Leukotrienes modulate secretion of progesterone and prostaglandins during the estrous cycle and early pregnancy in cattle: an *in vivo* study

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Abstract

Recently, we showed that leukotrienes (LTs) regulate ovarian cell function *in vitro*. The aim of this study was to examine the role of LTs in corpus luteum (CL) function during both the estrous cycle and early pregnancy *in vivo*. mRNA expression of LT receptors (*BLT* for LTB_4 and *CYSLT* for LTC_4), and 5-lipoxygenase (*5-LO*) in CL tissue and their localization in the ovary were studied during the estrous cycle and early pregnancy. Moreover, concentrations of LTs (LTB_4 and C_4) in the CL tissue and blood were measured. *5-LO* and *BLT* mRNA expression increased on days 16–18 of the cycle, whereas *CYSLT* mRNA expression increased on days 16–18 of the pregnancy. The level of LTB_4 was evaluated during pregnancy compared with the level of LTC_4 , which increased during CL regression. LT antagonists influenced the duration of the estrous cycle: the LTC_4 antagonist (azelastine) prolonged the luteal phase, whereas the LTB_4 antagonist (dapsone) caused earlier luteolysis *in vivo*. Dapsone decreased progesterone (P_4) secretion and azelastine increased P_4 secretion during the estrous cycle. In summary, LT action in the bovine reproductive tract is dependent on LT type: LTB_4 is luteotropic during the estrous cycle and supports early pregnancy, whereas LTC_4 is luteolytic, regarded as undesirable in early pregnancy. LTs are produced/secreted in the CL tissue, influence prostaglandin function, and serve as important factors during the estrous cycle and early pregnancy in cattle.

Reproduction (2010) 140 767–776

Introduction

The corpus luteum (CL) is the principal source of progesterone (P_4) in mammals. P_4 supports the secretory functions of the endometrium, which sustain early embryonic development, implantation, and placentation (Niswender *et al.* 2000). In cows, the main signal for pregnancy recognition and CL maintenance is interferon τ (IFNT; Thatcher *et al.* 2001, Spencer *et al.* 2007, Bazer *et al.* 2008). Nevertheless, the bovine conceptus produces numerous signals during early pregnancy including steroids, prostaglandins (PGs), and other proteins (Lewis *et al.* 1982, Eley *et al.* 1983, Thatcher *et al.* 1984). Uterine and ovarian PGs are considered to be important factors for regulating reproductive events such as ovulation, luteolysis, embryo implantation, and maintaining pregnancy (Weems *et al.* 2006). Generally, PGE_2 acts in luteotropic and luteoprotective ways, which leads to lengthening of the lifespan of the CL and P_4 production (Weems *et al.* 1997, Kotwica *et al.* 2003), whereas $PGF_{2\alpha}$ is the main luteolytic agent in ruminants (McCracken *et al.* 1999). Thus, in signaling from the conceptus to the maternal system for maternal

recognition of pregnancy, the correct contact between IFNT and all ovarian and uterine factors, such as P_4 and PGs, plays a pivotal role (Arosh *et al.* 2004, Weems *et al.* 2006). In this study, we examine the action of leukotrienes (LTs) as another potential trigger of reproductive processes in cattle.

LTs are synthesized by 5-lipoxygenase (*5-LO*) and are commonly known as potential inflammatory factors that cause edema in respiratory tract diseases, but they also have roles in reproduction and may enhance the action of PGs (Samuelsson 2000). Receptors for LTs are classified into two separate groups with respect to structure and cell location: BLT for LTB_4 and CYSLT for cysteinyl LTs (LTC_4 , LTD_4 , and LTE_4 ; Izumi *et al.* 2002). In humans, there are two isoforms of receptor for LTB_4 (LTB_4R and LTB_4R2 , also known as BLT1 and BLT2; Tager & Luster 2003) and at least two receptors for cysteinyl LTs (CYSLTR1 and CYSLTR2; Jones & Rodger 1999), whereas there are no data about the protein expression/localization of LT receptors in the bovine reproductive tract. LTs play a role in the bovine reproductive tract: intraluteal infusion of $PGF_{2\alpha}$ by a microdialysis system on day 12 of the estrous cycle was

shown to increase the levels of both LTB_4 and C_4 in CL perfusate (Blair *et al.* 1997). The secretory functions of bovine CL cells were shown to be regulated not only by PGs but also by LTs (Milvae *et al.* 1986, Korzekwa *et al.* 2010a, 2010b). We recently showed that mRNA for 5-LO and LT receptors is expressed in ovarian cell types, including steroidogenic and endothelial CL cells and granulosa cells (Korzekwa *et al.* 2010b). LTs are found to be auto/paracrine factors modulating the secretory functions of ovarian cells depending on the stage of the cycle and type of LT. LTB_4 seems to play a luteotropic role in the CL, stimulating P_4 and PGE_2 secretions, whereas LTC_4 stimulates the secretion of luteolytic $PGF_{2\alpha}$ and may enhance the luteolytic cascade within the bovine CL steroidogenic cells (Korzekwa *et al.* 2010a). These *in vitro* results need to be supported by *in vivo* studies to help us understand the complete effects of LT action on the bovine reproductive tract during the estrous cycle. Moreover, there is a lack of knowledge about the role of LTs in early pregnancy.

The main goal of this study was to determine and compare the actions of endogenous LTs at the end of luteal phase of the estrous cycle and during early pregnancy in cattle. We monitored the concentrations of selected LTs, B_4 and C_4 , in the plasma and CL tissue and we evaluated their production and expression in the CL tissue during the estrous cycle compared with early pregnancy. Furthermore, we examined the influence of endogenous LTs on the CL lifespan and function, as well as secretory function in the reproductive tract on day 16 of cycle/pregnancy by measuring P_4 and PG (PGE_2 and PGFM) concentrations in the blood samples after infusion of selective antagonists of LTB_4 and C_4 into the aorta abdominalis.

Results

Experiment 1: LT mRNA expression and production in the bovine CL tissue and its localization in the ovary during the estrous cycle and in early pregnancy

Figure 1 shows mRNA expression for LTRs (*BLT* and *CYSLT*) and 5-LO in the CL tissue during the estrous cycle and early pregnancy. *BLT* mRNA expression was upregulated on days 16–18 of pregnancy ($P<0.05$), whereas *CYSLT* mRNA expression was higher on days 8–10 and 16–18 of the estrous cycle ($P<0.05$). 5-LO mRNA expression was upregulated on days 16–18 of pregnancy ($P<0.05$).

Light microscopic observations for immunohistochemical staining of *BLT* and *CYSLT* and 5-LO localization in ovarian tissue on days 8–10 of the estrous cycle as a representative period are shown in Fig. 2. *BLT* and *CYSLT* and 5-LO were not only present in theca and stromal cells and in the area of preantral follicles but also in epithelial cells, smooth muscle cells of ovarian aortas, and in steroidogenic luteal cells of the CL.

LTB_4 concentration remained unchanged throughout the cycle (days 2–4, 8–10, and 16–18, $P>0.05$; Fig. 3) but increased on days 8–10 and 16–18 of pregnancy ($P<0.05$). LTC_4 concentration was higher on days 16–18 of the cycle and lower on days 8–10 and 16–18 of pregnancy ($P<0.05$).

Experiment 2: LTB_4 and C_4 concentrations in the blood during the estrous cycle and early pregnancy

Figure 4 shows LTB_4 and C_4 concentrations during the estrous cycle and early pregnancy in blood collected from jugular vein on days 0, 2, 8, 10, 15, 16, 17, and 21.

During the cycle, the level of LTC_4 increased between days 15 and 17 of the estrous cycle ($P<0.05$), whereas

Figure 1 Pattern of expression of (a) *BLT/LTR1*, (b) *CYSLT/LTR11*, and (c) *5-LO* mRNA on the selected days of the estrous cycle (white bars) and pregnancy (black bars) in the bovine CL tissue. Data are expressed as arbitrary units of respective mRNA/GAPDH mRNA. Different letters (a, b, and c) indicate statistical differences ($P<0.05$) in the quantitative mRNA expression between groups of cyclic or pregnant animals and statistical differences in the quantitative mRNA expression between groups of animals on the same days of the estrous cycle and pregnancy, as determined by two-way ANOVA followed by Bonferroni's multiple comparison test.

Figure 2 Representative immunohistochemical localization of BLT (A and D), CYSLT (B and E), and 5-LO (C) in ovary on days 16–18 of the estrous cycle, and a section incubated with rabbit IgG as a negative control (F). Bars: 20 and 100 µm. Arrows indicate the most intensive histochemical reactions. T, theca cells; PAF, preantral follicle; S, stromal cells; V, vessel; A, aorta; EC, endothelial cells; E, epithelial cells; M, smooth muscle cells; L, luteal CL cells.

the level of LTB_4 during the cycle remained unchanged ($P > 0.05$). During pregnancy, the level of LTC_4 was stable ($P > 0.05$), whereas the level of LTB_4 increased from day 8 ($P < 0.05$).

Experiment 3: effects of LT antagonists on P_4 and PGs output and CL lifespan in vivo

Preliminary study: establishing the effective dose of LTB_4 and LTC_4 antagonists

P_4 concentrations in blood plasma of control and experimental cows during the 24 h following LT infusion are shown in Fig. 5a and b. Azelastine at doses of 10 and 100 µg did not influence P_4 concentration in blood plasma in comparison with the control cows (Fig. 5a; $P > 0.05$). Azelastine at a dose of 250 µg elevated secretion of P_4 in the blood plasma compared with the control cows between 10 and 18 h following infusion ($P < 0.05$; Fig. 5a). Although dapsone at a dose of 10 µg temporarily increased the level of P_4 at 2, 3, 8, and 18 h of the experiment ($P < 0.05$; Fig. 5b), a dose of 100 µg did not influence secretion of P_4 in the blood plasma compared with the control cows ($P > 0.05$; Fig. 5b). Dapsone at a dose of 250 µg inhibited P_4 secretion from

1 h after infusion ($P < 0.05$; Fig. 5b). Additionally, in order to establish the effective dose of LT antagonists, the PG levels were measured (data not shown because no changes were observed following doses of 10 and 100 µg for both LT antagonists).

Effects of LT antagonists on P_4 and PG secretion, and on the lifespan of the CL during the estrous cycle and early pregnancy

The effects of azelastine and dapsone infusion (250 µg doses, as selected in the preliminary study) on the lifespan of the CL during the a) estrous cycle and b) early pregnancy are shown in Fig. 6. Azelastine at a dose of 250 µg was capable of prolonging the luteal phase of the estrous cycle for more than 30 days by increasing the lifespan of the CL ($P < 0.05$; Fig. 6a). On the contrary, dapsone at a dose of 250 µg shortened the length of the estrous cycle to 17 ± 0.8 days compared with saline-treated cows (21 ± 0.9 , $P < 0.05$; Fig. 6a). Azelastine did not influence CL function during early pregnancy ($P > 0.05$; Fig. 6b). Dapsone decreased the level of P_4 from day 21 to day 30–32 (from 10.25 ± 1.5 to 2.5 ± 1.1 ng/ml) of pregnancy ($P > 0.05$; Fig. 6b).

Figure 3 Concentrations of (a) LTB₄ and (b) LTC₄ in the CL tissue on the selected days of the estrous cycle (white bars) and pregnancy (black bars). Data are expressed as ng/g of the CL tissue. Different letters (a and b) indicate statistical differences ($P < 0.05$) in the LT concentration between groups of cyclic or pregnant animals, as determined by two-way ANOVA followed by Bonferroni's multiple comparison test.

and the absence of pregnancy was confirmed (by ultrasonography, USG) on day 36.

Figure 7 shows P₄, PGE₂, and PGFM concentrations in blood plasma of control and experimental cows after infusion of azelastine and dapsone (250 µg) on day 16 of the estrous cycle (Fig. 7a–c) and pregnancy (Fig. 7d–f). During the estrous cycle, azelastine stimulated P₄ output between 10 and 18 h after infusion ($P < 0.05$; Fig. 7a). Dapsone inhibited P₄ output from 1 h after infusion ($P < 0.05$; Fig. 7a). During pregnancy, neither antagonist influenced secreted P₄ in blood plasma ($P > 0.05$; Fig. 7d).

During the estrous cycle, azelastine at a dose of 250 µg stimulated PGE₂ output at 0.5 h after infusion and from 10 h onward ($P < 0.05$; Fig. 7b). However, dapsone at a dose of 250 µg did not influence secreted PGE₂ in blood plasma compared with the control cows and PGE₂ output during the first 24 h of experiment in the cycle ($P > 0.05$; Fig. 7b). During pregnancy, the same LTB₄ antagonist decreased PGE₂ output and secretion at 1 h after infusion and from 4 h onward ($P < 0.05$; Fig. 7e), whereas azelastine did not alter PGE₂ secretion in blood plasma compared with the control cows and PGE₂ output during the 24 h after infusion (Fig. 7e; $P > 0.05$).

Dapsone increased PGFM output in comparison to the control cows during the 24 h following infusion in the estrous cycle ($P < 0.05$; Fig. 7c). On the other hand,

azelastine did not influence PGFM concentration in blood plasma in comparison to the control cows (Fig. 7c; $P > 0.05$). In pregnant cows, dapsone increased PGFM concentration in comparison to the control cows during the 24 h of the experiment ($P < 0.05$; Fig. 7f), whereas PGFM output was not affected by azelastine ($P > 0.05$; Fig. 7f).

Discussion

In the present study, we found that LTs are produced and released in the bovine CL, and they modulate the action of P₄ and PGs in the bovine reproductive tract during the estrous cycle and early pregnancy. Our results revealed that LTs influence the lifespan of the CL during the estrous cycle and early pregnancy depending on the type of LT.

The highest levels of mRNA expression for *LTRs* and *5-LO* in the CL tissue were observed on days 16–18 of both the estrous cycle and early pregnancy. We have previously described a similar pattern of mRNA expression of *LTRs* and *5-LO* and production/secretion of LTs during the cycle in the two main cell populations of the bovine CL: steroidogenic and endothelial cells (Korzekwa *et al.* 2010a, 2010b). The highest mRNA expression levels for *CYSLT* and *5-LO* were found on days 14–16 of the cycle in endothelial CL cells, whereas *BLT* mRNA expression did not differ among cell types, which indicated that endothelial cells have the greatest potential for LT production among ovarian cells and are the main source of LTs in the bovine ovary (Korzekwa *et al.* 2010a, 2010b). Nevertheless, Chegini & Rao (1988) showed numerous binding sites for LTC₄ in steroidogenic CL cells between 1.5 and 3 months of pregnancy in cattle. Immunostaining for *LTRs* and *5-LO* showed that they were localized to ovarian vessels and luteal cells of the CL but also in ovarian follicles, which suggests a role for LTs in granulosa cells. According to the hypothesis of Brännström & Enskog (2002), immune

Figure 4 Profiles of LTB₄ and C₄ levels in peripheral blood plasma of cows collected on selected days of the cycle/pregnancy. Asterisks indicate statistical differences ($P < 0.05$) between LTB₄- and LTC₄-treated groups at the same time of the experiment, as determined by one-way ANOVA followed by repeated measurement ANOVA tests followed by Bonferroni's multiple comparison test.

Figure 5 Concentrations of progesterone in peripheral blood plasma of cows infused with saline (gray bars) and various doses of the LT antagonists (a) azelastine and (b) dapsone. All reagents were infused into the aorta abdominalis. Asterisks indicate statistical differences ($P < 0.05$) between control and treated groups in the same time of collection, as determined by repeated measurement ANOVA tests followed by Bonferroni's multiple comparison test.

cells (macrophages, monocytes, and leukocytes) infiltrate the ovary and secrete cytokines during the ovulation. Cytokines affect nonsteroidogenic ovarian cells, causing the production/secretion of ovulation mediators, such as metabolites of arachidonic acid, i.e. PGs and LTs. Thus, LTs acting as autocrine or paracrine factors within the bovine CL may be involved not only in processes connected with vasculature, but also with steroidogenic and granulosa cell functions (Korzekwa *et al.* 2010a, 2010b).

Our data indicate that endogenous LTC_4 inhibits P_4 secretion in the estrous cycle, since azelastine elevated P_4 output. On the contrary, endogenous LTB_4 action enhances P_4 secretion, since dapsone inhibited P_4 output in the estrous cycle. Nevertheless, the role of LTs in early pregnancy seems to be directly connected with PGs because blockade of endogenous LTs by both inhibitors had no effect on changes in P_4 levels during the 24 h after infusion. Although LTs affected the CL lifespan in early pregnancy, the effect of LT action required a longer time after blockade of endogenous LTs by injection of LT antagonists. It is obvious that some factors mediate the action of PGs (Niswender *et al.* 2000). LTs of ovarian and/or uterine origin are suggested to be one of these mediators. It would be interesting to know whether LTs directly influence P_4 production/secretion in the CL, both during the estrous cycle and in the process of embryo implantation during early pregnancy. Nevertheless, based on these *in vivo* results

we are not able to fully understand the molecular interactions between LTs and P_4 . Recent studies have shown that hydroxysteroid (11- β) dehydrogenase 1 (HSD11B1) action is dependent on PGs and P_4 in the period of embryo implantation in cattle (Simmons *et al.* 2010). Further studies are needed to determine whether LTs affect the expression of HSD11B1. We showed that the role of LTs in the estrous cycle is strictly type dependent, since LTC_4 inhibited P_4 action, whereas LTB_4 promoted P_4 action in the bovine reproductive tract *in vivo*. In addition, LT levels measured in plasma during the estrous cycle and early pregnancy confirmed the specification of endogenous LTs dependently on its type in the reproductive tract. The LTB_4 level was elevated during early pregnancy, whereas the LTC_4 level was highest on days 15–17 in plasma and days 16–18 in the CL tissue during the estrous cycle. The levels of both PGs changed after infusion of LTs, both in the estrous cycle and in early pregnancy during the 24 h of the experiment. The LTC_4 antagonist elevated PGE_2 output in the estrous cycle, whereas LTB_4 antagonist elevated PGFM output both in the cycle and pregnancy. An *in vitro* study carried out on steroidogenic and endothelial bovine CL cells on days 14–16 of the cycle showed that LTs increased secretion of PGE_2 as well as $PGF_{2\alpha}$ (Korzekwa *et al.* 2010b). Moreover, it is likely that the effect of LT secretion on steroidogenesis in the bovine CL is indirect and is mediated/supported by PGs and/or

Figure 6 Progesterone levels in peripheral blood plasma of cows during (a) the estrous cycle and (b) early pregnancy after infusion of saline (gray bars), 250 μ g azelastine (line), or 250 μ g dapsone (dotted line). All reagents were infused into the aorta abdominalis. Asterisks indicate statistical differences ($P < 0.05$) between control and treated groups in the same time of experiment, as determined by repeated measurement ANOVA tests followed by Bonferroni's multiple comparison test.

Figure 7 Concentrations of (a and d) progesterone, (b and e) prostaglandin E_2 , and (c and f) 13,14-dihydro,15-keto- $PGF_{2\alpha}$ in peripheral blood plasma of cows infused with saline (gray bars), 250 μ g azelastine (line), or 250 μ g dapsone (dotted line) on day 16 of the estrous cycle and early pregnancy. All reagents were infused into the aorta abdominalis. Asterisks indicate statistical differences ($P < 0.05$) between control and treated groups in the same time of experiment, as determined by repeated measurement ANOVA tests followed by Bonferroni's multiple comparison test.

cytokines during the estrous cycle. Based on the results, we postulate that LT function in early pregnancy is rather directly connected with the implantation and development of the embryo, and at least with the action of PGs. The results obtained showed the opposing action of the endogenous LTs, LTB_4 and C_4 , in the bovine reproductive tract, because the LT antagonists used blocked LT action. Such a contrary action of LTs may be the consequence of other factors that are involved in transcriptional and translational modifications of LTs, and may influence the role of LTs in reproductive processes (Murphy & Gijón 2007, Wittwer & Hersberger 2007). Therefore, further studies are needed to determine the role of LTs in the reproductive tract.

Our results showed that LTB_4 , not LTC_4 , is involved in early pregnancy in cattle because LTB_4 receptor mRNA expression and levels in the CL tissue were higher in pregnancy than in the estrous cycle. In particular, LTB_4 may interact with or mediate PGE_2 and/or IFNT in process of embryo implantation and modulate the action of PGE_2 , which plays a pivotal role in early pregnancy (Weems *et al.* 1997). Indeed, a previous study has shown that the highest level of LTA_4 hydrolase gene expression (which converts LTA_4 to LTB_4 and LTC_4) was observed in human CL cells at the mid-luteal stage and during pregnancy (Hattori *et al.* 1998). A high concentration of LTB_4 in human fetal membranes was shown by Ticconi *et al.* (1998) and LTB_4 enhanced human placental trophoblast cell function (Sato *et al.* 2008).

The data obtained in the current study strengthen the new concept that LTs are auto- and/or paracrine factors regulating reproductive processes in the CL during the estrous cycle and early pregnancy. The mechanisms controlling development, functionality, and regression of

the bovine CL involve many factors produced both inside and outside the CL (Niswender *et al.* 2000). We have demonstrated the production and secretion of LTs in the CL tissue and their localization in the ovary, and the influence of LTs on PG function during the estrous cycle and early pregnancy. Thus, LTB_4 is luteotropic during the estrous cycle and supports early pregnancy, whereas LTC_4 is luteolytic and its action is not desirable in early pregnancy.

Materials and Methods

All procedures were approved by the Local Animal Care and Use Committee, Olsztyn, Poland (agreement no. 58/2005/N).

A total of 84 healthy Holstein/Polish Black and White (75/25% respectively) cows (3 or 4 lactation) were used for the collection of the ovaries with CL in experiment 1, monitoring of the LT level in experiment 2, and checking the effects of LT antagonists on hormone output and CL lifespan in experiment 3. The animals were eliminated by the owners (Experimental Animal Farm of Polish Academy of Sciences in Baranowo and Agriculture Farm in Cieszymowo, Poland) from the dairy herds because of their lower milk production (years 2007–2009). The estrus of the cows was synchronized using two 5 mg $PGF_{2\alpha}$ analog (dinoprost, Dinolytic; Upjohn-Pharmacia N V S A) i.m. injections with an 11-day interval, as recommended by the vendor. The development of follicles and changes in CL structure during the estrous cycle were monitored by a veterinarian via *per rectum* USG examination using a Draminski Animalprofi Scanner (Draminski Electronics in Agriculture, Olsztyn, Poland) and confirmed by observing signs of estrus (i.e. vaginal mucus and standing behavior). The onset of estrus was taken as day 0 of the estrous cycle. Only the cows with signs of estrus were chosen for the study.

Experimental procedure

Experiment 1: LT mRNA expression and production in the bovine CL tissue and localization in the ovary during the estrous cycle and in early pregnancy

The aim of the experiment was to compare whether there are changes in LT production in the bovine CL during the estrous cycle and early pregnancy.

Bovine ovaries ($n=20$) were obtained from cows divided into two groups: pregnant and cyclic animals. The animals allocated to the pregnant group underwent artificial insemination with semen from the same bull. Ovarian tissue was obtained at a local slaughterhouse (Zakłady Miesne 'Warmia', Biskupiec, Poland) within 20 min of exsanguination and was transported on ice to the laboratory within 40 min. Estimation of the stages of the estrous cycle/pregnancy was monitored as mentioned (the reproductive history of all animals was known) and confirmed by macroscopic observation of the ovaries and uterus after slaughter (Miyamoto *et al.* 2000). Pregnancy was confirmed by flushing the uterus for embryo collection (Woclawek-Potocka *et al.* 2009).

Corpora lutea samples were collected on days 2–4 ($n=4$), 8–10 ($n=4$), and 16–18 ($n=4$) of the estrous cycle and days 8–10 ($n=4$) and 16–18 ($n=4$) of pregnancy. The days of the estrous cycle for evaluation of CL functionality were additionally confirmed by measurement of P_4 concentration in the peripheral blood. Pregnancy was confirmed as described previously (Woclawek-Potocka *et al.* 2009).

mRNA expression was quantitatively measured by real-time RT-PCR for 5-LO and LTB_4 and LTC_4 receptors (BLT and CYSLT respectively) as described previously (Korzekwa *et al.* 2010a). Concentrations of P_4 (control of CL stage, data not shown), LTB_4 , and LTC_4 were measured by enzyme immunoassay (EIA) in the CL tissues after extraction according to Korzekwa *et al.* (2008a). Immunohistochemistry was conducted in ovarian tissue for BLT, CYSLT, and 5-LO for observation of tissue compartments where LTs are produced.

Experiment 2: LTB_4 and C_4 concentrations in the blood during the estrous cycle and early pregnancy

The aim of the experiment was to follow and compare the changes in LTs concentration during the estrous cycle and early pregnancy. Peripheral blood samples were collected into tubes with 5 μ l EDTA and 1% aspirin solution (pH 7.3) from the jugular vein by syringe with needle on days 0, 2, 8, 10, 15, 16, 17, and 21 of the estrous cycle or pregnancy. Blood plasma was separated by centrifugation (2000 g; 10 min at 4 °C) and stored at –20 °C until P_4 , LTB_4 , and LTC_4 determinations were made ($n=12$).

Experiment 3: effects of LT antagonists on P_4 and PG output and CL lifespan in vivo

LT antagonists were used to test the hypothesis that LTs differentially affect the CL ($n=52$) function and lifespan during the estrous cycle and early pregnancy.

Preliminary study: establishing the effective dose of LTB_4 and LTC_4 antagonists. On day 15 of the subsequent estrous cycle,

catheters were inserted into the aorta abdominalis through the coccygeal artery of the cows for infusion of either saline ($n=4$) or LTB_4 and C_4 antagonists. A second catheter was inserted into the jugular vein for frequent collection of blood samples (Skarzynski *et al.* 2003a).

The LT antagonists were infused into the aorta abdominalis on day 16 of the estrous cycle: azelastine (an antagonist of LTC_4 ; azelastine hydrochloride, LKT-A9818-M100; Alexis Biochemicals, Lausen, Switzerland) and dapsone (an antagonist of LTB_4 ; 4,4'-diaminodiphenyl sulfone, LKT-ALX-270-090; Alexis Biochemicals) each in three doses: 10, 100, and 250 μ g ($n=4$ for each dose of LT antagonist) in 20 ml of saline. Peripheral blood samples were collected from the jugular vein before and for 24 h after treatment (18 times, beginning 2 h before the infusions as follows: –2, –1, –0.5, 0, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 21, and 24 h). After day 16 of the estrous cycle, blood was collected once daily until day 22 ($n=28$). The concentrations of P_4 and PGs (PGE_2 and PGFM; data not shown) in plasma samples were measured. Standing behavior was checked every 12 h after treatment to confirm the onset of estrus.

Effects of LT antagonists on P_4 and PG secretion and CL lifespan during the estrous cycle and early pregnancy

To determine the effects of LT antagonists on CL lifespan and P_4 levels during pregnancy, peripheral blood samples were collected from the jugular vein on days 0, 6, 9, 12, 15, 16, 17, 18, and 21 of the estrous cycle and days 0, 6, 9, 12, 15, 16, 17, 18, 21, 26, 32, and 36 of the pregnancy (embryo presence/absence was confirmed on day 32 of pregnancy by USG). The concentrations of P_4 in the plasma samples were measured after centrifugation, as in experiment 2.

To check the effect of blockade of endogenous LT actions on CL function during the estrous cycle and early pregnancy, saline ($n=8$) and 250 μ g (the dose selected in the preliminary study) dapsone ($n=8$) or azelastine ($n=8$) were infused into the aorta abdominalis on day 16 of the estrous cycle ($n=24$) or pregnancy ($n=24$, including 12 animals used from the preliminary study) according to the experimental design from the preliminary study. Peripheral blood samples were collected from the jugular vein before and for 24 h after infusion (18 times, beginning 2 h before the infusions). The concentrations of P_4 , PGE_2 , and PGFM in the plasma samples were measured.

Immunocytochemistry for LTB_4 receptor (BLT), LTC_4 receptor (CYSLT), and 5-LO protein

Cross sections of ovarian tissue samples were fixed in 4% paraformaldehyde in 0.1 M PBS (pH=7.4), washed in 0.1 M PBS, and cryoprotected in 18% sucrose. Immunostaining was carried out on consecutive sections. Afterwards, 10 μ m cryostat sections were stained for specific LT receptors and 5-LO. To block endogenous peroxidase, the sections were treated with hydrogen peroxide in methanol and washed in 0.1 M PBS. Then they were blocked with 10% normal goat serum for 1 h at room temperature, incubated overnight at room temperature with primary rabbit MAB raised against LTB_4 receptor (GTx71293; GeneTex Inc., Irvine, CA, USA), LTC_4 receptor

(APO6853PU-N; Acris GmbH, Herford, Germany), and 5-LO (160402 Cayman; Cayman Chemical Co., Ann Arbor, MI, USA), all diluted at 1:100, washed in PBS, incubated for 1 h at room temperature with 1:200 biotinylated anti-rabbit antibody (AK-5001 Vectastain ABC kit; Vector Laboratories, Peterborough, UK), washed, incubated for 45 min with ABC reagent in PBS, and washed again. Proteins were visualized by incubating the sections in 0.3 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co. Ltd) in 0.01% hydrogen peroxide in Tris-buffered saline (pH=7.2) for 2–3 min. Finally, sections were dehydrated and cover-slipped with DPX mounting medium (Park Scientific Ltd, Northampton, UK). To test the specificity of immunohistochemical staining, two types of controls were performed: i) the primary antibody was omitted during the immunostaining procedure; and ii) the primary antibody was substituted with nonspecific IgG. The observations and photographs were made using a light microscope (Nikon FXA, Tokyo, Japan). Classification of ovarian follicles was described according to Rodgers & Irving-Rodgers (2010).

Total RNA isolation

Total RNA was extracted from ovaries using Trizol reagent according to the manufacturer's instructions. One microgram of each sample of total RNA was reverse transcribed using the SuperScript First-Strand Synthesis System for RT-PCR (11904-018; Invitrogen), as described in the supplier's protocol.

Real-time PCR quantification

Quantitative fluorescence real-time PCR was performed using the Applied Biosystems 7300 System (Applied Biosystems, Foster City, CA, USA) with an SYBR Green PCR master mix (4367659 Power SYBR Green PCR Master Mix; Applied Biosystems) following the manufacturer's instructions. Real-time PCR (25 µl) included 12.5 µl SYBR Green PCR Master Mix, 0.5 µM each of sense and antisense primers, and reverse-transcribed cDNA (1 µl cDNA). The primers for 5-LO, BLT, and CYSLT mRNA expression were as detailed in a previous paper (Korzekwa *et al.* 2010a). For quantification, standard curves consisting of serial dilutions of the appropriate purified cDNA were plotted. Amplification was preceded by an initial denaturation step (15 min at 95 °C). The PCR programs for each gene were performed as follows: 40 cycles of denaturation (15 s at 95 °C), annealing (30 s at 56 °C), and elongation (60 s at 72 °C). After PCR, melting curves were acquired by stepwise increases at a temperature of 50–95 °C to ensure that a single product was amplified in the reaction. Control reactions in the absence of RT were performed to test for genomic DNA contamination. The specificity of the PCR products for examined genes was confirmed by gel electrophoresis and sequencing. The sequence homology obtained in the experiment was 99%. Dissociation curve analysis was performed after each real-time experiment to confirm the presence of only one product and the absence of the formation of primer dimers. Data were normalized to a calibrator sample using the $\Delta\Delta C_t$ method. Samples were run in triplicate, data are shown as the average fold increase, with S.E.M., and were

expressed relative to *GAPDH* as a housekeeping gene, which was expressed at the same levels (C_t values) for all examined genes and stages of the estrous cycle and pregnancy.

Extraction of hormones from the CL tissue

LTs were extracted from the CL tissue according to the method described previously (Korzekwa *et al.* 2008a).

Hormone determination

Measurements of P_4 and PGE_2 in the CL tissue and plasma were performed using a direct EIA as described previously (Skarzynski *et al.* 2003a, 2003b, Korzekwa *et al.* 2004). Antiserum to P_4 was donated by Dr S Okrasa (University of Warmia and Mazury, Olsztyn, Poland). The standard curve ranged from 0.39 to 100 ng/ml and the effective dose for 50% inhibition (ED_{50}) of the assay was 4.5 ng/ml. The intra- and interassay coefficients of variation (CV) values were 5.5 and 8.5% respectively. The PGE_2 standard curve ranged from 0.07 to 20 ng/ml and the ID_{50} of the assay was 1.25 ng/ml. The intra- and interassay CV values averaged 6.9 and 9.7% respectively.

The concentrations of PGFM in the plasma samples were determined with a direct EIA, as described previously (Skarzynski *et al.* 2003a). The anti-PGFM serum (WS4468-5) was donated by Dr W J Silvia (University of Kentucky, Lexington, KY, USA). The PGFM standard curve was produced for PGFM concentrations ranging from 32.5 to 8000 pg/ml and the ID_{50} of the assay was 315 pg/ml. The intra- and interassay CV values were on average 7.6 and 10.4% respectively.

The concentrations of LTB_4 and C_4 were determined in the culture media using commercially available EIA kits (520211, LTC_4 EIA kit; 520111, LTB_4 EIA kit; Cayman Chemical Co.) according to Korzekwa *et al.* (2008b). The LTB_4 standard curve ranged from 1.96 to 1000 pg/ml, and the effective dose for 50% inhibition (ID_{50}) of the assay was 2.5 pg/ml. The intra- and interassay CV values were on average 4.1 and 6.2% respectively. The LTC_4 standard curve ranged from 0.98 to 500 pg/ml and the effective dose for 50% inhibition (ID_{50}) of the assay was 1.85 pg/ml. The intra- and interassay CV values were on average 4.9 and 7.4% respectively.

Statistical analysis

In experiment 1, the determination of every measurement for each group was performed in triplicate. The statistical significance of differences in 5-LO, BLT, and CYSLT expression and the statistical significance of differences in hormone concentrations were analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test (ANOVA; GraphPAD PRISM Version 4.00, San Diego, CA, USA), if the initial ANOVA was significant ($P < 0.05$). The intensity of the histochemical reactions was estimated by the measurement of optical density (0–450) using Olympus DP SOFT Software (Olympus, Warsaw, Poland). Three slides of each sample were stained and examined under a light microscope (Nikon Microphot FXA).

LT concentration and differences during the estrous cycle and in early pregnancy in experiments 2 and 3, as well the analyses of P_4 , PGE_2 , and PGFM in the plasma samples,

collected before, during, and after administration of LT antagonists, were performed using a repeated measures design approach with treatments and the time of sample collection (hours, or days of the cycle or pregnancy) being fixed effects with all interactions included repeated measurement ANOVA tests followed by Bonferroni's multiple comparison test (GraphPAD PRISM Version 5.00). $P < 0.05$ was considered significant.

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.

Funding

This research was supported by grants-in-aid for scientific research from the Polish Ministry of Scientific Research and Higher Education (2P06D 004 30 and N N311 01 3837).

Acknowledgements

The authors are indebted to thank D V Przemysław Warmowski for assistance during *in vivo* experiment and USG examination, and Ewa Rewinska for assistance during *in vivo* experiment and EIA determinations in experiment 3. We thank Dr Stanisław Okrasa of the Warmia and Mazury University in Olsztyn (Poland) for P₄ antiserum, Dr W J Silvia (University of Kentucky, Lexington, KY, USA) for PGFM antiserum, and the firm Draminski (Olsztyn, Poland) for use of the USG scanner to monitor the phase of the estrous cycle in experimental cows. The authors also thank the heads of the animal farms, Mr Maciej Baurycza (Animal Farm of Cieszymowo) and Mr Henryk Jabłoński (Experimental Animal Farm of the Polish Academy of Sciences in Baranowo) for their excellent cooperation and for allowing us to use the animals for this study, and Mr Marek Domin, the owner of the slaughterhouse (Zakłady Miesne 'Warmia,' Biskupiec, Poland) for permitting collection of the material.

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Received 30 April 2010

First decision 7 June 2010

Revised manuscript received 1 August 2010

Accepted 20 August 2010