Canine placenta: a source of prepartal prostaglandins during normal and antiprogesterin-induced parturition

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Abstract

Expression of cyclooxygenase 2 (COX2, now known as PTGS2), prostaglandin E2 synthase (PTGES, PGES), and prostaglandin F2 α synthase (PGFS), of the respective receptors PTGFR (FP), PTGER2 (EP2), and PTGER4 (EP4) and of the progesterone receptor (PGR, PR) was assessed by real-time PCR, immunohistochemistry (IHC), or *in situ* hybridization (ISH) in utero/placental tissue samples collected from three to five bitches on days 8–12 (pre-implantation), 18–25 (post-implantation), and 35–40 (mid-gestation) of pregnancy and during the prepartal luteolysis. Additionally, ten mid-pregnant bitches were treated with the antiprogesterin aglepristone (10 mg/kg bw (2 $\times$ /24 h)); ovariectomy was 24 and 72 h after the second treatment. Plasma progesterone and 15-ketodihydro-PGF2 α (PGFM) concentrations were determined by RIA. Expression of the PGR was highest before implantation and primarily located to the endometrium; expression in the placenta was restricted to the decidual cells. PTGS2 was constantly low expressed until mid-gestation; a strong upregulation occurred at prepartal luteolysis concomitant with an increase in PGFM. PGFS was upregulated after implantation and significantly elevated through early and mid-gestation. PTGES showed a gradual increase and a strong prepartal upregulation. PTGFR, PTGER2, and PTGER4 were downregulated after implantation; a gradual upregulation of PTGFR and PTGER2 occurred towards parturition. ISH and IHC co-localized PGFS, PTGFR, PTGES, and PTGS2 in the trophoblast and endometrium. The changes following application of aglepristone were in the same direction as those observed from mid-gestation to prepartal luteolysis. These data suggest that the prepartal increase of PGF2 α results from a strong upregulation of PTGS2 in the fetal trophoblast with the withdrawal of progesterone having a signalling function and the decidual cells playing a key role in the underlying cell-to-cell crosstalk.

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Introduction

In the dog, corpora lutea (CL) reach full functional capacity between days 20 and 30 after ovulation; thereafter, the more gradual decrease indicates luteal regression. The resulting course of progesterone (P₄) concentrations in peripheral plasma is almost identical in pregnant and nonpregnant dogs until about day 60 of luteal lifespan when the more gradual decline observed so far turns into a steep one as a precondition for parturition, which generally occurs 63 days after mating. In the nonpregnant dog, however, the gradual decline continues to levels <1 ng/ml about 10–20 days later (Concannon *et al.* 1989,

Hoffmann *et al.* 1994). These deviating patterns in P₄ secretion may be seen as an indication for different mechanisms regulating a) gradual luteal regression in nonpregnant dogs and b) prepartal luteolysis.

Both luteotropic and luteolytic mechanisms in pregnant and nonpregnant dogs have been addressed in a number of papers. Gonadotropic support is required for luteal maintenance in pregnant and nonpregnant bitches during somewhat more than the second half of luteal lifespan with prolactin rather than LH being the major luteotropic factor (Okkens *et al.* 1990). Yet, luteal regression occurs in spite of an increased availability of prolactin (Gräf 1978) and LH (Hoffmann & Schneider 1993).

Recently, the role of prostaglandins in luteal regression in the nonpregnant bitch has been addressed (Hoffmann *et al.* 2004, Kowalewski *et al.* 2006a, 2008a). As hysterectomy does not interfere with normal ovarian function (Hoffmann *et al.* 1992), a role of CL-derived prostaglandin F $_{2\alpha}$ (PGF $_{2\alpha}$) acting as a luteolytic agent via para-/autocrine mechanisms was postulated, also in analogy to the situation observed in other species, i.e. cattle (Diaz *et al.* 2002). However, the results obtained rather support a luteotropic effect of PGE $_2$ during the first third of luteal lifespan (Kowalewski *et al.* 2008b) than a luteolytic role of PGF $_{2\alpha}$.

The observed luteolytic response of the canine CL to systemic application of PGF $_{2\alpha}$ agonists (Concannon & Hansel 1977, Romagnoli *et al.* 1991) does not contradict this hypothesis as the CL rather constantly express the PGF $_{2\alpha}$ receptor (PTGFR) following its formation around day 15 after ovulation (Kowalewski *et al.* 2008a).

In pregnant bitches for the immediate prepartal release of PGF $_{2\alpha}$, a clear role in respect to inducing myometrial contractions could be deduced (Nohr *et al.* 1993, Hoffmann *et al.* 1999). Indications on a luteolytic role are equivocal and only relate to the observations by Hoffmann *et al.* (1996), who reported about a somewhat prolonged pregnancy in two dogs following application of the cyclooxygenase inhibitor indomethacin.

The situation is further hampered by the fact that there are no data on the origin of the prepartally released PGF $_{2\alpha}$ and the underlying mechanisms triggering this release.

Cyclooxygenase 2 (PTGS2) is the essential enzyme allowing for the formation of PGH $_2$, the common precursor of PGF $_{2\alpha}$ and PGE $_2$. The reduction of PGH $_2$ by 9,11-endoperoxidase reductase activity of prostaglandin F $_{2\alpha}$ synthase (PGFS) is the major route for PGF $_{2\alpha}$ synthesis leading to the synthesis of PGF $_{2\alpha}$ directly from PGH $_2$. However, alternatively PGF $_{2\alpha}$ could also be formed via 9-ketoprostaglandin reductase or 11-ketoprostaglandin reductase activity using the PGH $_2$ -derived PGE $_2$ and PGD $_2$ as substrates respectively (Asselin & Fortier 2000, Madore *et al.* 2003).

As the canine PGFS cDNA sequence was originally cloned and described based on the mRNA isolated from utero/placental cross sections of late pregnant dogs (Kowalewski *et al.* 2008a), there is a distinct likelihood that the utero/placental unit could be the origin of the prepartal PGF $_{2\alpha}$ release. This would resemble the situation in cattle, where strongly upregulated PTGS2 levels are observed during parturition in the uninucleated trophoblast cells indicating that the fetal part of the placenta (cotyledon) may be involved in the prepartal output of PGF $_{2\alpha}$ and PGE $_2$ in this species (Schuler *et al.* 2006).

Both prostaglandins show strong functional interrelationships; thus, PGE $_2$ acts on softening the cervix prior to and during the uterine contractions, triggered by the action of PGF $_{2\alpha}$ (Stys *et al.* 1981, Fuchs *et al.* 1984). Additionally, it has been suggested that PGE $_2$ plays an essential role in controlling the differentiation

of endometrial stromal cells into decidual cells (decidualization) and hence in implantation, as has been shown in mice (Pakrasi & Jain 2008).

Taking together the information available so far, it was hypothesized that in the dog placenta-derived prostaglandins might play a role in the prepartal preparation of the genital tract possibly also contributing to prepartal luteolysis. We therefore tested for the expression of PTGS2, PGFS, prostaglandin E $_2$ synthase (PTGES), and the respective PGF $_{2\alpha}$ and PGE $_2$ receptors (PTGFR, PTGER2, and PTGER4). As the decidual cells of maternal origin are the only cells in canine placenta expressing the progesterone receptor (PGR; Vermeirsh *et al.* 2000), an immediate functional interrelationship between CL-derived P $_4$ and placental function is suggested. Therefore, also expression of the PGR was assessed as well as the concentrations of P $_4$ and 15-ketodihydro-PGF $_{2\alpha}$ (PGFM), the major metabolite of PGF $_{2\alpha}$, in peripheral plasma. In order to gain further information on the underlying mechanism, the same parameters were assessed after application of the PGR blocker aglepristone to terminate pregnancy.

Results

Normal pregnancy and parturition

Progesterone and PGFM concentrations

As was already shown for P $_4$ (Kowalewski *et al.* 2009), mean progesterone concentrations were: 35.71 ± 7.9 ng/ml in the pre-implantation period, 29.73 ± 13.23 ng/ml in the post-implantation period, 13.32 ± 8.66 ng/ml at mid-gestation, and 2.07 ± 0.99 ng/ml during the prepartal progesterone decline; the effect of time was highly significant ($P < 0.0019$; Fig. 1A). The course of the PGFM concentrations also showed a highly significant effect of time ($P < 0.0001$) with highest values occurring during prepartal luteolysis revealing an inverse relationship in the course of P $_4$ and PGFM

Figure 1 Progesterone (P $_4$; ng/ml) and prostaglandin F $_{2\alpha}$ metabolite (PGFM; nmol/l) profiles in peripheral blood plasma of bitches during pregnancy and normal luteolysis (A) and during aglepristone-induced luteolysis (B).

concentrations (Fig. 1A). The mean PGFM concentrations were: 0.85 ± 0.26 nmol/l in the pre-implantation period, 4.82 ± 2.3 nmol/l in the post-implantation period, 5.44 ± 2.2 nmol/l at mid-gestation, and 18.2 ± 3.67 nmol/l during prepartal luteolysis (Fig. 1A).

Expression of PGR mRNA and immunohistochemical localization in the utero/placental unit

Expression of mRNA revealed a highly significant effect of time ($P < 0.0001$). It was highest in the pre-implantation period (Fig. 2A) and decreased significantly ($P < 0.001$) thereafter with no further changes observed towards the end of gestation (Fig. 2A). In the pre-implantation group, immunohistochemistry (IHC) located PGR to the endometrial stroma and to nuclei of both glandular and superficial epithelial cells, as well as in the smooth muscle cells of the myometrium (Fig. 3A). Following formation of the placenta, decidual cells were the only cells of the placental labyrinth strongly expressing the PGR (Fig. 3C). No signals could be detected in the endothelial cells of maternal and fetal vessels or in the trophoblast (Fig. 3C). No or only weak signals were seen in the maternal stroma and epithelial cells of deep endometrial glands and in the superficial endometrial glands referred to as glandular chambers of the utero/placental unit (not shown). The myometrial PGR signals remained detectable throughout pregnancy.

Expression of PTGS2 mRNA and immunohistochemical localization in utero/placental unit

Real-time RT-PCR revealed a significant ($P < 0.0005$) effect of time; expression was low until prepartal luteolysis when a highly significant ($P < 0.001$) increase in PTGS2 mRNA expression occurred (Fig. 4A). As observed by IHC, the endometrium of the pre-implantation group stained

Figure 2 Expression of progesterone receptor (PGR) as determined by real-time (TaqMan) PCR during pregnancy and normal luteolysis (A) and during aglepristone-induced luteolysis (B; compared with the mid-gestation group). RGE, relative gene expression (mean $\pm$ s.d.); bars with different asterisks differ with $P < 0.001$.

negative, while strong signals were observed in the myometrium at all stages of pregnancy (Fig. 3B).

Following formation of the placenta, a distinct staining for PTGS2 was localized to the invading trophoblast surrounding large maternal vessels at the base of the placental labyrinth; at the time of prepartal luteolysis, it had spread over the entire trophoblast (Fig. 3D). No staining was seen in the endothelial cells of maternal or fetal vessels or stromal cells. Only occasionally, some decidual cells showed a weak staining (Fig. 3D). With the beginning of implantation, some positive signals were also detected in the endometrial glands, especially in the deep endometrial glandular epithelium (not shown).

Expression of PGFS and PTGFR mRNA and localization in the utero/placental unit

Expression of *PGFS* and *PTGFR* mRNA showed a significant effect of time ($P < 0.0008$ and $P < 0.02$ respectively). Expression of *PGFS* mRNA was lowest during the pre-implantation period, followed by an increase ($P < 0.05$) in the post-implantation and mid-gestation periods (Fig. 4C). It decreased thereafter until prior to parturition by ~ 3.7 -fold.

Expression of *PTGFR* mRNA revealed a biphasic expression pattern (Fig. 4E) and was similarly high in the pre-implantation period and during prepartal luteolysis, but distinctly lower in the post-implantation ($P < 0.05$) and mid-gestation periods (Fig. 4E).

Cellular localization of *PGFS* and *PTGFR* mRNA by *in situ* hybridization (ISH) reflected localization of PTGS2. Virtually, all trophoblast cells stained positive during prepartal luteolysis, especially the invading trophoblast at the base of the placental labyrinth and in the area of the glandular chambers (Fig. 5A and B) where glandular epithelial cells were also positive.

Expression of PTGES, PTGER2, and PTGER4 mRNA and localization in the utero/placental unit

Expression patterns of *PTGES* and of the PGE2 receptors, *PTGER2* and *PTGER4*, showed a significant effect of time ($P < 0.0001$, $P < 0.0001$, and $P < 0.05$ respectively). *PTGES* mRNA showed the lowest expression during the pre-implantation period, a gradual increase until mid-gestation and was significantly elevated ($P < 0.001$) during prepartal luteolysis (Fig. 6A).

The biphasic expression pattern of *PTGER2* (Fig. 6C) with a significant upregulation during the pre-implantation period and prepartal luteolysis ($P < 0.001$ and $P < 0.05$ respectively) resembled that of *PTGFR* (Fig. 4E). Expression of *PTGER4* mRNA was highest during the pre-implantation period and significantly lower ($P < 0.05$) at prepartal luteolysis (Fig. 6E).

ISH located *PTGES* mRNA in the same cells as *PGFS* mRNA with strongest signals observed in the trophoblast cells (Fig. 5C). The same location was observed for the mRNA of *PTGER2* and *PTGER4* (not shown).

Figure 3 Immunohistochemical localization of progesterone receptor (PGR) and PTGS2 in canine endometrium at the pre-implantation stage of pregnancy (A and B) and in the placenta during normal and aglepristone-induced luteolysis (C–F). The insets to A and B show the myometrial expression of PGR and PTGS2 respectively. (A and B) Pre-implantation PGR is localized in nuclei of endometrial luminal epithelial cells (●) and epithelial cells of superficial (□) and deep (■) endometrial glands; in contrast, PTGS2 is exclusively expressed in myometrium (inset to B). □, glandular covering layer (connective tissue). (C–F) In the placental labyrinth during normal and aglepristone-induced luteolysis, maternal blood vessels (●) with characteristic big endothelial cells and fetal blood vessels (□) are staining negatively for PGR and PTGS2. Maternal decidua cells (■) show only weak and/or sporadic PTGS2 signals, but very strong PGR immunoreactivity. Fetal trophoblast (□) is the only component of the placental labyrinth staining strongly for PTGS2.

Induced parturition

As parturition was induced around days 40–45, the expression of all factors assessed was compared with the mid-gestation group, which was used as a nontreated control in the statistical evaluation applying the Dunnett's multiple comparison test. For all factors, the observed changes in mRNA expression following application of the antiprogesterin were in the same direction as those observed from mid-gestation to prepartal luteolysis. Thus, there was a significant upregulation of *PTGS2* ($P < 0.001$; Fig. 4B), *PTGES* ($P < 0.01$), and *PTGER2* ($P < 0.01$; Fig. 6B and D). The increase of *PTGFR* ($P > 0.05$) was not significant; all effects were more distinct at 24 h than at 72 h after treatment (Figs 4B and F and 6B and D).

Expression of *PGFS* and *PTGER4* was decreased ($P < 0.01$ and $P < 0.05$ respectively) with the effect being more pronounced at 72 h than at 24 h after treatment (Figs 4D and 6F). No changes in the *PGR* mRNA expression were observed (Fig. 2B).

IHC (Fig. 3E and F) and ISH localized expression of all factors assessed in the same cell types as in nonantiprogesterin-treated dogs.

Similarly, changes in PGFM and P_4 plasma levels showed a significant effect of time ($P < 0.0047$ and $P < 0.0163$ respectively). The initial concentration of PGFM increased from 2.75 ± 0.68 to 5.04 ± 1.36 nmol/l at the second treatment with the antiprogesterin. It was high 48 h later (9.33 ± 1.1 nmol/l) and decreased by 72 h (4.97 ± 1.5 nmol/l). P_4 decreased significantly ($P < 0.01$) from 15.11 ± 6.7 ng/ml at the first treatment to 5.1 ± 2.7 ng/ml and 1.2 ± 0.6 ng/ml 24 and 72 h respectively after the second alizine treatment (Fig. 1B).

Discussion

The data obtained clearly indicate that all major components of the prostaglandin system are expressed in the utero/placental unit of the pregnant dog. However, the quantitative data obtained by real-time RT-PCR do

Figure 4 Expression of *PTGS2*, *PGFS*, and *PTGFR* as determined by real-time (TaqMan) PCR during pregnancy and normal luteolysis (A, C, and E) and during aglepristone-induced luteolysis (B, D, and F; compared with the mid-gestation group). RGE, relative gene expression (mean $\pm$ s.d.); bars with different asterisks differ with $P < 0.001$ (A) or $P < 0.01$ (D), or $P < 0.05$ (C, E, and F). Figure B, bars with different letters differ with: a versus b, $P < 0.001$; a versus c $P < 0.05$.

not allow a distinction between the uterus and/or the placenta (trophoblast) as the site of origin except for the pre-implantation period when the placenta is not yet formed. During this period, *PTGS2*, *PGFS*, and *PTGES* are expressed on a low level and IHC located *PTGS2* solely to the myometrium. The relatively high expression of the $\text{PGF2}\alpha$ receptor *PTGFR* and of the PGE2 receptors, *PTGER2* and *PTGER4*, may be seen as signs of a high responsiveness of the uterus to $\text{PGF2}\alpha$ and PGE2 during the pre-implantation period.

Also expression of the PGR was high during the pre-implantation period and IHC located nuclear staining predominantly to uterine epithelial cells. Thus, the responsiveness of the epithelium to P_4 is maintained at least until implantation, securing the production of uterine milk (embryotrophe) and hence embryonic survival.

Following formation of the placenta, IHC localized expression of *PTGS2* to the epithelial trophoblast cells; staining was initially restricted to the invading trophoblast surrounding the large maternal vessels at the base of the placental labyrinth but had spread over the entire trophoblast in the period of prepartal luteolysis, an observation accompanied by a dramatic increase in the expression of the respective mRNA and coinciding with a substantial prepartal increase of PGFM. This points towards a functional interrelationship and the role of placental *PTGS2* as a rate-limiting factor in the provision of prepartal prostaglandins. Such a role of *PTGS2* as a rate-limiting factor has also been observed in the horse (Boerboom *et al.* 2004), where blocking of endometrial *PTGS2* expression by the conceptus at day 15 of early pregnancy prevented $\text{PGF2}\alpha$ -induced luteal regression allowing for continuation of pregnancy; along this line is the observation that neither *PGFS* nor *PTGES* – the essential downstream enzymes – were significantly upregulated both during the time of luteolysis in cyclic

animals and during early pregnancy. Hence, an important *PTGS2*-related mechanism leading to the suppression of uterine luteolytic $\text{PGF2}\alpha$ output during pregnancy in a horse has been postulated (Boerboom *et al.* 2004).

Based on our data and concerning the canine placenta, this increase must mainly originate in the trophoblast cells as ISH co-localized expression of *PGFS* to these cells. The observation that *PGFS* mRNA expression is higher during the post-implantation and mid-gestation periods may indicate that the expression of *PGFS* is regulated at the post-transcriptional level resulting in the low PGFM levels detected in peripheral plasma. On the other hand, decreased mRNA levels observed at prepartal luteolysis could be indicative for enhanced substrate turnover due to the increased *PTGS2* availability during this time. However, since no corresponding data on the *PGFS* expression at the protein level are yet available, no final conclusion can be drawn.

Though the reduction of PGH2 by 9,11-endoperoxidase reductase activity is the major route for $\text{PGF2}\alpha$ synthesis, it cannot be ruled out for sure that the formation of $\text{PGF2}\alpha$ is through the alternative pathway via 9-ketoprostaglandin reductase or 11-ketoprostaglandin reductase activity using PGH2 -derived PGE2 and PGD2 as substrates respectively (Asselin & Fortier 2000, Madore *et al.* 2003). Such an alternative $\text{PGF2}\alpha$ -biosynthetic pathway has been identified in cattle (Madore *et al.* 2003) in which at least three closely related enzymes possessing the *PGFS* activity have been characterized within the AKR1C subclass of aldo-keto reductases family (reviewed by Madore *et al.* (2003)); none of them is, however, upregulated during the time of luteolysis. As reported by the same group, also another previously described enzyme 20 α -hydroxysteroid dehydrogenase (AKR1B5) is capable of converting PGH2 into $\text{PGF2}\alpha$ and reveals an enhanced endometrial

Figure 5 Localization of *PGFS* (A), *PTGFR* (B), and *PTGES* (C) by *in situ* hybridization (ISH) in canine placenta during luteolysis. Strong mRNA signals for *PGFS*, *PTGFR*, and *PTGES* are observed in fetal trophoblast ($\Rightarrow$), specifically that invading large maternal blood vessels ($\blacktriangleright$). $\blacksquare$, Superficial endometrial glands (glandular chambers). The same mRNA localization pattern was observed after the aglepristone treatment.

expression in cyclic cows around the time of luteolysis (Madore *et al.* 2003). Similarly, in the rabbit the aldoketo reductase activity is associated with both the PGE 9-reductase and 20 α -hydroxysteroid dehydrogenase activity (Wintergalen *et al.* 1995).

Following these observations, further studies concerning the alternative PGF2 α -biosynthetic pathways in canine placental compartment should be considered.

Similar to PGF2 α , PGE2 also seems to originate from the trophoblast cells as ISH located mRNA expression of *PTGES* to these cells, with the expression being significantly elevated during prepartal luteolysis. As there are no data on the expression on the protein level and PGE2 plasma concentration, this observation must be interpreted very carefully and only seen as a hint that the increased availability of *PTGS2* during prepartal luteolysis is associated with a basic increased capacity to release PGE2 by the placental compartment during prepartal luteolysis. Within the utero/placental unit, the trophoblast seems to be the major target for PGF2 α and PGE2 as ISH localized their receptors primarily to these cells.

Interestingly, both *PTGFR* and *PTGER2* mRNA were upregulated during prepartal luteolysis, while *PTGER4* mRNA was downregulated. This allows the conclusion that prepartal trophoblast function is affected by PGF2 α and PGE2, triggering different signalling cascades possibly in relation to the mechanisms allowing for final placental maturation and release. The expression of PGE2 receptors, EP1 and EP3, was either not detectable or only very weak in the canine corpus luteum (Kowalewski *et al.* 2008b) pointing towards its organ/species-specific expression as has also been shown for the mouse CL (Segi *et al.* 2003). Hence, our conclusions on the role of PGE2 in regulation of canine placenta function would require further information on the expression of these receptors in the canine uterine/placental compartment.

Taken together, our data suggest a paracrine/autocrine role of the *PTGS2*-derived arachidonic acid metabolites in the processes of canine decidualization and placentation. Such an autocrine/paracrine effect of PGF2 α has been previously proposed in bovine placentomes from early to late gestation (Arosh *et al.* 2004). Similarly, PGE2 has been shown to be involved in the autocrine/paracrine regulation of decidualization in mice (Pakrasi & Jain 2008). During the later stages of pregnancy and prepartal luteolysis, they might contribute to the final maturation and release of the placenta.

In addition, the increased availability of PGE2 in the prepartal period could be utilized as a substrate for PGF2 α synthesis (Asselin & Fortier 2000, Madore *et al.* 2003); further studies are, however, necessary to support this conclusion.

As we have recently reported (Kowalewski *et al.* 2009) and except for the upregulation of *PTGER2* mRNA, the expression patterns of luteal *STAR*, *HSD3B*, *PTGS2*, *PGFS*, *PTGES*, *PTGER4*, and of the *PGR* observed during antiprogesterin (aglepristone)-induced luteolysis resemble those of physiological luteal regression. Hence, the hypothesis was forwarded (Kowalewski *et al.* 2009) that luteal P₄ acts as an autocrine/paracrine factor within a

Figure 6 Expression of *PTGES*, *PTGER2*, and *PTGER4* as determined by real-time (TaqMan) PCR during pregnancy and normal luteolysis (A, C, and E) and during aglepristone-induced luteolysis (B, D, and F; compared with the mid-gestation group). RGE, relative gene expression (mean $\pm$ s.d.); bars with different asterisks differ with $P < 0.001$ (A) or $P < 0.01$ (B and D), or $P < 0.05$ (E and F). Figure C, bars with different letters differ with: a versus b, $P < 0.001$; b versus c, $P < 0.05$.

positive loop feedback mechanism involving STAR and HSD3B, and that luteal regression is not an actively controlled but rather permissive process related to aging of the CL as observed by electron microscopy (Hoffmann *et al.* 2004).

Following treatment with aglepristone on day 58 of pregnancy, Baan *et al.* (2008) observed an increase of PGFM parallel to a decrease of P_4 levels. Our studies with aglepristone treatment between days 40 and 45 of pregnancy confirm this observation. Interestingly, also in the utero/placental unit, all changes observed concerning mRNA expression of the prostaglandin system after treatment with the antiprogesterone aglepristone were in the same direction as observed during prepartal luteolysis. As the PGR is only expressed by the maternal stroma-derived decidual cells as observed by Vermeirsch *et al.* (2000) and confirmed in this study, these cells must have an important signalling function in respect to induction of luteolysis and prepartal preparation of the utero/placental unit for parturition.

The induction of prepartal luteolysis might then be brought upon by the fact that CL-derived P_4 levels reach a critical lower threshold during the course of luteal regression affecting the crosstalk between the maternal decidual cells and the fetal trophoblast, leading to prepartal activation of the prostaglandin system as evidenced by upregulation of *PTGS2* during prepartal luteolysis.

Our results also provide an explanation of the observation that aglepristone-induced parturition in the dog may require or may not require ecobolic support (Hoffmann *et al.* 1999, Baan *et al.* 2005). As provision of adequate amounts of *PTGS2* seems to be restricted to the immediate phase prior to parturition, the time point of treatment with the antiprogesterone seems to be the critical issue.

In summary, these observations point to an essential role of P_4 not only in respect to maintenance of pregnancy

by securing uterine quiescence, but also by acting as an important autocrine/paracrine and also endocrine factor in respect to luteal regression and luteolysis.

Materials and Methods

Animals and tissue samples

Animal experiments were performed in accordance with animal welfare legislation (permit no. II 25.3-19c20-15c GI 18/14 and VIG3-19c20/15c GI 18,14 (Gießen) and permit no. Ankara 2006/06 (Faculty of Veterinary Medicine, University of Ankara)), and all utero/placental tissue samples were obtained together with the collection of CL as described earlier (Kowalewski *et al.* 2009).

Normal pregnancies

Eighteen clinically healthy pregnant bitches of different breeds (aged 2–8 years) were divided into four groups, and utero/placental unit samples were collected via ovariohysterectomy (OHE) on one of the following days of pregnancy:

- Group 1; pre-implantation, days 8–12, $n=5$.
- Group 2; post-implantation, days 18–25, $n=5$.
- Group 3; mid-gestation, days 35–40, $n=5$.
- Group 4; prepartal, progesterone decline, $n=3$.

In group 4, P_4 concentrations were monitored every 6 h beginning on day 58 of pregnancy, and OHE was performed when P_4 continued to decrease below 3 ng/ml in two consecutive measurements.

Induced abortion

Ten bitches were treated with the antiprogesterone aglepristone between 40 and 45 days of pregnancy using the dose recommended for induction of abortion (10 mg/kg bw, 2 times 24 h apart). OHE was performed 24 h ($n=5$) and 72 h ($n=5$) after the second injection.

Tissue preservation and determination of P_4 and PGFM

Immediately after their removal, tissue samples were washed with PBS, trimmed off the surrounding connective tissue, immersed for 24 h in RNAlater (Ambion Biotechnologie GmbH, Wiesbaden, Germany), and then stored at -80°C until further use. For IHC and ISH, tissue samples were fixed for 24 h in 10% neutral phosphate-buffered formalin, washed with PBS, dehydrated in a graded ethanol series, and embedded in paraffin-equivalent Histo-Comp (Vogel, Giessen, Germany).

Established RIA procedures were used for the assay of peripheral blood plasma progesterone (Hoffmann *et al.* 1973) and the prostaglandin $F_{2\alpha}$ metabolite PGFM (Kindahl *et al.* 1976, Granström & Kindahl 1982).

RNA extraction, semi-quantitative real-time (TaqMan) PCR, and data evaluation

Total RNA was isolated from utero/placental unit samples using TRIzol Reagent (Gibco-BRL, Life Technologies) and treated with DNase I recombinant, RNase-free, to eliminate genomic DNA contaminations (Roche Molecular Biochemicals) according to the manufacturer's instructions.

The primers were ordered from MWG Biotech AG; the 6-carboxyfluorescein (6-FAM) and 6-carboxytetra methylrhodamine (TAMRA)-labeled probes were from Eurogentec, B-4102 Seraing, Belgium. Semi-quantitative real-time PCR experiments and the comparative C_t method ($\Delta\Delta C_t$ method) for the relative quantification of genes were performed as previously described (Kowalewski *et al.* 2006a). The results are expressed as the fold change in gene expression over the calibrator.

Sequences for primers and TaqMan probes were as follows:

<i>PTGS2</i> (forward):	5'-GGA GCA TAA CAG AGT GTG TGA TGT G-3';
<i>PTGS2</i> (reverse):	5'-AAG TAT TAG CCT GCT CGT CTG GAA T-3';
<i>PTGS2</i> (TaqMan probe):	5'-CGC TCA TCA TCC CAT TCT GGG TGC T-3';
<i>PTGES</i> (forward):	5'-CTG TCA TCA CCG GCC AAG T-3';
<i>PTGES</i> (reverse):	5'-CCT GGT CAC TCC GGC AAT A-3';
<i>PTGES</i> (TaqMan probe):	5'-ACG CCC TGA GAC ACG GAG GCC T-3';
<i>PTGER2</i> (forward):	5'-CAC CCT GCT GCT GCT TCT C-3';
<i>PTGER2</i> (reverse):	5'-CGG TGC ATG CGG ATG AG-3';
<i>PTGER2</i> (TaqMan probe):	5'-TGC TCG CCT GCA ACT TTC AGC GTC-3';
<i>PTGER4</i> (forward):	5'-AAA TCA GCA AAA ACC CAG ACT TG-3';
<i>PTGER4</i> (reverse):	5'-GCA CGG TCT TCC GCA GAA-3';
<i>PTGER4</i> (TaqMan probe):	5'-ATC CGA ATT GCT GCT GTG AAC CCT ATC C-3';
<i>PGFS</i> (forward):	5'-AGG GCT TGC CAA GTC TAT TGG-3';

<i>PGFS</i> (reverse):	5'-GCC TTG GCT TGC TCA GGA T-3';
<i>PGFS</i> (TaqMan probe):	5'-TCC AAC TTT AAC CGC AGG CAG CTG G-3';
<i>PTGFR</i> (forward):	5'-ACC AGT CGA ACA TCC TTT GCA-3';
<i>PTGFR</i> (reverse):	5'-GGC CAT CAC ACT GCC TAG AAA-3';
<i>PTGFR</i> (TaqMan probe):	5'-CAT GGT GTT CTC CGG TCT GTG CCC-3';
<i>PGR</i> (forward):	5'-CGA GTC ATT ACC TCA GAA GAT TTG TTT-3';
<i>PGR</i> (reverse):	5'-CTT CCA TTG CCC TTT TAA AGA AGA-3';
<i>PGR</i> (TaqMan probe):	5'-AAG CAT CAG GCT GTC ATT ATG GTG TCC TAA CTT-3';
<i>GAPDH</i> (forward):	5'-GCT GCC AAA TAT GAC GAC ATC A-3';
<i>GAPDH</i> (reverse):	5'-GTA GCC CAG GAT GCC TTT GAG-3';
<i>GAPDH</i> (TaqMan probe):	5'-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3'.

Immunohistochemical staining

Owing to the restricted availability of canine-specific and/or cross-reacting antibodies, the immunohistochemical detection of target genes was limited to *PTGS2* and the *PGR*.

Utero/placental cross sections were cut (4 μm thick) and mounted on SuperFrost Plus microscope slides (Menzel-Gläser; Braunschweig, Germany). The standard immunoperoxidase detection method applied was as previously described (Kowalewski *et al.* 2006a). The antibodies used were: monoclonal mouse anti-rat *PTGS2* IgG, clone 33, BD Pharmingen (Heidelberg, Germany) and mouse mAb generated against amino acids 922–933 (C-terminal) of the human *PGR*, clone 10A9, Immunotech, Hamburg, Germany. An isotype-specific irrelevant mAb IgG1 (Dianova, Hamburg, Germany) was used as a negative control. The biotinylated horse anti-mouse IgG BA2000 was from Vector Laboratories Inc., Burlingame, CA, USA. The peroxidase activity was detected with NovaRed substrate kit according to the manufacturer's instructions (Vector Laboratories). Finally, sections were counterstained with hematoxylin and mounted with Histokit (Assistant, Osterode, Germany).

In situ hybridization

The following primers derived from canine-specific gene sequences were used in RT-PCR to generate templates for subsequent cRNA probe synthesis:

<i>PGFS</i> (forward):	5'-GAT CTC TGT GCC ACA TGG GAG-3';
<i>PGFS</i> (reverse):	5'-TGG GTC CTT CAG GAG AAC TGG-3';
<i>PTGFR</i> (forward):	5'-TGT GCC CAC TTT TTC TAG GC-3';
<i>PTGFR</i> (reverse):	5'-TCT TCC CAG TCT TTG ATG TG-3';
<i>PTGES</i> (forward):	5'-ACC ATC TAC CCC TTC CTG T-3';
<i>PTGES</i> (reverse):	5'-CTG CTT CCC AGA CGA TCT -3';

PTGER2 (forward): 5'-TTC TCC TGG CTA TTA TGA CC-3';
PTGER2 (reverse): 5'-ATC TAC TGG CGT TTG ACT G-3';
PTGER4 (forward): 5'-GGT ACG GGT GTT CAT CAA C-3';
PTGER4 (reverse): 5'-AGA GGA GGG TCT GAG ATG TG-3'.

The length of the amplicons was: 235, 291, 214, 273, and 323 bp for PGFS, PTGFR, PTGES, *PTGER2*, and *PTGER4* respectively.

The further procedure was as previously described (Kowalewski *et al.* 2006b). Briefly, the PCR products were cloned into the pGEM-T plasmid (Promega). Digestion of the harvested pGEM-T plasmid clones containing the respective inserts was by using the restriction enzymes *Nco*I (antisense cRNA) and *Not*I (sense cRNA; New England Biolabs, Frankfurt, Germany). The synthesis of digoxigenin (DIG)-labeled cRNA was achieved with DIG RNA labeling kit from Roche Molecular Biochemicals. Semi-quantitation of the DIG-labeled cRNA was by dot blot analysis of serial dilutions of cRNA probes on a positively charged Nylon Membrane (Roche Molecular Biochemicals). Paraffin-embedded utero/placental cross sections were mounted on the SuperFrost Plus slides (Menzel Glaeser, Braunschweig, Germany), dewaxed, digested with 70 µg/ml proteinase K (Sigma-Aldrich Chemie GmbH), and postfixed with 4% paraformaldehyde and then processed according to the procedure by Lewis & Wells (1992) and Klonisch *et al.* (1999). Detection of the DIG-labeled cRNA probes was by alkaline phosphatase-conjugated sheep anti-DIG Fab fragments (Roche Molecular Biochemicals) diluted 1:5000 in 1% ovine serum followed by incubation with the substrate 5-bromo-4-chloro-3-indolyl phosphate in the presence of nitroblue tetrazolium (Roche Molecular Biochemicals).

Statistical analysis

To test for an effect of time in the respective group, a parametric one-way ANOVA was applied. Multiple comparison post-tests were performed in case of $P < 0.05$. Those were Tukey–Kramer multiple comparison test in experiments measuring the expression of target genes during the course of pregnancy and Dunnett's multiple comparison test in experiments showing the expression of genes after aglepristone-induced luteolysis. In the latter, the results present the fold change in gene expression compared to its expression at mid-gestation. Owing to the uneven distribution of the real-time data obtained for the *PTGS2*, *PGFS*, and *PTGER4*, data were analyzed using the Kruskal–Wallis test (a nonparametric ANOVA) followed by Dunn's multiple comparison test. Numerical data were presented as the mean $\pm$ s.d. For all tests, the statistical software program, GraphPad 3.06 (GraphPad Software, Inc., San Diego, CA, USA) was used.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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