Depletion of vitamin C from pig corpora lutea by prostaglandin $F_{2\alpha}$ -induced secretion of the vitamin

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The luteolytic effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) are thought to be mediated in part by the promotion of an increasingly oxidative cellular environment. Loss of antioxidants is one mechanism by which $PGF_{2\alpha}$ might induce or exacerbate oxidative damage within the corpus luteum. This study was performed to establish whether depletion of vitamin C is an acute effect of $PGF_{2\alpha}$ on the pig corpus luteum and to gain insight into the mechanism of luteal vitamin C loss at luteolysis. Gilts ($n = 4$) were anaesthetized and both utero–ovarian veins and an ear vein were catheterized. Each corpus luteum on the treated ovary received an intraluteal injection of $PGF_{2\alpha}$ (1 μ g) followed by a sustained release implant containing 100 μ g of the prostaglandin. The other ovary served as the control and each corpus luteum received corresponding volumes of injection vehicle and blank implant. Blood was collected from the ear vein and both utero–ovarian veins every 15 min beginning 15 min before the onset of treatment. Collection of blood stopped when animals were ovariectomized and corpora lutea were collected at 2 h after treatment. Progesterone and vitamin C (ascorbate) concentrations were measured in tissue and plasma samples. $PGF_{2\alpha}$ -treated luteal tissue had similar progesterone, but significantly lower ascorbate, concentrations when compared with control corpora lutea. $PGF_{2\alpha}$ treatment resulted in a rapid and sustained increase in plasma ascorbate within the treatment-side utero–ovarian vein, while the control utero–ovarian vein and ear vein showed little change in plasma ascorbate during the experimental period. No effect of $PGF_{2\alpha}$ on plasma progesterone was evident. This finding suggests that $PGF_{2\alpha}$ depletes the pig corpus luteum of vitamin C by inducing secretion of the vitamin into the bloodstream. Further studies are necessary to determine whether the depletion of vitamin C that is induced by $PGF_{2\alpha}$ contributes to the demise of the pig corpus luteum.

Introduction

Recent studies have established a role for reactive oxygen species in the loss of steroidogenesis and destruction of the corpus luteum at the end of the nonfertile oestrous cycle. Luteolysis is associated with an accumulation of superoxide and peroxide radicals within the corpus luteum (Sawada and Carlson, 1989; Riley and Behrman, 1991). Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a putative luteolysin in many species including pigs, is known to induce accumulation of such reactive oxygen species within the corpus luteum (Sawada and Carlson, 1989). Exposure of luteal cells to these oxidants results in oxidation and loss of fluidity in cellular membranes (Sawada and Carlson, 1991) and interruption of progesterone production (Behrman and Preston, 1989; Behrman and Aten, 1991; Gatzuli *et al.*,

1991). These studies support an oxidant-mediated mechanism of $PGF_{2\alpha}$ action on the corpus luteum.

Vitamin C or ascorbate is a multifunctional antioxidant that is synthesized by the liver in pigs and sequestered within the corpus luteum via an ATP-dependent process (Stansfield and Flint, 1967; Musicki *et al.*, 1996). The concentrations of vitamin C found in the functional pig corpus luteum are as much as 100 fold greater than systemic plasma concentrations (Petroff *et al.*, 1997) and rival the ascorbate concentrations found in the adrenal gland, the richest source of vitamin C in the body (Levine and Morita, 1985). In a recent study, we found that concentrations of vitamin C in regressing pig corpora lutea were greatly reduced in comparison with corpora lutea collected during the mid-luteal phase and early pregnancy (Petroff *et al.*, 1997). Furthermore, exogenous $PGF_{2\alpha}$ rapidly causes such depletion of luteal ascorbate in intact and hypophysectomized rats (Sato *et al.*, 1974; Aten *et al.*, 1992; Musicki *et al.*, 1996).

Several mechanisms might be responsible for depletion of luteal ascorbate stores after treatment with $PGF_{2\alpha}$. The vitamin

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may be irreversibly oxidized while neutralizing the reactive oxygen species that accumulate in luteal tissue after exposure to PGF_{2α} (Sawada and Carlson, 1989). Periods of luteal ascorbate depletion are associated with an accumulation of lipid peroxides, suggesting that the vitamin does play a significant role as a luteal antioxidant (Aten *et al.*, 1992). PGF_{2α} may also deplete luteal vitamin C stores indirectly by stimulating LH release from the pituitary gland (Sato *et al.*, 1974). Vitamin C concentrations decrease in the rat corpus luteum after treatment with LH (Stansfield and Flint, 1967; Musicki *et al.*, 1996) and this phenomenon was the basis of a commonly used bioassay for LH (Parlow, 1958). Lastly, PGF_{2α} may induce secretion of luteal stores of vitamin C. PGF_{2α} treatment of cultured rat luteal cells that have been preloaded with radiolabelled ascorbate causes a significant increase in vitamin C secretion (Musicki *et al.*, 1996). The aims of this experiment were to determine whether PGF_{2α} causes a loss of luteal ascorbate from the pig corpus luteum *in vivo*, to determine whether such a loss involves secretion of luteal ascorbate into the bloodstream, and over what time period this reduction in luteal ascorbate occurs.

Materials and Methods

Experimental model

Landrace × Duroc gilts ($n = 4$), weighing from 120 to 140 kg, were used in this study. The onset of oestrus was determined by daily observation (08:30 h) in the presence of intact boars. Surgical procedures were performed on day 13 after the onset of oestrus (day 0). Corpora lutea of pigs are not consistently responsive to PGF_{2α} before day 12 (see Estill *et al.* (1993) for review); day 13 was therefore chosen as the day of treatment. The within animal control guarded against any confounding effect of endogenous prostaglandin. This protocol was approved by the Animal Care and Use Committee at the Ohio State University.

A surgical plane of anaesthesia was maintained throughout the experiment using halothane inhalation. After exposure of the reproductive tract via a midventral incision, both utero-ovarian veins were catheterized with polyethylene catheters (i.d. 0.35 mm, o.d. 0.58 mm; Clay Adams, Paraisippany, NJ) using a modification of the technique of Ottobre *et al.* (1980). An ear vein was also catheterized using an 18 g × 1.25" over-the-needle i.v. catheter (Terumo Medical Corp., Elkton, MD) for the collection of systemic blood samples. All catheters were flushed with heparinized saline (100 i.u. ml⁻¹; Elkins-Sinn, Inc., Cherry Hill, NJ) after emplacement and collection of each blood sample. Correct placement of the catheter was tested by palpation and by comparison of utero-ovarian vein plasma progesterone concentrations with those of systemic plasma within each animal. Utero-ovarian veins that yielded blood samples containing concentrations of progesterone similar to systemic (ear vein) samples at all time points were considered to have been improperly cannulated. Blood samples were collected from both utero-ovarian veins and the ear vein at -15, 0, 15, 30, 45, 60, 75, 90, 105, and 120 min relative to the onset of treatment. Blood collections required < 2 min for each time point.

Ovaries were randomly designated as receiving PGF_{2α} or control treatment. PGF_{2α} treatment entailed intraluteal injections of 1 µg PGF_{2α} (Lutalyse; Upjohn Co., Kalamazoo, MI) delivered in 250 µl normal saline to all corpora lutea on that ovary followed by implantation of each corpus luteum with approximately 100 µg PGF_{2α} (tris salt, Sigma Chemical Co., St Louis, MO) as a 50 µl sustained-release Silastic implant (Ford and Christenson, 1991). Such intraluteal PGF_{2α} implants have been shown to induce luteal regression and loss of tissue progesterone in pig corpora lutea (Ford and Christenson, 1991; Hehne *et al.*, 1994; Christenson *et al.*, 1995). The procedure was modified by adding an injection of PGF_{2α} in saline to the luteal implant protocol, as leaching of PGF_{2α} from the implant may have been somewhat delayed. Since we anticipated that PGF_{2α} would cause an acute change in concentrations of utero-ovarian venous ascorbic acid (i.e. within 2 h), it was necessary to ensure that the treatment had immediate access to the luteal cells. This treatment protocol permitted acute and prolonged increases in PGF_{2α} within treated corpora lutea. Corpora lutea on the remaining (control) ovary received an intraluteal injection of normal saline and a blank Silastic implant. The order of treatment was randomly determined. Intraluteal injections were completed by 5 min and implants delivered by 15 min from the onset of treatment.

Immediately after collection of each set of blood samples, plasma was isolated by centrifugation (2500 g for 10 min at 4°C). Plasma from each blood sample was divided into two aliquots. One aliquot was immediately placed on dry ice and maintained at -70°C until assayed for progesterone. The second aliquot was acidified with a trichloroacetic acid (TCA) solution (Dabrowski and Hinterleitner, 1989), transferred to dry ice and maintained at -70°C until assayed for vitamin C. Two hours after the onset of treatment both ovaries were removed. Corpora lutea were immediately dissected, examined for the presence of implants and stored at -70°C until homogenization and assay for progesterone and vitamin C.

Progesterone radioimmunoassay

The concentrations of progesterone in plasma and tissue samples were measured by radioimmunoassay using a proven and specific antibody (GDN 337; donated by G. Niswender, Colorado State University). For analysis of luteal progesterone, two corpora lutea per ovary were pooled during homogenization in Tris-buffered saline. The progesterone assay had been validated in our laboratory for use with primate serum and luteal incubate (Johnson *et al.*, 1988). A standard curve with a slope of approximately -2.0 after log/logit transformation was first established using 2.5–500 pg progesterone standards. This assay was validated for use with pig plasma and luteal homogenate by showing that when increasing volumes of plasma or homogenate were extracted and assayed for progesterone content, values were parallel to the standard curve. During the homogenate validation, the slope of the standard curve was -1.83, while a standard curve prepared using luteal homogenate yielded a slope of -1.61. Similarly, during the plasma validation the slopes of both the standard curve and the plasma curve were -2.2. The efficiency of extraction of [³H]progesterone from plasma and tissue samples was

83.3 ± 0.8% and 86.7 ± 1.0%, respectively. All plasma samples from each animal were run in duplicate within the same assay. The intra-assay coefficient of variation for plasma samples was 7.9 ± 5.5%. Tissue progesterone concentrations were determined in a single assay with an intra-assay coefficient of variation of 9.4%.

Measurement of vitamin C

For the measurement of total vitamin C (ascorbic acid + dehydroascorbic acid), plasma samples were vortexed and tissue samples were homogenized with a 5% (w/v) TCA solution containing 250 mmol HClO₄ l⁻¹ and 0.08% (w/v) EDTA. The homogenates were centrifuged at 29 000 g for 30 min at 4°C. For analysis of tissue vitamin C, two corpora lutea per ovary were pooled during homogenization. The concentrations of total vitamin C in the resulting supernatants were determined colorimetrically, with correction for interference, as described by Dabrowski and Hinterleitner (1989). All samples were assayed in duplicate. This assay has been used previously in our laboratory to measure vitamin C concentrations in pig liver, kidney and ovary (Mahan *et al.*, 1994; Petroff *et al.*, 1997).

Statistical analyses

Mean tissue concentrations of progesterone and vitamin C in control and PGF_{2α}-treated corpora lutea were compared using a one-way ANOVA with animal being the block and PGF_{2α} the relevant treatment. Plasma samples from utero-ovarian veins draining PGF_{2α}-treated and control ovaries as well as ear vein plasma samples were analysed for both progesterone and vitamin C endpoints using a repeated measures design. These data were analysed for an effect of time within treatment. Individual least squares means were compared using the probability of difference function of Statistical Analysis System (SAS). Differences with a *P* value < 0.05 were considered significant.

Results

Tissue progesterone concentrations

Concentrations of progesterone were similar in control and PGF_{2α}-treated corpora lutea (Fig. 1). These high concentrations of progesterone are similar to those seen in fully functional corpora lutea (Petroff *et al.*, 1997). Moreover, the PGF_{2α} implants used in this study required 12 h to elicit the changes in luteal progesterone in a previous study (Hehnke *et al.*, 1994). Thus, luteal function was probably not compromised by 2 h after treatment in the present study.

Tissue vitamin C concentrations

Mean concentrations of total vitamin C within PGF_{2α}-treated corpora lutea were significantly (*P* < 0.05) decreased in comparison with controls at 2 h after treatment (Fig. 2). Vitamin C concentrations in control corpora lutea were similar

Fig. 1. Progesterone content of luteal tissue after treatment with prostaglandin F_{2α} (PGF) or vehicle. Each bar represents the mean ± SEM of the progesterone content of luteal tissue from four gilts at the end of the experimental procedure. CL: corpus luteum.

Fig. 2. Content of vitamin C in luteal tissue after treatment with prostaglandin F_{2α} (PGF) or vehicle. Each bar represents the mean ± SEM of the vitamin C content of luteal tissue from four gilts at the end of the experimental procedure. CL: corpus luteum. *Significantly different from control (*P* < 0.05).

to those previously reported in mid-cycle corpora lutea (Petroff *et al.*, 1997).

Plasma progesterone concentrations

Data from the control utero-ovarian vein of one pig were excluded as progesterone concentrations did not differ from those of systemic plasma, indicating a suspect cannula placement. Concentrations of progesterone in plasma from the utero-ovarian veins from both treated and control sides varied greatly from one time point to the next. Plasma from the control side utero-ovarian vein appeared to contain greater concentrations of progesterone throughout the experiment (Fig. 3a). However, this was not attributable to any treatment effect, as progesterone concentrations did not change significantly from zero hour controls for either of the utero-ovarian veins. Similarly, systemic concentrations of progesterone did not change significantly after PGF_{2α} treatment (Fig. 3b).

Plasma vitamin C concentrations

Data from the control utero-ovarian vein of one pig were excluded due to suspect cannulation. Concentrations of

Fig. 3. Concentrations of progesterone in plasma from utero-ovarian veins draining (○) control and (●) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-treated ovaries (a) and ear vein plasma (b) collected before and during intraluteal treatment with $PGF_{2\alpha}$ and vehicle. Values are least squares means for data from four gilts. SEM = 242 (a); 5.4 (b).

Fig. 4. Vitamin C concentrations in plasma from an ear vein (●) and utero-ovarian veins draining (■) control and (▲) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-treated ovaries before and throughout intraluteal treatment with $PGF_{2\alpha}$ and vehicle. Values are least square means for data from four gilts. SEM = 2.82. *Significantly different from their respective zero hour controls ($P < 0.05$).

progesterone in the plasma collected from this vessel were not much higher than systemic values. Ascorbate concentrations in plasma derived from this vessel did not differ from systemic plasma values, either. Vitamin C concentrations were quite similar in plasma collected from the ear vein and both utero-ovarian veins before treatment with $PGF_{2\alpha}$ (Fig. 4). Vitamin C concentrations in the utero-ovarian venous plasma from the $PGF_{2\alpha}$ -treated side rose significantly from pretreatment values by 15 min and remained high until 75 min after treatment. These concentrations were significantly greater than those present in both the ear vein plasma and plasma collected from the control utero-ovarian vein. Maximal concentrations of vitamin C in the plasma from the utero-ovarian vein of the treated side were more than twice pretreatment values. Although ascorbate concentrations appeared to rise slightly with time in both systemic plasma and plasma from the control

side utero-ovarian vein, this effect was not significant. Overall, intraluteal $PGF_{2\alpha}$ treatment specifically induced a significant release of the antioxidant into the venous effluent.

Discussion

The high concentrations of vitamin C found in the fully functional corpus luteum have led many workers to investigate its role in luteal function. Ascorbate is known to serve as a cofactor in collagen synthesis, an important process in the rapidly growing corpus luteum (Luck *et al.*, 1995). Vitamin C is also involved in steroidogenesis and peptide hormone production (Luck *et al.*, 1995) and may thereby directly promote luteal function (Jenkins, 1961; Biswas and Deb, 1970; Luck and Jungclas, 1987a, b; Byrd *et al.*, 1993). Vitamin C further serves as a cellular anti-oxidant, functioning especially in combination with vitamin E (Packer *et al.*, 1979) to neutralize reactive oxygen species produced by processes such as cellular respiration and steroidogenesis. Interruption of these functions of vitamin C would result in the reversal of tissue growth, waning progesterone production, and the increasingly oxidative cellular environment seen during regression of the corpus luteum.

Previous studies have established that concentrations of vitamin C are reduced in regressing corpora lutea, but remain high if luteal function continues due to rescue of the corpus luteum in the event of pregnancy (Petroff *et al.*, 1997; Sheldrick and Flint, 1989; Luck and Zhao, 1993). This reduction in luteal vitamin C is largely due to a decrease in both ascorbate and its oxidized form, dehydroascorbate. Thus, no significant changes in vitamin C metabolism were noted during natural luteolysis.

In the present study, treatment of functional pig corpora lutea *in vivo* with prostaglandin $F_{2\alpha}$, the putative luteolysin in pigs, resulted in a rapid loss of luteal ascorbate. This finding is in agreement with previous studies in which rats were treated systemically with $PGF_{2\alpha}$ (Sato *et al.*, 1974; Aten *et al.*, 1992), although a portion of this effect may have been attributable to $PGF_{2\alpha}$ -mediated increases in serum LH in previous studies. The within-animal control and local nature of $PGF_{2\alpha}$ administration used in the present study enabled us to demonstrate significant ascorbate depletion within the corpus luteum that was attributable solely to a direct action of $PGF_{2\alpha}$ *in vivo*. This finding is in agreement with a recent study performed using cultured rat luteal cells (Musicki *et al.*, 1996). In the present study, the loss of luteal vitamin C preceded any change in luteal function. On the basis of the results of Hehnke *et al.* (1994) and Christenson *et al.* (1995), a decrease in progesterone production by corpora lutea was anticipated to occur between 6 and 12 h after treatment with $PGF_{2\alpha}$. Thus, depletion of luteal ascorbate stores appears to be an early change in response to $PGF_{2\alpha}$ in pig corpora lutea, and this depletion of ascorbate could be a component in the sequence of luteolytic events.

$PGF_{2\alpha}$ appears to deplete the pig corpus luteum of vitamin C by inducing secretion of the vitamin into the bloodstream. This is indicated by the rapid and profound increase in plasma ascorbate seen in the venous effluent from $PGF_{2\alpha}$ -treated ovaries in the present study. Musicki *et al.* (1996) documented that $PGF_{2\alpha}$ stimulates secretion and inhibits uptake of vitamin

C by cultured rat luteal cells. Although the loss of tissue ascorbate could be explained by inhibition of uptake or stimulation of secretion, the abrupt increase in utero-ovarian concentrations of vitamin C after PGF_{2α} treatment cannot be accounted for by inhibition of uptake.

This study documents an acute depletion of vitamin C from the pig corpus luteum after intraluteal administration of PGF_{2α} *in vivo*. This loss of luteal ascorbate preceded any change in luteal function as assessed by tissue and plasma progesterone concentrations. The depletion of luteal vitamin C stores was associated with rapid and sustained release of the vitamin into the venous effluent of the treated ovary. It appears therefore that PGF_{2α} action depletes the pig corpus luteum of vitamin C by inducing secretion of the vitamin into the bloodstream. Further studies are necessary to determine whether the depletion of vitamin C that is induced by PGF_{2α} contributes to the demise of the pig corpus luteum.

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