The cooperative action of angiotensin II with subluteolytic administration of PGF_{2α} in inducing luteolysis and oestrus in the cow

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There is evidence that the potent vasoconstrictor angiotensin II (Ang II) regulates luteal functions. In the present study, the effect of an intraluteal injection of Ang II alone or in combination with pretreatment with a subluteolytic i.m. dose of PGF_{2α} on the concentration of plasma progesterone, corpus luteum regression and duration of the oestrous cycle was investigated. Cows were assigned randomly to receive an intraluteal injection of either: (i) 500 µl saline 30 min after i.m. administration of saline (control, $n = 7$); (ii) 500 µl saline 30 min after i.m. administration of a subluteolytic dose (125 µg) of a PGF_{2α} analogue (1/4 PGF_{2α}, $n = 5$); (iii) 2 mg Ang II in 500 µl saline 30 min after i.m. administration of saline (Ang II, $n = 5$); or (iv) 2 mg Ang II in 500 µl saline 30 min after i.m. administration of a subluteolytic dose (125 µg) of the PGF_{2α} analogue (1/4 PGF_{2α}/Ang II, $n = 6$) on day 12 of the

oestrous cycle. There were no significant changes in plasma progesterone concentrations in the control, 1/4 PGF_{2α} or Ang II treatment groups. Treatment with 1/4 PGF_{2α}/Ang II decreased plasma progesterone concentration, and induced luteolysis and oestrus. The onset of oestrus in cows treated with full-dose (500 µg) PGF_{2α} (3.1 ± 0.2 (mean $\pm$ SEM) days after treatment) was significantly earlier than that in cows treated with 1/4 PGF_{2α}/Ang II (4.8 ± 0.2 days after treatment) ($P < 0.05$). The results from the present study demonstrate that an intraluteal injection of Ang II after i.m. administration of a subluteolytic dose of PGF_{2α} analogue induces luteolysis and oestrus. Thus, these results support the contention that Ang II is directly correlated with the process of luteal regression in cows.

Introduction

PGF_{2α} is a luteolytic factor in cows. An injection of PGF_{2α} or its analogue induces a cascade of luteolysis, and the corpus luteum undergoes functional and structural regression.

There is evidence that the potent vasoconstrictor angiotensin II (Ang II) regulates ovarian functions. Ang II is a peptide that is produced and released by luteal endothelial cells (Hayashi *et al.*, 2000), and it may participate in the cascade of luteolysis as a local regulator. Hayashi *et al.* (2001) showed that there are increases in Ang II release and in the expression of angiotensin-converting enzyme mRNA within the corpus luteum after a luteolytic injection of PGF_{2α} analogue. Ang II inhibits progesterone production stimulated by LH in bovine luteal cells in culture (Stirling *et al.*, 1990). Furthermore, an *in vitro* microdialysis system implanted in bovine luteal explants from the mid-luteal phase of the oestrous cycle was used to show that PGF_{2α} stimulates the release of Ang II and that Ang II decreases

progesterone release (Hayashi and Miyamoto, 1999). These results indicate that the luteolytic effect of PGF_{2α} may be mediated in part by Ang II. In addition, expression of Ang II receptor mRNA was detected in the bovine corpus luteum and the luteal endothelial cells (Hayashi *et al.*, 2000).

However, another vasoconstrictive peptide, endothelin 1 (ET-1), has a local luteolytic action that is similar to that of Ang II in the bovine corpus luteum (Girsh *et al.*, 1996a,b; Miyamoto *et al.*, 1997). Miyamoto *et al.* (2001) reported that an intraluteal injection of ET-1 after i.m. administration of a subluteolytic dose of a PGF_{2α} analogue resulted in a rapid decrease in plasma progesterone concentrations during the mid-luteal phase in cows. In addition, an intraluteal injection of the endothelin receptor ET_A antagonist before an i.m. injection of PGF_{2α} reduced the luteolytic effect of PGF_{2α} (Hinckley and Milvae, 2001). These findings support a possible role of ET-1 in mediating PGF_{2α}-induced luteolysis.

The aim of the present study was to investigate the effect of an intraluteal injection of Ang II alone or in combination with pretreatment with a subluteolytic i.m. dose of PGF_{2α} on the concentrations of plasma progesterone and Ang II, corpus luteum regression and the duration of the oestrous cycle.

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Materials and Methods

Animals

Thirty multiparous, lactating Holstein cows were used to determine concentrations of plasma progesterone and Ang II during the experimental periods. All cows had undergone at least two oestrous cycles of normal duration (21–22 days) before being used in the experiments. The day of oestrus was designated as day 0.

Experimental design

On day 12 of the oestrous cycle, cows were assigned randomly to receive one of four types of intraluteal injection: (i) 500 μ l saline 30 min after i.m. administration of saline (control, $n = 7$); (ii) 500 μ l saline 30 min after i.m. administration of a subluteolytic dose (125 μ g) of a PGF_{2 α} analogue (cloprostenol, Estrumate; Sumitomo Pharm. Co., Osaka) (1/4 PGF_{2 α} , $n = 5$); (iii) 2 mg Ang II (Peptide Institute Inc., Osaka) in 500 μ l saline (Ang II, $n = 5$) 30 min after i.m. administration of saline; or (iv) 2 mg Ang II in 500 μ l saline 30 min after i.m. administration of a subluteolytic dose (125 μ g) of a PGF_{2 α} analogue (1/4 PGF_{2 α} /Ang II, $n = 6$). The dose of Ang II was selected on the basis of the results of a preliminary experiment. Intraluteal injection of Ang II was examined at different doses (0.5, 1.0 and 2.0 mg) after i.m. administration of a subluteolytic dose (125 μ g) of a PGF_{2 α} analogue and 2.0 mg of Ang II was the only dose in this model that induced a significant decrease in progesterone release and luteolysis. Thus, for further experiments, 2.0 mg Ang II was used.

For intraluteal injections of Ang II or saline, a 16-gauge customized needle, 23 cm in length, was inserted through the thinnest abdominal wall ipsilateral to the corpus luteum to the peritoneal cavity. The ovary with a corpus luteum was grasped by a hand inserted through the rectum. A 1 ml disposable syringe was filled with either 500 μ l saline or Ang II in saline according to the experimental design, and with 200 μ l of air (a dead volume of the customized needle). The needle was inserted through the ovarian parenchyma to approach the corpus luteum, so that the injection of 500 μ l fluid into the corpus luteum was conducted via the rear side of the ovary. Luteolysis was induced in seven cows by administering a full-dose (500 μ g) of PGF_{2 α} analogue (full-dose PGF_{2 α}) i.m. to compare the changes in plasma progesterone concentration. The times of the intraluteal injection or i.m. treatments were designated as 0 h, and 20 ml blood samples were collected by caudal venepuncture at –48.0, –24.0, 0.0, 0.25, 0.5, 1.0, 2.0, 6.0, 12.0, 24.0 and 48.0 h, and at 1 day intervals for a further 7 days. Plasma was obtained by centrifugation at 900 g for 20 min at 4°C and was stored at –30°C until assayed.

Hormone determination

Plasma concentrations of progesterone and Ang II were determined in duplicate by second antibody enzyme-immunoassay after extraction using 96-well ELISA plates

(NUNC-Immuno Plate; NUNC™ Brand Products, Roskilde).

Plasma progesterone concentrations were assayed after extraction by diethyl ether. The enzymeimmunoassay for progesterone was described by Miyamoto *et al.* (1992). The standard curve ranged from 0.05 to 50.0 ng ml^{–1}, and the ED₅₀ value of the assay was 2.4 ng ml^{–1}. The intra- and interassay coefficients of variation were on average 6.2% and 9.3%, respectively.

For extraction of Ang II, the plasma samples (7 ml) were diluted with 5 ml of distilled water and the pH was adjusted to 2.5 with 5.0 mol HCl l^{–1}. All samples were then applied to a Sep-Pak C₁₈ cartridge (Waters, Milford, MA) as described by Miyamoto *et al.* (1997). The residue was evaporated and then dissolved in 200 μ l assay buffer (42.0 mmol Na₂HPO₄ l^{–1}, 8.0 mmol KH₂PO₄ l^{–1}, 20.0 mmol NaCl l^{–1}, 4.8 mmol EDTA l^{–1}, 0.05% (w/v) BSA, pH 7.5) for the Ang II enzyme-immunoassay. Thus, the samples were concentrated 35-fold for plasma as a result of this process, which allowed determination of peptide concentrations in enzymeimmunoassay within the range of the standard curve. The recovery rate of Ang II that had been added to the plasma was 75%. The enzymeimmunoassay for Ang II was carried out as described by Hayashi and Miyamoto (1999). The standard curve for Ang II ranged from 2.5 to 5000 pg ml l^{–1}, and the ED₅₀ value of the assay was 95 pg ml l^{–1}. The intra- and interassay coefficients of variation were 6.4 and 7.4%, respectively.

Statistical analysis

The mean concentrations of progesterone and Ang II in plasma collected at different time points were compared with the values at the same time point by repeated measures ANOVA followed by the *t* test with Bonferroni method. The duration of the oestrous cycle of cows that received the 1/4 PGF_{2 α} /Ang II treatment was compared with that of cows receiving full-dose PGF_{2 α} treatment by ANOVA followed by the Student's *t* test. Differences were considered significant at $P < 0.05$.

Results

Effect of Ang II and PGF_{2 α} on plasma progesterone concentrations

There was no significant change in plasma progesterone concentration after an intraluteal injection of Ang II (Fig. 1). Administration of 1/4 PGF_{2 α} did not affect plasma progesterone concentration as in the control and Ang II groups (Fig. 1). In contrast, treatment with 1/4 PGF_{2 α} /Ang II induced a rapid decrease in plasma progesterone concentrations after Ang II injection (Fig. 1). Plasma progesterone concentrations of cows in the 1/4 PGF_{2 α} /Ang II treatment groups were lower than those in the other treatment groups ($P < 0.05$; Fig. 1).

Effect of 1/4 PGF_{2 α} /Ang II and full-dose PGF_{2 α} treatments on the onset of oestrus

The change in plasma progesterone with time in the

Fig. 1. Effects of angiotensin II (Ang II) and $\text{PGF}_{2\alpha}$ on mean plasma progesterone concentrations in cows. Cows received: (i) 500 μl saline 30 min after i.m. administration of saline (control, $n = 7$, $\blacktriangle$); (ii) 500 μl saline 30 min after i.m. administration of a sublytic dose (125 μg) of a $\text{PGF}_{2\alpha}$ analogue (1/4 $\text{PGF}_{2\alpha}$, $n = 5$, $\diamond$); (iii) 2 mg Ang II in 500 μl saline 30 min after i.m. administration of saline (Ang II, $n = 5$, $\blacksquare$); or (iv) 2 mg Ang II in 500 μl saline 30 min after i.m. administration of a sublytic dose (125 μg) of the $\text{PGF}_{2\alpha}$ analogue (1/4 $\text{PGF}_{2\alpha}$ /Ang II, $n = 6$, $\circ$). *Values are significantly different from other treatments at the same time point ($P < 0.05$).

cows treated with 1/4 $\text{PGF}_{2\alpha}$ /Ang II was not different from that in the cows treated with the full-dose $\text{PGF}_{2\alpha}$. Both treatments induced luteolysis (Fig. 2). However, the onset of oestrus in cows treated with full-dose $\text{PGF}_{2\alpha}$ (3.1 ± 0.2 (mean $\pm$ SEM) days after treatment) was significantly earlier than that in cows treated with 1/4 $\text{PGF}_{2\alpha}$ /Ang II (4.8 ± 0.2 days after treatment) ($P < 0.05$; Fig. 2). However, in cows in the control group and in cows treated with Ang II or 1/4 $\text{PGF}_{2\alpha}$ alone, the onset of oestrus occurred 9.6 ± 0.2 days after treatment (the duration of the oestrous cycle in these cows was 21.6 ± 0.2 days). Therefore, these treatments had no effect on the duration of the oestrous cycle.

Effect of treatments on plasma Ang II concentrations

Intraluteal injection of Ang II alone resulted in a transient and very acute increase in the concentration of plasma Ang II at 0.25 h after treatment ($P < 0.05$) followed by a rapid return to the basal value (Fig. 3). There were no significant changes in plasma Ang II concentration in the other treatment groups (Fig. 3).

Discussion

The results of the present study demonstrate that an intraluteal injection of Ang II after i.m. administration of a sublytic dose of $\text{PGF}_{2\alpha}$ led to a decrease in plasma progesterone concentrations, and induced luteolysis and

Fig. 2. Concentrations of plasma progesterone in cows treated with (a) 1/4 $\text{PGF}_{2\alpha}$ /angiotensin II (Ang II) ($\circ$; $n = 6$) and (b) full-dose $\text{PGF}_{2\alpha}$ ($\blacklozenge$; $n = 7$) (mean $\pm$ SEM). Arrows indicate oestrus. In cows treated with 1/4 $\text{PGF}_{2\alpha}$ /Ang II, the onset of oestrus occurred 4.8 ± 0.2 (mean $\pm$ SEM) days after treatment, and in the cows treated with full-dose $\text{PGF}_{2\alpha}$, the onset of oestrus occurred 3.1 ± 0.2 (mean $\pm$ SEM) days after treatment. These times were significantly different ($P < 0.05$).

oestrus in cows. This is the first study to demonstrate the ability of Ang II to induce luteolysis in cows.

The change in plasma progesterone concentrations with time in cows treated with 1/4 $\text{PGF}_{2\alpha}$ /Ang II was not different from that in the cows treated with the full-dose $\text{PGF}_{2\alpha}$. Both treatments induced luteolysis. Although the injection of sublytic $\text{PGF}_{2\alpha}$ alone did not induce luteolysis, the 1/4 $\text{PGF}_{2\alpha}$ /Ang II treatment rapidly reduced plasma progesterone concentration. These results indicate that Ang II is needed for the cascade mechanisms of luteolysis in the corpus luteum.

In cows treated with a luteolytic injection of $\text{PGF}_{2\alpha}$ analogue i.m., the blood flow within the mid-cycle corpus luteum increased rapidly at 0.5–2.0 h, decreased at 4 h to the basal value observed at 0 h, and then decreased further from 8 to 48 h (Acosta *et al.*, 2002). Moreover, treatment

Fig. 3. Effects of angiotensin II (Ang II) and $\text{PGF}_{2\alpha}$ on mean concentrations of plasma Ang II. Cows received: (i) 500 μl saline 30 min after i.m. administration of saline (control, $n = 7$, $\blacktriangle$); (ii) 500 μl saline 30 min after i.m. administration of a subluteolytic dose (125 μg) of a $\text{PGF}_{2\alpha}$ analogue (1/4 $\text{PGF}_{2\alpha}$, $n = 5$, $\diamond$); (iii) 2 mg Ang II in 500 μl saline 30 min after i.m. administration of saline (Ang II, $n = 5$, $\blacksquare$); or (iv) 2 mg Ang II in 500 μl saline 30 min after i.m. administration of a subluteolytic dose (125 μg) of the $\text{PGF}_{2\alpha}$ analogue (1/4 $\text{PGF}_{2\alpha}$ /Ang II, $n = 6$, $\circ$). *Value is significantly different from other treatments at the same time point ($P < 0.05$).

with the subluteolytic dose of a $\text{PGF}_{2\alpha}$ analogue that was used in the present study also induced an acute increase in blood flow within the mid-cycle corpus luteum at 0.5–2.0 h after i.m. injection, although the increase was less than that observed with full-dose $\text{PGF}_{2\alpha}$ treatment (K. Hayashi and A. Miyamoto, unpublished). Therefore, the first increase in blood flow may be a key part of the local mechanism that controls luteal regression in this model. However, Hayashi and Miyamoto (1999) showed that Ang II results in a decrease in progesterone release in a microdialysis system implanted in bovine luteal explants from the mid-luteal phase of the oestrous cycle *in vitro*. In particular, an infusion of Ang II after $\text{PGF}_{2\alpha}$ or a concomitant infusion of Ang II with $\text{PGF}_{2\alpha}$ inhibited progesterone release more rapidly than did an infusion of Ang II alone (Hayashi and Miyamoto, 1999). Cellular mechanisms involved in the actions of Ang II in luteal cells are mediated, at least in part, by an increase in the intracellular concentration of free calcium ($[\text{Ca}^{2+}]_i$) at least in rats (Pepperell *et al.*, 1993). It is likely that $\text{PGF}_{2\alpha}$ activated this mechanism first (Davis *et al.*, 1987) and then Ang II may have further stimulated the increase of $[\text{Ca}^{2+}]_i$, resulting in a pronounced inhibition of progesterone. These findings indicate that the effect of $\text{PGF}_{2\alpha}$ triggers the first step of the complex cascade of luteolysis, which involves an acute change of vascular function within the corpus luteum.

An intraluteal injection of Ang II resulted in a transient increase in Ang II concentration in plasma at 0.25 h after treatment followed by a rapid return to the basal value. In contrast, 1/4 $\text{PGF}_{2\alpha}$ /Ang II treatment caused no such increase in the plasma concentration of Ang II. Although it is unclear why Ang II alone, rather than in combination with $\text{PGF}_{2\alpha}$, caused an acute increase in plasma Ang II concentration, the results from the present study indicate that the injected Ang II remained in the corpus luteum as a result of subluteolytic $\text{PGF}_{2\alpha}$ treatment. This finding may be one of the reasons why Ang II can induce luteolysis with subluteolytic $\text{PGF}_{2\alpha}$ treatment, although we cannot exclude the possibility that the peak of Ang II in plasma of cows treated with 1/4 $\text{PGF}_{2\alpha}$ /Ang II occurred at a different time so that we could not find the peak due to a very short half-life of Ang II.

The cows treated with 1/4 $\text{PGF}_{2\alpha}$ /Ang II returned to oestrus 4.8 days after treatment, whereas cows treated with full-dose $\text{PGF}_{2\alpha}$ returned to oestrus 3.1 days after treatment. It is unclear whether this delay in oestrus is due to direct or indirect effects. The possible mechanisms involved in the effects on luteal as well as follicular functions remain to be elucidated.

It has been shown that the intraluteal release of ET-1 (Ohtani *et al.*, 1998) and Ang II (Hayashi *et al.*, 2001) increases in the corpus luteum of cows after a luteolytic $\text{PGF}_{2\alpha}$ injection. In addition, ewes that received an i.m. injection of ET-1 preceded by a subluteolytic dose of $\text{PGF}_{2\alpha}$ displayed a rapid decrease in plasma progesterone concentrations (Hinckley and Milvae, 2001). Ang II also stimulates ET-1 release through the activation of protein kinase C in endothelial cells (Emori *et al.*, 1991). Thus, a strong positive feedback relationship among $\text{PGF}_{2\alpha}$, Ang II and ET-1 might be established during the early stage of luteolysis within the corpus luteum.

In conclusion, the results from the present study demonstrate that an intraluteal injection of Ang II after a subluteolytic dose of a $\text{PGF}_{2\alpha}$ analogue i.m. induces luteolysis and oestrus. Thus, these results support the contention that Ang II is directly correlated with the process of regression of the corpus luteum in cows.

The authors thank K. Okuda (Okayama University, Japan) for progesterone antiserum and K. Wakabayashi (Gunma University, Japan) for angiotensin II antiserum. This study was supported by Grants-in-Aid for Scientific Research (11660276 and 12556046) of the Japan Society for the Promotion of Science (JSPS), and the Novartis Foundation (Japan) for the Promotion of Science. K. Hayashi is supported by JSPS Research Fellowships for Young Scientists.

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Received 21 January 2002.

First decision 22 February 2002.

Revised manuscript received 25 April 2002.

Accepted 16 May 2002.