkin *et al.* 2008). In addition, the release of all PG examined is strikingly concomitant, making it likely that the general rather than the specific synthases account for the vast increase in PG. Others have demonstrated an increase in *PTGS2* in luminal epithelium due to pregnancy (Arosh *et al.* 2004b), although our data show that *PTGS2* was not significantly upregulated in accordance with Kim *et al.* (2003). Undoubtedly, our mRNA expression analyses neglect translational peculiarities as well as differences due to location of expression, but others have specifically focused on this aspect in detail (reviewed in Fortier *et al.* (2008)).

During the phase of maternal recognition of pregnancy, the decrease in epithelial *PTGS2* has been reported (Arosh *et al.* 2004b). Moreover, it has been discussed that the relative production of PGE₂ and PGF_{2α} may be more important than the absolute levels of individual-secreted PG, in the choice between embryo implantation and resumption of the estrous cycle (Asselin *et al.* 1997a). IFNT has been made responsible for the reduced production of PGF_{2α} relative to PGE₂ evidenced by numerous *in vitro* studies (Asselin *et al.* 1997a, 1997c, Parent *et al.* 2002, Emond *et al.* 2004, Tithof *et al.* 2007, Godkin *et al.* 2008). However, we find clear evidence that there is no difference in PGF_{2α}/PGE₂ ratio between cyclic and pregnant uteri *in vivo*. The differences reported might therefore resemble *in vitro* culture situations in which a) the relative contribution of PG production by the different cell populations (Kim & Fortier 1995, Godkin *et al.* 2008) used did not resemble the actual *in vivo* situation or b) the exposure of IFNT does not take into account the PG contribution of the embryo.

Luminal PG can arise from both endometrial as well as embryo secretion. *PTGS2* was demonstrated in trophoblast cells (Charpigny *et al.* 1997), and the supernatant of blastocysts contained considerable amount of PG in cattle and sheep (Marcus 1981, Charpigny *et al.* 1997).

Bovine blastocysts produced substantial amounts of PG, with higher abundance of $\text{PGF}_{2\alpha}$ than PGE_2 (Shemesh *et al.* 1979, Lewis *et al.* 1982). Wilson *et al.* (1992) reported a tenfold higher PGE_2 than 6-keto $\text{PGF}_{1\alpha}$ production of embryos cultured *in vitro*. As species-specific peculiarity, bovine blastocysts were shown to produce primarily $\text{PGF}_{2\alpha}$, while ovine embryos produced mainly 6-keto $\text{PGF}_{1\alpha}$ and $\text{PGF}_{2\alpha}$ (Lewis 1989).

It is difficult to dissect the embryo from the endometrial contribution, but we may estimate from the metabolic signature of the PG. Both 15-keto $\text{PGF}_{2\alpha}$ and 15-keto PGE_2 comport with their respective hormone during the cycle. This most probably reflects the maternal metabolic turnover, as in agreement with protein expression HPGD is steadily expressed during the luteal phase of the cycle (Parent *et al.* 2006). In general, the embryos resembled an equal or higher mRNA transcript abundance per μg RNA of general (*PTGS2*) and specific PG synthases (*PTGFS*, *PTGES*, and *PTGIS*) than the endometrium. The pregnancy-induced increase leads to a general increase in both PG and PG metabolites, followed by a vast increase in PG hormones solely. In our study, embryos displayed much lower quantities of *HPGD* transcripts than the endometrium, which is in line with the endometrium having been shown to produce much higher quantities of PGFM than blastocysts (Lewis *et al.* 1982). It was speculated that the endometrium could metabolize both its own and the blastocyst's PG by high levels of *HPGD* (Lewis *et al.* 1982). But given the high amounts of hormone present in the uterine lumen, we rather conclude that the prominent increase in $\text{PGF}_{2\alpha}$ and PGE_2 during pregnancy without accompanying rise in PGFM metabolites most probably reflects (at least part of) the embryonic contribution to uterine PG. If so, the reported IFNT-induced maternal shift of PG synthesis would accomplish local importance in the endometrium, whereas the maternal contribution to the intrauterine PG milieu would quantitatively be of subordinate importance.

Consequently, the endometrium must undergo major changes mediated through IFNT concerning the specific receptors for further pregnancy-specific signal transduction (Arosh *et al.* 2003, 2004a). PGE_2 is produced by both the endometrium and the embryo as seen from high embryonic expression of *PTGES*. As reported earlier, the action is primarily directed towards the endometrium due to low-receptor concentration of the embryo and much higher endometrial *PTGER2* (Arosh *et al.* 2003). $\text{PGF}_{2\alpha}$ may act paracrine or autocrine on the embryo due to remarkably high embryonal *PGFR* expression. The embryo seems therefore highly sensitive towards $\text{PGF}_{2\alpha}$, which may likely be of physiological benefit so far underestimated.

PGI_2 displayed the predominant amount of PG during the preimplantation period, increasing particularly at day 15. An endocrine luteotropic effect of PGI_2 is conceivable by means of counter-current passage

(Milvae & Hansel 1980). More locally, in mice in a first line of action, PGI_2 increases endometrial vascular permeability as potent vasoactive agent thus mediating implantation (Moncada 1983). The invasive implantation sites in mice specifically express high levels of *PTGIS* (Lim *et al.* 1999). Though the increase in PGI_2 in the present study is prior to implantation coincident with the beginning of elongation (Robinson *et al.* 2006), an effect on the permeability of the maternal vasculature seems nonetheless likely. Via uterine PGI_2 receptor, PGI_2 in the uterine lumen could lead to an increased blood flow to the pregnant uterus by vasodilatation (Schramm *et al.* 1984). Only recently, an antiapoptotic role has been assigned to PGI_2 in preimplantation embryo development (Pakrasi & Jain 2008). Unlike in mice however, we could not detect an accompanied increase in PGI_2 by enhanced endometrial *PTGIS* transcript abundance. As the positional effect of the embryo was not considered by the flushing method, we might have overlooked a differential expression at the endometrial surface in contact with the trophoblast. At day 18 though, as the conceptus is fully expanded, both uterine horns are in contact with the trophoblast. Our data are consistent with the lack of increase of *PTGIS* protein in superficial endometrium during the respective early pregnancy period in sheep (Cammis *et al.* 2006).

Peroxisome proliferator-activated receptors (PPARA, B/D, G) have been identified as nuclear receptors for PGI_2 (Forman *et al.* 1997). Specifically, PPAR δ /RXRA has been associated with the control of implantation in the mouse (Lim *et al.* 1999), whereas PPAR γ has been implicated in the process of placental development and differentiation (Tarrade *et al.* 2001). In bovine embryos, PPAR γ , co-localized with RXR, appeared in both trophoblast and inner cell mass (Mohan *et al.* 2002). In a recent study, the regulation of the ovine endometrial PPARs has been demonstrated, where, consistent with our findings, PPAR γ appeared next to PPAR δ in both endometrium and embryo (Cammis *et al.* 2006). It is therefore reasonable to assume that although the membrane receptor *PTGIR* was almost absent, nuclear receptors for PGI_2 are present on bovine preimplantation embryos allowing PGI_2 action in a para- or intracrine manner. A perinuclear localization of *PTGS2* in trophoblast cells (Morita *et al.* 1995) furthermore implicates local stimulatory effects on the embryo.

The termination of *PTGS2* expression on the embryo coincides with implantation in sheep (Charpigny *et al.* 1997). Consistent with our data of no further rise after days 15–18, day 19 bovine blastocysts secreted PG less abundantly than those of day 16 (Lewis *et al.* 1982), obviously peaking around day 18 (Wilson *et al.* 1992) depending on the specific PG in focus. The principal actors and temporal sequential role of embryo-derived PG are therefore tightly regulated and exert a predominant role as signaling molecules during the early preimplantation phase.

In summary, PG are highly abundant in the pregnant preimplantation bovine uterus lumen. The impact of the embryo obviously generates an intrauterine environment in which high concentrations of PGI₂ and PGF_{2α} by far outrange PGE₂. This may be facilitated by either sole or combined action of PG transporters redirecting cyclic PG and enhanced endometrial and/or trophoblast synthesis. Of specific relevance are both the effect of PG on the endometrium as well as the paracrine and/or autocrine role, specifically emphasizing PGI₂ and PGF_{2α}, acting on abundantly expressed trophoblast receptors. The embryo thus generates a beneficial environment supporting its elongation and development as well as ensuring inhibition of luteolysis. Uterine PGI₂ and PGF_{2α} might play a so far underestimated role during early pregnancy with beneficial rather than detrimental effects on fertilization success.

Materials and Methods

Pretreatment of animals and collection of endometrial and trophoblast tissue samples

Cyclic Simmental heifers 23 months of age on average were cycle synchronized by an i.m. injection of a single dose of 500 mg cloprostenol (Estrumate; Essex Tierarznei, Munich, Germany) at diestrus. Animals were observed for sexual behavior (i.e. toleration, sweating, and vaginal mucus) to determine standing heat, which occurred around 60 h after Estrumate injection. Blood samples were taken at day of synchronization, day 0 of the estrous cycle and just before slaughtering to determine serum P₄ (Prakash *et al.* 1987) and estradiol-17β (E₂; Meyer *et al.* 1990) levels. The pregnant groups of animals were inseminated with cryo-conserved sperm at estrus (AI) and slaughtered at days 12, 15, and 18 of gestation respectively ($n=5$ per group). For these three groups, control groups ($n=5-7$ per group) were inseminated with the supernatant of centrifuged sperm derived from the same bull and also slaughtered at days 12, 15, and 18 of the estrous cycle respectively. The obtained uteri were flushed with 100 ml PBS (~21 °C). Animals were excluded from the study if no intact conceptus was detected in case of AI. Flushing fluid was centrifuged at 800 *g* for 10 min at 5 °C and stored at -20 °C until further investigation. The uteri were opened longitudinally and from the middle part of the ipsilateral uterine horn, intercaruncular endometrium was sampled as described previously (Bauersachs *et al.* 2005). All experiments were conducted with the permission from the local veterinary authorities and were in accord with the accepted standards of humane animal care.

Determination of PG and TXB₂ concentrations in bovine uterus fluid by LC-MS/MS

The content of PGE₂, PGD₂, TXB₂, PGF_{2α}, and 6-keto PGF_{1α} in bovine uterus fluid was determined by LC-electrospray ionization (ESI)-MS/MS as described previously (Degousee *et al.* 2008). PG and TXB₂ were separated with a Synergi Hydro-RP

Table 3 Sequences and accession numbers of the PCR primers for assayed genes and length, annealing (AT), fluorescence acquisition (FA), and melting point (MP) temperature of the PCR products.

Gene	Reference (acc. no.)	Forward primer (5'-3')	Reverse primer (5'-3')	PCR product (bp)	AT (°C)	FA (°C)	MP (°C)
Polyubiquitin	NM_174133	AGATCCAGGATAAGGAAGGCAT	GCTCCACCTCCAGGGTGAT	198	60	83	88
PLA2C4A	NM_001075864	ACAGAGTCTTGGGAGTTCTGG	ATGTTACGTGCTTTGGGTC	143	60	76	83
PLA2G6	XM_001251754	ATCATCCAGCTGCTCATCG	AAGTACACACCGCATGTAGG	149	60	88	92
PTGS2	NM_174445	TCGAGGTGTATGTAGTGTGA	GTCTGGGCAAGAAATGCAA	359	60	82	87
SLCO2A1 (PGT)	NM_174829	CTGCCACATCATCATC	CAGGGAGAGTTGTGGAC	130	64	82	87
AKR1B1	NM_001012519	ATACAAGCCGGCGGTTAAC	TGCTGCAATCGCTTTGATC	188	60	78	82
PTGFR	NM_181025	AGCCTTGCCATGCTATCC	TAGTCCATTGATGAGGTGCC	127	64	77	81
PTGES	NM_174443	TCCTGGTCTTCTTCCTGG	CCAGACAATCTCGAGGG	132	64	86	91
PTGER2	NM_174588	CTACTTTGCCCTTTCCATACC	GATGAAGCACACCTGCC	210	60	85	90
PTGER4	NM_174589	CGATAGTGTAGCGCTACC	AGCCCGCATAGTAGGAG	220	60	89	93
HPGD	NM_001034419	TGATCAGTGGAACTACCTGG	TGAGATTAGACCCATCC	183	60	87	91
PTGIS	NM_174444	CGTCTCAGTGAAGAGCTC	TTCTGGGAGCTCAGGAAGG	150	64	88	92
PTGIR	NM_001015622	GTTTCAATGCCTCAACC	GTTTCCCTTTTCCCTC	200	60	76	84
PPARG	NM_181024	AAGACGACAGACAAATCACCG	ACTGACACCCCTGGAAGATG	142	60	78	82

column (150×2 mm I.D., 4 µm particle size, and 80 Å pore size from Phenomenex, Aschaffenburg, Germany) and determined with an API 4000 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany). Briefly, a stock solution with 100 µg/ml PGE₂, PGD₂, TXB₂, PGF_{2α}, and 6-keto PGF_{1α} was prepared in methanol. The stock solution was further diluted with methanol to obtain working standards with the following concentration ranges: PGE₂, PGD₂, TXB₂, and 6-keto PGF_{1α} from 0.100 to 1250 ng/ml, PGF_{2α} from 0.4 to 5000 ng/ml. Samples for the standard curve and quality controls were prepared with 200 µl PBS, 100 µl 0.15 M EDTA, 80 µl 45 mM H₃PO₄, 20 µl working standards, and 20 µl internal standard solution containing [²H₄]-PGE₂, [²H₄]-PGD₂, [²H₄]-TXB₂, [²H₄]-6-keto PGF_{2α}, and [²H₄]-PGF_{2α} at a concentration of 25 ng/ml. Bovine uterus fluid samples were prepared similarly, instead of 200 µl PBS and 20 µl working standard, 200 µl sample and 20 µl methanol were added. PGE₂, PGD₂, TXB₂, PGF_{2α}, 6-keto-PGF_{1α}, and internal standards were extracted with liquid-liquid extraction. Therefore, the prepared samples were extracted twice with ethyl acetate. The organic phase was removed at a temperature of 45 °C under a gentle stream of nitrogen. The residues were reconstituted with 50 µl acetonitrile/water/formic acid (20:80:0.0025, v/v, pH 4.0), centrifuged for 2 min at 10 000 g, and then transferred to glass vials (Macherey-Nagel, Düren, Germany) prior to injection into the LC-MS/MS system. Mass transitions used for quantification of the respective substances were m/z 351.2 → m/z 271.2 for PGE₂ and for PGD₂, m/z 353.2 → m/z 309.1 for PGF_{2α}, m/z 369.2 → m/z 168.9 for TXB₂, and m/z 369.3 → m/z 163.2 for 6-keto-PGF_{1α}. Quantification was performed with Analyst Software V1.4.2 (Applied Biosystems) using the internal standard method (isotope-dilution mass spectrometry). Ratios of analyte peak area and internal standard peak area (y-axis) were plotted against concentration (x-axis), and calibration curves for each PG and TXB₂ were calculated by least-square regression with 1/concentration² weighting. The variations in accuracy were <12%. The intra-day and inter-day variabilities were <10%. The lower limit of quantification was 10 pg/ml for PGE₂, PGD₂, TXB₂, and 6-keto PGF_{1α} respectively and 40 pg/ml for PGF_{2α}.

Determination of PG PGFM and PGE₂ and concentrations in bovine uterus fluid by ELISA

PGFM and PGE₂ were evaluated directly in uterine flushing fluids without extraction. The determination of PGFM was undertaken as described earlier (Meyer *et al.* 1989). The determination of PGE₂ was done using commercially available high-sensitivity competitive ELISA kits (Assay Design, Inc., Ann Arbor, MI, USA). The standard curve ranged from 7.8 to 1000 pg/ml, and the ED50 of the assay was 40–50 pg/ml. The intra- and inter-assay coefficient of variation values were 10 and 12% respectively.

RNA extraction and RT-qPCR of tissue samples

Total RNA from endometrial tissue samples was isolated and subjected to quality control using the Bioanalyzer 2100 (Agilent Technology, Palo Alto, CA, USA). A two-step RT-qPCR was undertaken as described recently (Bauersachs *et al.* 2006).

RT-qPCRs using the LightCycler DNA Master SYBR Green I protocol (Roche) were performed. The primers used to amplify specific fragments referring to selected regulated genes are depicted in Table 3. The annealing temperature, the appropriate fluorescence acquisition points for quantification within the fourth step of the amplification segment, and the melting points are outlined. The cycle number (C_t) required for achieving a definite SYBR Green fluorescence signal was calculated by the second derivative maximum method (Light-Cycler software version 3.5.28). The C_t is correlated inversely with the logarithm of the initial template concentration. The C_t determined for the target genes were normalized against the housekeeping gene ubiquitin (ΔC_t). In order to avoid negative digits while allowing an estimation of a relative comparison between two genes, data are presented as means ± s.e.m. subtracted from the arbitrary value 20 (ΔC_t). Thus, a high ΔC_t proportionally resembles high-transcript abundance (Livak & Schmittgen 2001).

Data analysis

Data are presented as means ± s.e.m. (n=4). For statistical analysis, the SAS program package release 9.1.3 (2002; SAS Institute, Inc., Cary, NC, USA) was used. The ΔC_t as log data for gene expression followed normal distribution, while the natural logarithm of LC-MS/MS data was used to ensure normal distribution. Comparing cyclic and pregnant endometrium, the model included the day, the status (cyclic versus pregnant), and their interaction. Differences between treatments were localized using the least significant difference test. Student's *t*-test was employed comparing the transcript abundance of embryos at different stages. Differences were considered significant at *P*<0.05. Differences between endometrial and embryonic gene expression were estimated as fold changes in means, but not considered for statistical analyses because of minor tissue inherent effects on RT-qPCR. Graphs were plotted with SigmaPlot 8.0 (SPSS Software GmbH, Munich, Germany).

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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