Effects of ingestion of pine needles (*Pinus ponderosa*) by late-pregnant beef cows on potential sensitive Ca^{2+} channel activity of caruncular arteries

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Cows were fed either a control ($n = 6$) or pine needle ($n = 12$) diet beginning on day 249 of pregnancy. On day 3 and day 5 of feeding, control-fed and pine needle-fed cows were slaughtered and placentomes were collected for *in vitro* perfusion of the caruncular artery. Potential sensitive Ca^{2+} channel (PSC) activity as well as the responsiveness to phenylephrine (α_1 -adrenergic agonist) and adrenaline (α_1 - and α_2 -adrenergic agonist) were determined. Selected gravid uterine tissues (endometrium and myometrium, as well as caruncular and cotyledonary tissues) and associated arteries (caruncular, intercaruncular and umbilical) were isolated, minced and portions either extracted immediately for measurement of PSC or frozen at -90°C until assayed for peroxidase activity or number of α_2 -adrenergic receptors and affinities or for both. *In vitro* perfused placentomes from day 5 pine needle-fed cows had greater ($P < 0.05$) PSC activity, as measured by the increase in perfusion pressure in response to a depolarizing dose of KCl, than day 3 pine needle-fed or control-fed cows (10.3 ± 2.5 versus 6.1 ± 1.2 , and 4.3 ± 0.7 kPa, respectively). Furthermore, day 5 pine needle-fed cows also exhibited greater ($P < 0.05$) contractile responses to adrenaline than day 3 pine needle-fed or control-fed cows. Contractile responses to phenylephrine were similar ($P > 0.1$) for all three treatment groups. The observed increase in PSC activity and responsiveness to adrenaline, however, was not reflected by increasing numbers or affinities of PSC or α_2 -adrenergic receptors on caruncular arteries. Peroxidase activity (pg mg^{-1} tissue) was higher ($P < 0.05$) in caruncular tissue of pine needle-fed cows (299 ± 39) than in control-fed (186 ± 40) cows. The observed increase in peroxidase activity in caruncular tissue of pine needle-fed cows may function to increase local catechol oestrogen synthesis, in an attempt to inhibit the vasoconstrictor effects of the substance(s) in pine needles.

Introduction

Ingestion of *Pinus ponderosa* needles by late pregnant beef cows initiates a premature parturition (James *et al.*, 1989). Stuart *et al.* (1989) suggested that uterine vascular insufficiency may initiate pine needle-induced parturition. They observed a profound reduction in caruncular arterial diameter with ischaemic necrosis of the associated caruncular tissue during post-mortem examinations of early-calving pine needle-fed cows. Christenson *et al.* (1992a) showed that plasma from pine needle-fed cows, but not from control-fed cows, contained a substance(s) that increased caruncular arterial smooth muscle tone (i.e. decreased diameter) *in vitro*. Furthermore, consumption of pine needles by late pregnant beef cows resulted in a progressive decrease ($> 70\%$ total) in uterine blood flow followed by a premature parturition (Christenson *et al.*, 1992b).

Regulation of uterine arterial smooth muscle tone (i.e. vessel diameter) results from the coordinated interaction of α_2 -adrenergic receptors, potential sensitive Ca^{2+} channels (PSC),

and catechol oestrogens (Ford, 1989). Catechol oestrogens, vasoactive metabolites of oestrogen, prevent α_2 -adrenergic receptor mediated changes in vessel diameter by specifically inhibiting Ca^{2+} uptake through PSC (Ford *et al.*, in press). Placental oestrogens are converted to their catechol forms by a soluble peroxidase (Rosazza *et al.*, 1989) that is found in high concentrations in uterine lymphatic fluid and caruncular arterial tissue during pregnancy (Ford *et al.*, 1991), where it probably mediates the observed preferential dilation of the caruncular arterial bed (Makowski *et al.*, 1968).

The present study was designed to determine the site (e.g. PSC, α_2 -adrenergic receptor, peroxidase) and mechanism of action through which a substance in pine needles elicits its effects on increasing uterine arterial tone (i.e. decreasing uterine blood flow).

Materials and Methods

Animals and treatments

Eighteen pregnant multiparous beef cows bred at 1 week intervals in groups of six were transported from Fort Keogh

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Livestock and Range Research Laboratory, Miles City, MT, to the Animal Reproduction Farm in Ames, IA. Each group of six cows was acclimatized to facilities and handling procedures for 3 weeks before assignment to either control or pine needle diets. During this adjustment period, cows were placed in stanchions for 3–4 h a day and fed 8.2 kg of chopped alfalfa hay; for the remainder of the day, cows were maintained in a paddock area. Water and a vitamin-mineral supplement (Master Mix All Purpose Min-Plus, Central Soya, Fort Wayne, IN) were available to cows *ad libitum* except during the daily feeding period. The pine needles used in this study were from the same batch as those used by Christenson *et al.* (1992b) which are known to induce a premature parturition (Short *et al.*, 1992). Feeding procedures did not differ from that used in the previous study (Christenson *et al.*, 1992b). Briefly, all cows were restricted to one-half their diet on day 247 of pregnancy (term = 280 days) and received no feed on the day before initiation of the control or pine needle diets. On day 249 of pregnancy (day 0), two cows from each group of six were randomly assigned to the control diet (8.2 kg chopped alfalfa hay) and four to the pine needle diet (5.5 kg of chopped alfalfa hay plus 2.7 kg of chopped pine needles). After either 2 or 4 days of feeding, one control-fed and two pine needle-fed cows were transported to the Animal Science abattoir to be slaughtered at 07:00 h the following day (i.e. day 3 and day 5 of treatment, respectively).

Immediately after slaughter, the gravid uterus (minus the calf) and the spleen were collected and placed on ice. Within 10 min of collection, two placentomes were dissected free from the gravid uterus of the control-fed cow, and one from each pine needle-fed cow. Placentomes were then placed in oxygenated Krebs–Ringer solution (4°C) until caruncular arterial perfusions were begun. Selected uterine and placental tissues (endometrial, myometrial, caruncular and cotyledonary) as well as arteries (caruncular, main uterine, intercaruncular and umbilical) were removed, minced and frozen (–90°C) until assayed for peroxidase activity. The splenic artery (systemic control artery) was collected and processed as above. A portion of the minced caruncular arteries and myometrial tissue was frozen in liquid nitrogen for measurement of α_2 -adrenergic receptors. Plasma membrane extracts were isolated from fresh splenic arteries, caruncular arteries, and myometrial tissues and then frozen at –90°C for subsequent determination of numbers of PSC.

In vitro perfusion

Within 30 min after collection, a placentome from the control-fed cow and a placentome from one of the two pine needle-fed cows were selected for immediate *in vitro* perfusion as previously described (Sauer *et al.*, 1989). The other placentome from the control-fed cow and the placentome from the remaining pine needle-fed cow were placed in chilled (4°C) continuously gassed (95% O₂:5% CO₂) Krebs–Ringer solution for perfusion later. The maximum time from tissue collection to the start of the second perfusion was 2.5 h. Previous studies demonstrated that placentome viability and arterial responsiveness to pharmacological agents remain constant when placentomes are used within 6.5 h of collection (Sauer *et al.*, 1989). No significant differences were noted between the responses of placentomes perfused at 30 min or 2.5 h; data obtained from these perfusions were therefore grouped for analysis.

Continuously gassed (95% O₂:5% CO₂) Krebs–Ringer solution (37°C) was perfused through the caruncular artery of each preparation at a rate of 5 ml min^{–1} resulting in the establishment of a baseline perfusion pressure of 7.9 ± 0.5 kPa for the placentomes obtained from the control-fed cows. Drugs and the vehicle (Krebs–Ringer) were perfused into the intraluminal flow with a syringe infusion pump at a rate of 0.5 ml min^{–1}. Changes in intraluminal pressure due to increasing or decreasing arterial diameter were measured by pressure transducers and depicted on a chart recorder.

After both placentomes had established a constant baseline perfusion pressure (about 30 min), they were simultaneously subjected to the following sequence of infusions: (1) membrane depolarizing concentration (0.2 mol l^{–1}) KCl for 5 min; (2) vehicle for 10 min; (3) 30 μ mol phenylephrine l^{–1} (α_1 -adrenergic receptor agonist) for 5 min; (4) vehicle for 10 min; (5) 30 μ mol adrenaline l^{–1} (α_1 -, α_2 -adrenergic receptor agonist) for 5 min; (6) vehicle for 25 min and (7) bolus dose (200 μ l) of KCl (250 mg ml^{–1}) given 20 min after the start of the vehicle perfusion. This sequence of pharmacological tests evaluated PSC activity (perfusions 1 and 7), phasic contractility (perfusion 3) and tonic contractility (perfusion 5). Vehicle infusions (2, 4, 6) separated pharmacologic tests allowing vessels to return to their original baseline perfusion pressure. Previous studies have shown no carryover effect of these agonists following an adequate vehicle perfusion period (Sauer *et al.*, 1989).

Assays

Measurement of PSC. Potential sensitive Ca²⁺ channels (PSC) were determined according to the protocol of Batra (1985). Plasma membrane fractions were prepared from fresh minced arteries homogenized in Hepes/sucrose buffer (0.01 mol l^{–1}/0.25 mol l^{–1}, pH 7.2). The homogenate was centrifuged at 1000 g for 10 min, the supernatant collected and centrifuged at 12 000 g for 15 min. The supernatant resulting from the second centrifugation was then centrifuged at 40 000 g for 1 h to obtain the plasma membrane fraction. The resulting supernatant was discarded and the pellet (plasma membrane fraction) was suspended in buffer (1 mg protein ml^{–1}) and stored at –90°C until used for ligand-binding studies. The standard binding assay was performed in KCl/Hepes buffer (100 mmol l^{–1}/20 mmol l^{–1}, pH 7.2) using [³H]nitrendipine concentrations between 0.02 to 1.0 nmol l^{–1} and 50 μ g membrane protein. Incubations were carried out at 25°C for 30 min. Nonspecific binding was determined using 25 μ mol nifedipine l^{–1}, and averaged 25% at the dissociation constant (K_d). Reactions were terminated by rapid filtration through Whatman GF/F glass fibre filters. The filters were washed three times with 4 ml buffer then placed in counting vials containing 10 ml Bio-Safe NA (Research Products International, Mount Pleasant, IL), and the radioactivity was measured in a liquid scintillation spectrophotometer.

Measurement of α_2 -adrenergic receptors. Measurement of α_2 -adrenergic receptors was performed using the selective α_2 -adrenergic receptor antagonist, [³H]rauwolscine. Plasma membrane fractions were prepared from pulverized vessels and myometrium and then stored at –90°C as previously described

Table 1. Responses of caruncular arteries from control-fed and pine needle-fed cows

Treatment	n	Infusate			
		KCl (0.2 mol l ⁻¹)	Phenylephrine (30 µmol l ⁻¹)	Adrenaline (30 µmol l ⁻¹)	Vehicle + bolus dose (0.2 mol l ⁻¹) KCl (200 µl)
Control-fed	6	3.2 ± 0.9 ^a	8.9 ± 3.1 ^a	11.9 ± 2.8 ^c	4.1 ± 0.7 ^a
Day 3 pine needle-fed	6	4.1 ± 0.9 ^a	7.1 ± 0.5 ^a	7.9 ± 1.9 ^c	6.1 ± 1.1 ^a
Day 5 pine needle-fed	5	6.4 ± 1.2 ^b	9.1 ± 2.7 ^a	17.5 ± 3.3 ^d	10.3 ± 2.7 ^b

Values are means ± SEM.

Data are expressed as kPa increase over baseline perfusion pressure which averaged 7.6 kPa for all cows.

^{a,b}Means within a column with different superscripts are significantly different ($P < 0.05$).

^{c,d}Means within a column with different superscripts are different ($P < 0.08$).

(Guenther *et al.*, 1988). Binding assays were performed in triplicate by incubating 0.1 ml membrane suspension (100–200 µg protein) for 20 min at 25°C with the tritiated ligand (diluted in 0.2 ml of 0.05 mol Tris l⁻¹, pH 7.6, containing 0.005 mol MgCl₂ l⁻¹ and 1% ascorbate) in concentrations ranging from 0.3 to 6 nmol l⁻¹. Nonspecific binding was determined by adding 10 µmol phentolamine l⁻¹ to a second set of incubates. Reactions were terminated and the radioactivity measured as described above for the PSC quantitation.

Analysis of binding data

Saturation binding curves for [³H]nitrendipine and [³H]-rauwolscine were determined with each membrane preparation by using five concentrations of each respective radioligand. The maximum number of binding sites (B_{max}) as well as the K_d value were obtained by Scatchard analysis, using a computer program (EBDA, Biomedical Computing Technology Information Center, Nashville, TN). Specific binding of [³H]nitrendipine and [³H]rauwolscine in arterial and myometrial membranes was rapid and saturable. Furthermore, individual Scatchard plots showed that there was a single population of high-affinity binding sites for each radioligand. Protein determinations were made with the Bio-Rad Protein Assay (Richmond, CA).

Measurement of peroxidase

Uterine peroxidase was extracted and assayed as described by Farley *et al.* (1992). Briefly, tissues were homogenized in 1 ml Tris-HCl buffer (0.01 mol l⁻¹, pH 7.2; 4°C) then centrifuged at 40 000 g for 30 min. The supernatant was collected and represented the soluble fraction. The pellets were rehomogenized in Tris-HCl containing 0.5 mol CaCl₂ l⁻¹ to solubilize the peroxidase located in the particulate fraction. The supernatant representing the Ca²⁺-extracted fraction was collected after centrifugation (10 000 g for 30 min). Soluble and extracted fractions were evaluated in duplicate at three dilutions for peroxidase activity using H₂O₂ and *o*-phenylenediamine as substrates. Horseradish peroxidase was used to generate a standard curve. The interassay CV for a pooled endometrial tissue extract included in each assay was 5.8%. Peroxidase activity was expressed as pg horseradish peroxidase equivalents mg⁻¹

tissue wet weight. As concentrations of soluble peroxidase were about ten times greater than membrane associated peroxidase for all tissues sampled, only soluble peroxidase activity is reported.

Statistical analysis

Contractile responses and changes in α_2 -adrenergic receptors, PSC, and peroxidase activity *in vitro* were analysed as a 2 × 2 factorial treatment arrangement. Owing to heterogeneity of variance, log transformations were carried out on perfusion responses before they were analysed. No differences were observed between day 3 and day 5 control responses; they were therefore pooled for subsequent analyses and depiction of data. Orthogonal comparisons were made between control-fed versus day 3 and day 5 pine needle-fed responses as well as day 3 versus day 5 pine needle-fed responses. When no differences were observed between day 3 and day 5 pine needle-fed cows, their results were also combined for depiction in tables and figures. One cow in the day 5 pine needle-fed group could not be used because it calved prematurely on day 4 of feeding.

Results

In vitro perfusion

Baseline perfusion pressure was similar for control-fed cows (7.9 ± 0.5 kPa) and for day 3 or day 5 pine needle-fed cows (6.8 ± 0.5 and 8.0 ± 0.4 kPa, respectively). Furthermore, caruncular arterial responses to drug perfusions were similar for day 3 pine needle-fed cows and those fed a control diet (Table 1). In contrast, caruncular arteries from day 5 pine needle-fed cows exhibited greater ($P < 0.05$) PSC activity, as shown by the increased perfusion pressures in response to perfusion or bolus administration of a depolarizing dose of KCl. In addition, caruncular arteries from day 5 pine needle-fed cows exhibited a slight ($P < 0.08$) increase in perfusion pressure in response to adrenaline than caruncular arteries from day 3 pine needle-fed and control-fed cows. Phasic contractility, as estimated by phenylephrine administration, was not different for caruncular arteries across all three treatment groups.

Fig. 1. Soluble peroxidase activity for (a) gravid uterine tissues and (b) arteries from control-fed ($n = 6$; □) and pine needle-fed ($n = 11$; ■) cows. Tissue type means within a panel with different superscripts are significantly different ($P < 0.05$) from other tissue types. *Means are different ($P < 0.05$) between pine needle-fed and control-fed cows.

Measurement of vascular and myometrial PSC and α_2 -adrenergic receptors

Numbers of PSC in caruncular arteries (B_{max} ; fmol mg⁻¹ protein) and binding affinities (K_d ; pmol l⁻¹) for control-fed (99 ± 9 and 143 ± 34) and pine needle-fed (88 ± 11 and 140 ± 25) cows were not different ($P > 0.1$). Similarly, no difference ($P > 0.1$) in numbers of α_2 -adrenergic receptors in caruncular arteries (B_{max} ; fmol mg⁻¹ protein) or binding affinities (K_d ; pmol l⁻¹) were observed between control-fed (30 ± 6 , 1.0 ± 0.2) and pine needle-fed (36 ± 5 , 1.7 ± 0.4) cows. Numbers of myometrial α_2 -adrenergic receptors (fmol mg⁻¹ protein) were higher ($P < 0.08$) in day 5 pine needle-fed cows (632 ± 127) than in both day 3 pine needle-fed (349 ± 90)

and control-fed (407 ± 91) cows which were not significantly different. The K_d value for α_2 -adrenergic receptors in myometrial tissues were similar across treatment groups (data not shown). Potential sensitive Ca^{2+} channel numbers (fmol mg⁻¹ protein) for the splenic arteries and myometrial tissue were also not significantly different between control (76 ± 17 and 144 ± 15 , respectively) and pine needle-fed (76 ± 15 and 150 ± 11 , respectively) cows.

Peroxidase activity in gravid uterine tissues and associated arteries

Uterine tissue peroxidase activity was highest in the endometrium, the purported site of peroxidase synthesis (Ford, 1992; Fig. 1a). Caruncular and cotyledonary tissue had similar peroxidase activities, but had lower values than endometrial tissue. Peroxidase activity was lowest in the myometrial tissue. The only treatment effect observed was an increase ($P < 0.04$) of peroxidase activity in caruncular tissue from pine needle-fed versus control-fed cows (Fig. 1a). Caruncular arterial peroxidase activity was similar ($P > 0.1$) to that found in the endometrium (Fig. 1a and 1b). When compared with the caruncular arteries, the intercaruncular artery, main uterine artery and splenic artery had lower ($P < 0.05$) peroxidase activity (Fig. 1b). The umbilical artery had markedly lower ($P < 0.05$) peroxidase activity compared with all the other arterial tissues sampled.

Discussion

Previous studies using this feeding regimen have shown that pine needle-induced parturition typically occurs between 6–11 days after initiation of the diet (Christenson *et al.*, 1992b; Short *et al.*, 1992). Increasing numbers of α_2 -adrenergic receptors in myometrium have been closely linked with increased contractility of myometrial smooth muscle (Roberts *et al.*, 1981; Marnet *et al.*, 1987; Ko *et al.*, 1990). Furthermore, treating cows with xylazine, an α_2 -adrenergic receptor agonist, caused an increase in intra-uterine pressure which could be inhibited with α_2 -adrenergic receptor antagonists (Rodriguez-Martinez *et al.*, 1987). The increasing numbers of myometrial α_2 -adrenergic receptors in day 5 pine needle-fed cows supports the assumption that these cows were preparing for the parturient process with increasing myometrial contractility.

We determined that consumption of pine needles by late pregnant beef cows elicited a progressive reduction in uterine blood flow (Christenson *et al.*, 1992b). Furthermore, using the *in vitro* perfused bovine placentome, we determined that uterine arterial tone and PSC activity were increased in placentomes perfused with plasma from pine needle-fed cows (Christenson *et al.*, 1992a). The study reported here was designed to determine the specific site of action of the pine needle factor(s) on the known mechanisms controlling uterine blood flow (Ford *et al.*, 1992). Blood flow to the uterus is known to be regulated by two Ca^{2+} -dependent mechanisms: (1) phasic contractility and (2) tone. Phasic contractility remains intact throughout pregnancy, allowing the short-term (3–10 min) shunting of blood away from the uterus and other abdominal viscera to the skeletal muscle during periods of acute maternal stress (Shnider *et al.*, 1979; Sauer *et al.*, 1989). Uterine arterial tone progressively decreases during pregnancy, allowing uterine blood flow to

increase to meet the needs of the growing fetal-placental unit (Ferrell and Ford, 1980; Guenther *et al.*, 1988). A phasic contraction results from α_1 -adrenergic receptor activation which stimulates the hydrolysis of phosphatidylinositol into inositol triphosphate and diacylglycerol. Inositol triphosphate releases Ca^{2+} from the sarcoplasmic reticulum, which activates calmodulin resulting in the phosphorylation of myosin light chain kinase and a transient shortening of the vascular smooth muscle cells (Rasmussen *et al.*, 1987). Pine needle feeding did not change the phasic response, confirming our earlier observation that plasma from pine needle-fed cows could not alter phasic responses (Christenson *et al.*, 1992a). It therefore seems convincing that feeding pine needles neither alters the receptors or second messenger systems involved in phasic contractile responses nor does it contain an α_1 -adrenergic receptor agonist.

Changes in uterine arterial tone are mediated by α_2 -adrenergic receptors, and their interaction with PSC and the oestrogen metabolites, catechol oestrogens. Adrenaline activation of α_1 - and α_2 -adrenergic receptors increases diacylglycerol (α_1 -response) and the opening of the PSC (α_2 -response) allowing extracellular Ca^{2+} to cycle across the cell membrane. The diacylglycerol and Ca^{2+} in turn activates protein kinase C which phosphorylates proteins in the actin domain, causing a long-term change in cell length (Rasmussen *et al.*, 1987; Ford *et al.*, 1992). In this study we observed an increase in tonic contractility (adrenaline response) in caruncular arteries from day 5 pine needle-fed cows but not day 3 pine needle-fed cows. This might suggest that it took longer than 3 days to alter the cellular constituents (i.e., PSC, α_2 -adrenergic receptor) involved in tone changes. However, upon determination of caruncular arterial PSC and α_2 -adrenergic receptor numbers and dissociation constants, there were no differences between groups. This result suggests that the PSC or another distal component in the tone pathway may be directly activated. Evaluation of PSC activity *in vitro* substantiates this premise as a marked increase in PSC activity was observed in day 5 pine needle-fed cows. This observation is consistent with results from our study which showed that plasma from cows consuming pine needles contained a substance which increased PSC activity (Christenson *et al.*, 1992a).

Uterine peroxidase has been demonstrated to convert oestradiol and oestrone to their vasoactive 2- and 4-hydroxylated 'catechol' metabolites (Rosazza *et al.*, 1989). Catechol oestrogens are the only oestrogens known to decrease uterine tone *in vitro* and increase uterine blood flow *in vivo* (Ford *et al.*, in press). Catechol oestrogens block PSC activity (Stice *et al.*, 1987) thereby preventing Ca^{2+} uptake and a long term vasoconstriction. Pine needle-fed cows had high concentrations of peroxidase in caruncular tissue, suggesting that in response to the decreasing blood supply, peroxidase production may have increased in an attempt to increase production of catechol oestrogen.

This study was the first to characterize and compare fully peroxidase activities of selected gravid bovine uterine tissues and associated arteries. Previous studies in pigs have shown that peroxidase activity was markedly higher in endometrial tissue than in other gravid uterine tissues (Ford, 1992). Furthermore, only endometrial tissue could synthesize and secrete peroxidase *in vitro* (Ford, 1992). In the study reported here, bovine endometrial tissue again had the greatest peroxidase activity of any tissue examined, suggesting that this tissue is the

site of synthesis in this species. Furthermore, as the distance from the endometrial tissue increases, peroxidase activity decreases, suggesting tissue diffusion or a specific lymphatic transport mechanism or both mechanisms. We reported high concentrations of peroxidase in lymphatic fluid from late pregnant cows (Ford *et al.*, 1991). Caruncular arterial peroxidase activity was high and similar to that observed in the endometrium, and may explain the preferential vasodilation of this vascular bed during pregnancy. As it is unlikely that the arterial tissue has the capacity for large scale production of peroxidase; it has been postulated (Ford *et al.*, in press) that lymphatic vessels transport peroxidase to the artery where it can bind to and ultimately affect uterine blood flow via metabolism of oestrogens to catechol oestrogens.

In summary, on the basis of the increase in PSC activity and tone induced by pine needles, and the lack of change in α_2 -adrenergic receptors or PSC numbers, we conclude that pine needles contain a substance(s) which interferes with the ability of catechol oestrogen to block PSC activity. This substance may be a PSC agonist, or it may inhibit catechol oestrogen synthesis or prevent catechol oestrogen from interacting with the PSC.

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