Intraluteal Administration of a Nitric Oxide Synthase Blocker Stimulates Progesterone and Oxytocin Secretion and Prolongs the Life Span of the Bovine Corpus Luteum (44514)

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Abstract. **To test the role of nitric oxide (NO) in secretory functions of bovine corpora lutea (CL), two groups of four Holstein heifers each were treated as follows: Group 1, Nω-Nitro-L-Arginine Methyl Ester (L-NAME), an inhibitor of nitric oxide synthase (NOS), on Day 11 or 12 of the cycle and Group 2, L-NAME on Days 17 and 18 of the cycle. All treatments were administered by an intraluteal microdialysis system (MDS). Drugs were infused for 4-hr periods on the designated days, and the treatment periods were preceded and followed by 4-hr control periods. Perfusate and jugular blood samples were collected at half-hour intervals. Perfusate samples were analyzed for progesterone (P4), oxytocin (OT), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and leukotriene C_4 (LTC_4); jugular plasma samples were analyzed for P_4, OT, and LH. Perfusion of L-NAME on Day 11 or 12 consistently increased P_4 concentration in the perfusate, but had no effect on the life span of the CL. Perfusion of L-NAME on Days 17–18 also elevated P_4 levels in the perfusate, and in addition, maintained P_4 levels in the plasma of three of the four treated animals through Day 25 of the cycle. L-NAME perfusion also increased OT release concomitant with P_4 into the perfusate at both the mid- and late-luteal phase treatments. For the most part, concentrations of LH, OT, and P_4 in the jugular plasma samples collected during the perfusions were unaffected by treatments. L-NAME perfusion caused small, but significant ($P < 0.05$) increases in perfusate $PGF_{2\alpha}$ and LTC_4 at Days 17 and 18 and in LTC_4 on Day 11 or 12. These data indicate that NO plays a direct luteolytic role in regression of the bovine CL.**

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The current concept that luteolysis in cattle is brought about by $PGF_{2\alpha}$ of uterine origin secreted in response to OT released by the CL is inadequate to explain many of the events that actually occur at the time of regression (1). Although luteolytic *in vivo*, $PGF_{2\alpha}$ *in vitro* does not inhibit basal P_4 production by the large luteal cells (LLC) and actually stimulates P_4 production by small luteal cells (SLC). There is very little OT present in the CL at the time of luteolysis, and several recent studies indicate that luteolysis can occur after depletion of luteal OT (2) and in the absence of measurable OT release from the luteal tissue (3). A number of studies indicate that the products of the lipoxygenase pathway of the arachidonic acid (AA) cascade, particularly LTC_4 play major roles in luteolysis. Intrauterine infusions of a blocker of the lipoxygenase pathway prolong the functional life of the CL (4), and lipoxygenase products, including LTC_4, markedly inhibit P_4 production *in vitro*. In a previous microdialysis study, leukotriene B_4 (LTB_4) and LTC_4 were found in the perfusate and rose prior to the decline in progesterone during luteolysis (3).

Bovine ovaries are richly supplied by adrenergic and

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peptidergic nerves (5) as well as nerves synthesizing NO (6). Neuropeptides such as neuropeptide Y, substance P, vasoactive intestinal peptide (7), and norepinephrine (2, 8) act on P_4 secretion in bovine CL. NO, a highly reactive free radical acts as an important regulator of many physiological events, including blood pressure, neurotransmission, and host defense (9, 10). NO is synthesized *via* the oxidation of L-arginine by nitric oxide synthase (NOS). This enzyme catalyzes the mixed function oxidation of a guanidino nitrogen atom of L-arginine to yield L-citrulline and NO (9). The presence of NO has been shown in ovaries of many mammalian species (11–20) including bovine cumulus cells (21). In rats and rabbits, NO influences follicular development, rupture, and atresia (11, 13–15, 20), and participates in the regulation of CL function (17, 19). In women undergoing *in vitro* fertilization, a positive correlation was found between circulating and follicular fluid nitrite/nitrate concentrations and follicular development (22, 23). Moreover, it was shown that the cells obtained from rat ovaries at all stages of follicular development (preantral, Graafian, ovulatory, and atretic) and luteinization synthesized NO in a linear manner over time, but the basal production of NO was 6- to 14-fold higher in cells obtained from luteinized ovaries than from cells obtained from ovaries at all other stages (16).

In vitro experimental evidence indicates that NO negatively regulates estradiol (E_2) and P_4 secretion in human granulosa cells (12) and rat luteinized ovarian cells (16), whereas inhibition of NOS activity significantly increases E_2 secretion without changes in P_4 production (12, 16). Furthermore, it was shown that NO increased PG production in perfused rabbit ovaries (20), and addition of NOS inhibitors to incubated luteal tissue from late pseudopregnant rats significantly diminished $PGF_{2\alpha}$ production (19). These *in vitro* studies suggest that NO functions as an important autocrine and/or paracrine agent in ovarian secretion, but there is little or no information about its actions *in vivo*, especially in ruminant animals in which the uterus plays a key role in luteolysis. Therefore, the aim of these experiments was to determine the role of NO in the secretory function of bovine CL during the middle and late luteal phases of the estrous cycle.

Materials and Methods

MDS Implantation. Normally cycling Holstein heifers (n = 8) were injected intramuscularly (i.m.) with 25 mg of $PGF_{2\alpha}$ (Lutalyse; Pharmacia and Upjohn, Bridgewater, NJ) during the luteal phase to induce luteolysis and estrus. The MDS was implanted, as described by Blair *et al.* (3), into the CL on Days 10 or 11 (n = 4) and 16 (n = 4) of the subsequent estrous cycle (Day 0 = estrus). The animals were premedicated with xylazine, 50 mg/animal i.m. (Rompun; Miles, Shawnee Mission, KS) and local anesthesia (epidural and at the site of incision), using 2% lidocaine hydrochloride (Lidocaine 2% injectable; Butler, Columbus, OH), was induced immediately prior to surgery. The ovaries were exteriorized through a flank laparotomy, and the MDS was threaded through the CL such that the dialysis tubing (Fresenius SPS 960; Frankfurt, Germany; MW cutoff = 1,000,000 Da; o.d. = 500 μm; i.d. = 340 μm) was localized within the CL. The MDS was fixed to the surface of the CL at the points of entrance and exit by tissue glue, and the ovary was replaced into the peritoneal cavity. The connecting tubes were exteriorized through a puncture in the paralumbar fossa and connected to Teflon tubing. One end of the Teflon tubing was connected to a syringe pump (KDS Scientific Model 100; Cole Palmer, Vernon Hills, IL), whereas the other was connected to a fraction collector (Model 2110; Bio Rad, Hercules, CA). Animals were kept in individual stalls in a temperature-controlled room, and the CL was perfused with Ringer's solution immediately after surgery. All infusions were at a flow rate of 3 ml/hr. All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee.

The recovery rates of hormones across the MDS were measured as previously described (24) and amounted to ≈ 0.1% for OT and LTC_4, 0.3% for $PGF_{2\alpha}$, and 1% for P_4.

Schedule of Experiments. *Experiment 1.* The CL of 4 animals were perfused on Day 11 or 12 of the cycle for 4 hr with 100 mg of Nω-Nitro-L-Arginine Methyl Ester (L-NAME; Cayman Chemical Co., Ann Arbor, MI), an inhibitor of NOS. Perfusate and jugular blood samples were collected every 30 min before, during, and after L-NAME infusion. Each 4-hr treatment period was preceded and followed by 4-hr control periods during which physiological saline solution was perfused at the same rate (3 ml/ hr). Blood samples were collected through catheters implanted into the jugular vein. Additional blood samples were taken once daily by jugular venipuncture through Day 22 of the estrous cycle to determine the functional life of the CL.

Experiment 2. The CL of 4 animals were perfused on Day 17 and again on Day 18 of the estrous cycle for 4 hr with 100 mg of L-NAME. As in Exp. 1, treatment periods were preceded and followed by 4-hr control periods. The perfusate and blood samples were collected with the same frequency as in Exp. 1. Moreover, blood samples were taken once daily by jugular venipuncture from Days 19–25 of the estrous cycle to determine the functional life of the CL.

All samples were stored at –80°C until hormone determinations were made.

Hormone Determinations. P_4 concentrations in perfusate and plasma samples were determined by radioimmunoassay (RIA) as previously described (25). The P_4 antiserum used had 11.64% cross-reaction with 5β-dihydroprogesterone, 1.97% with 5α-dihydroprogesterone, 2.64% with 20β-OH-progesterone, 1.90% with 20α-OH-progesterone, 3.22% with 17α-OH-progesterone, 0.09% with pregnenolone, 0.96% with corticosterone, 0.03% with testosterone, 0.01% with cortisol, and less than 0.01% with estrone, 17β-estradiol, 17α-estradiol, estriol, androstendione, 5α-dihydrotestosterone, dehydroepiandrostendione, and cholesterol. The assay sensitivity was 0.15 ng/ml, and

the intra- and interassay coefficients of variation (CVs) were 5.30% and 9.44%, respectively.

OT concentrations in perfusate and plasma samples were measured by RIA as previously described (26, 27). The detection limit of the assay was 0.34 pg/ml, and intra- and interassay CVs were 4.6% and 9.5%, respectively.

LH concentration in plasma samples was determined by RIA as described by Thompson *et al.* (28). The assay sensitivity was 0.44 ng/ml and intra- and interassay CVs were 3.7% and 9.6%, respectively.

LTC_4 and $PGF_{2\alpha}$ concentrations were determined in perfusate samples using commercially available enzyme immunoassay kits (Cayman Chemical Co.) according to instructions of the manufacturer. The assay sensitivities were 9.81 pg/ml for LTC_4 and 4.51 pg/ml for $PGF_{2\alpha}$, respectively. The intra- and interassay CVs were 4.1% and 9.0% for LTC_4 and 5.4% and 8.7% for $PGF_{2\alpha}$, respectively.

Data Analysis. Experimental data are shown as mean ± SEM. Treatment differences in each experiment were assessed by one-way analysis of variance (ANOVA) using Bonferroni's Multiple Comparison Test (Graph Pad Prism). Differences with $P < 0.05$ were regarded as statistically significant.

Results

Experiment 1. Perfusion of L-NAME on Day 11 or 12 of the cycle caused marked synchronous increases in P_4 and OT ($P < 0.001$) in the perfusate samples (Fig. 1). L-NAME perfusion also caused a relatively small, but significant ($P < 0.001$) increase in LTC_4 concentration, but did not change the $PGF_{2\alpha}$ level ($P > 0.05$) in the perfusate samples (Fig. 1). No changes were observed in P_4, OT, and LH concentrations in plasma samples collected during perfusion (Fig. 2). Plasma P_4 concentrations of all treated animals declined between Days 18 and 22 (Fig. 3).

Experiment 2. L-NAME perfusion on Days 17 and 18 also caused increases in P_4 concentrations ($P < 0.001$) in perfusate samples on both Days 17 and 18 (Fig. 4a), even though the level of P_4 in the plasma decreased slightly on both Day 17 ($P < 0.05$) and Day 18 ($P < 0.01$; Fig. 4b). The concentrations of P_4 in plasma samples collected during Days 19–25 of the estrous cycle were maintained above 3 ng/ml in three of the four treated animals (Fig. 5). Increased levels of both LTC_4 ($P < 0.01$) and $PGF_{2\alpha}$ ($P < 0.05$) were found in the perfusate samples (Fig. 6). As was the case at Day 11 or 12, L-NAME perfusions increased OT concentrations ($P < 0.001$) in the perfusate samples (Fig. 7) on Days 17 and 18 of the cycle, but did not affect the concentration of OT or LH in the plasma samples (unpublished data).

Discussion

The abilities of infused L-NAME to cause marked increases in P_4 release at Day 11 or 12 and at Days 17 and 18, and to prolong the functional life of the CL to at least 25 days, when administered at Days 17 and 18, indicate that

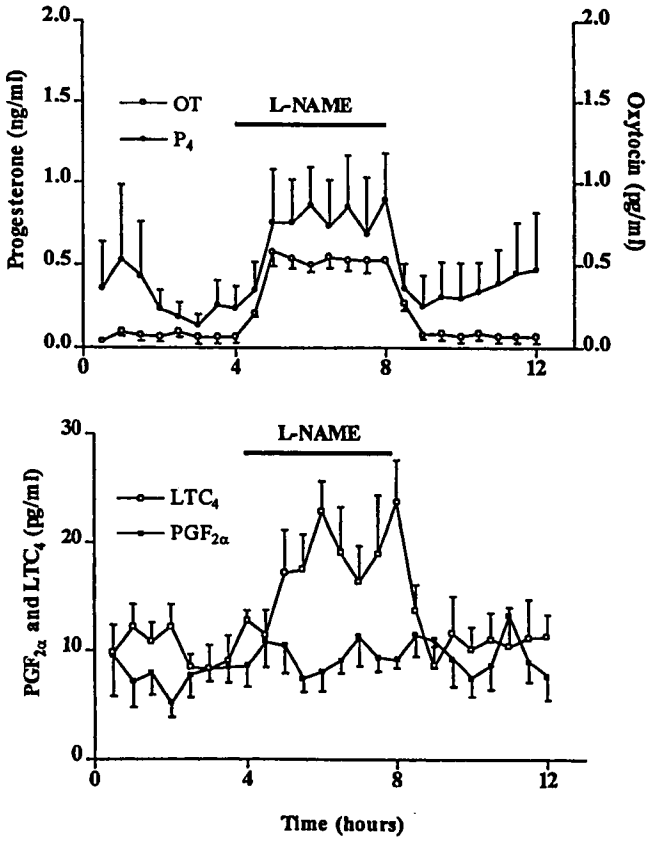

Figure 1. Concentrations of progesterone (P_4), oxytocin (OT), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and leukotriene C_4 (LTC_4) in perfusate samples during L-NAME infusion on Day 11 or 12 of the estrous cycle.

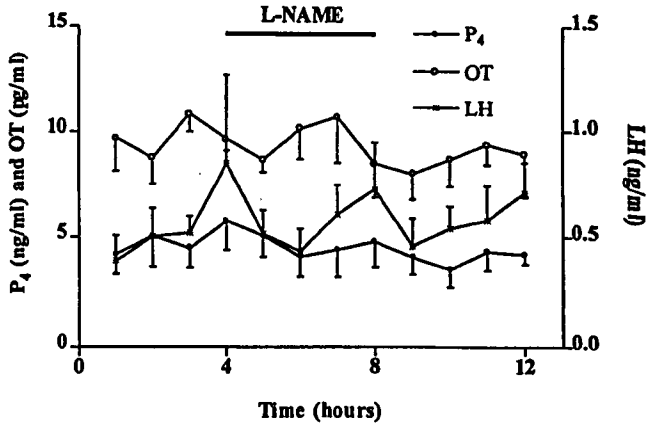

Figure 2. Concentrations of progesterone (P_4), oxytocin (OT), and luteotropic hormone (LH) in plasma samples collected during L-NAME infusion on Day 11 or 12 of the estrous cycle.

NO plays an important role at the ovarian level in inhibiting P_4 production and initiating luteal regression. NO inhibited both P_4 and E_2 secretion in human granulosa cells (12) and luteinized rat ovarian cells (16) and inhibited P_4 secretion in cultured bovine luteal cells (29). NO has also been shown to inhibit several P450 steroidogenic enzymes (30). It is also known that NO activates heme-containing enzymes, among them a cyclooxygenase (COX) which is rate-limiting in the

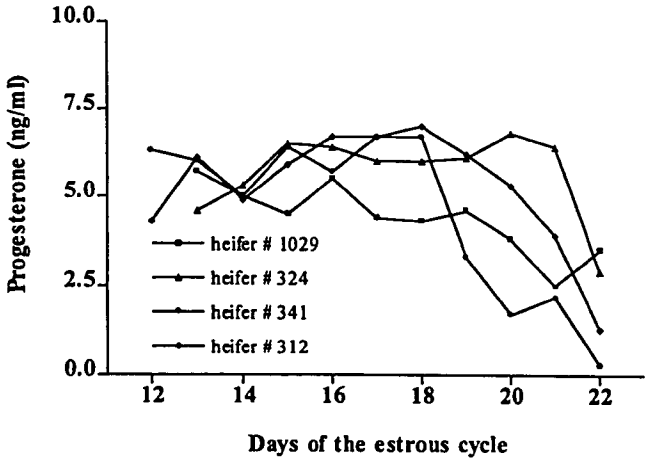

Figure 3. Plasma progesterone concentrations in samples collected once daily after L-NAME infusion on Day 11 or 12 to Day 22 of the estrous cycle.

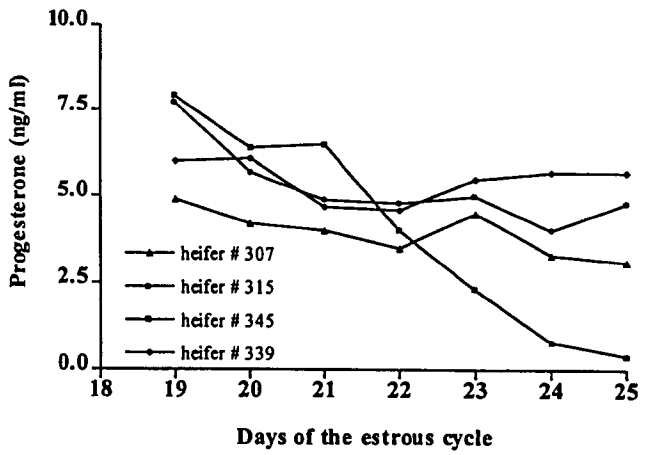

Figure 5. Concentration of progesterone in plasma samples collected once daily after L-NAME infusion on Days 17 and 18 of the estrous cycle.

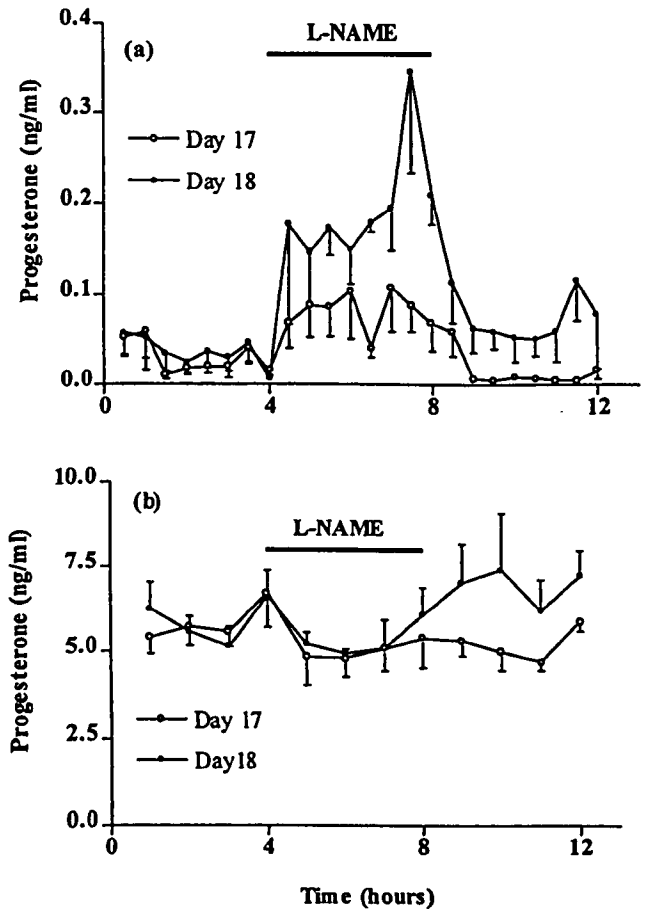

Figure 4. Concentration of progesterone in (a) perfusate and (b) blood plasma samples during L-NAME infusion on Days 17 and 18 of the estrous cycle.

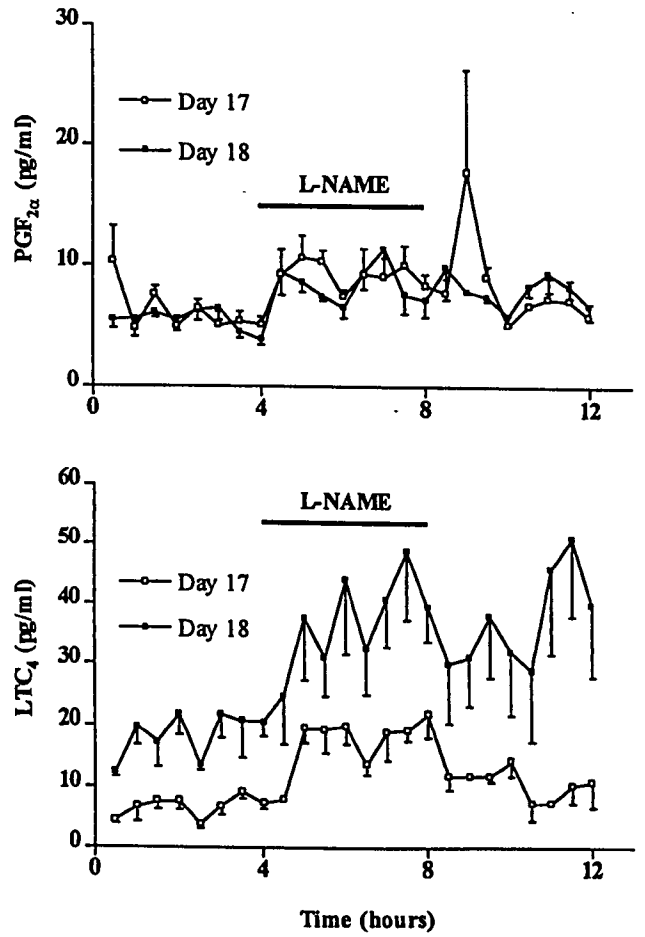

Figure 6. Concentrations of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and leukotriene C_4 (LTC_4) in perfusate samples during L-NAME infusion on Days 17 and 18 of the estrous cycle.

biosynthesis of prostaglandins, tromboxane A_2, and prostacyclin (9, 31). A most interesting feature of these data is the precise coincidence of the changes in secretion of P_4 and OT into the perfusate before, during, and after L-NAME administration at mid- (Day 11 or 12) and late (Days 17–18) luteal phases of the cycle. A linkage between P_4 secretion and secretory granule exocytosis from the LLC was first reported for the ewe in 1974 (32) and later for the cow (33). Since the secretory granules of the LLC are known to contain OT (34), it would appear that L-NAME, through NO inhibition, may act primarily on the LLC. However, it

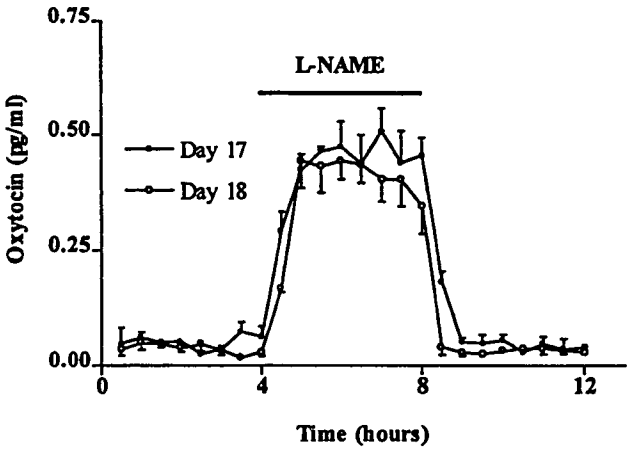

Figure 7. Concentration of oxytocin (OT) in perfusate samples during L-NAME infusion on Days 17 or 18 of the estrous cycle.

should be noted that SLC secrete P_4 in response to LH, even though they contain few, if any secretory granules.

The fact that infusion of L-NAME on Days 17 and 18 elevated P_4 and prevented luteolysis at the normal time, despite the fact that it increased both $PGF_{2\alpha}$ and LTC_4 in the perfusate, suggests that NO exerts its luteolytic effects directly on the CL. This local luteolytic effect might involve direct effects on the luteal cells, a mobilization of endothelin which has been shown to have luteolytic properties (35), or a combination of both. Activation of endothelial nitric oxide synthase (eNOS) by endothelins results in release of NO that mediates the vasorelaxing effects of bovine endothelial cells, which are numerous in bovine luteal tissue.

The administration of L-NAME may reduce or abolish the vasorelaxing effect of NO and endothelin. However, the marked increases in P_4 and OX in the present study were precisely limited to the period of L-NAME infusion, and it seems unlikely that they were influenced by previous accumulations due to vasoconstriction. Blood flow alterations play important roles in luteal regression. A marked decline in blood flow occurs during both normal and $PGF_{2\alpha}$-induced luteolysis (1). Sclerotic changes involving hypertrophy and hyperplasia of cells in the walls of small arterioles leading to a reduced blood flow have been associated with luteal regression in cattle (1).

The increase in $PGF_{2\alpha}$ and LTC_4 in the perfusate after L-NAME administration was a surprising result. However, accumulating evidence suggests that NO, and endothelin-1 as well, exert their effects by enhancing or mediating the effects of $PGF_{2\alpha}$ and LTC_4 rather than by controlling their production (36, 37). The ability of $PGF_{2\alpha}$ to cause an initial increase in P_4 production *in vivo* after intraluteal administration of $PGF_{2\alpha}$ by microdialysis was recently demonstrated (38), and $PGF_{2\alpha}$ is known to increase P_4 production by bovine luteal cells *in vitro*.

For the most part, concentrations of LH, OT, and P_4 in the jugular plasma were not affected by changes in concentrations in the perfusate samples, although the functional life of the CL was prolonged after L-NAME administration on Days 17 and 18, as indicated by continued high plasma P_4 levels until Day 25. Plasma P_4 concentrations in normal Holstein heifers begin to decline on about Day 18 of the cycle and are usually less than 1 ng/ml by Day 20 (1, 4). P_4 plasma concentrations had clearly declined in all of the animals treated with L-NAME by Day 22 (Fig. 2). The small but significant ($P < 0.05$) decline in plasma P_4 levels during L-NAME perfusion on Days 17 and 18 did not appear to be due to decreased LH secretion. It is known that NO increases LHRH release in rats (39, 40) and that LH stimulates P_4 secretion by the bovine CL (1, 41).

In summary, intraluteal infusion of the NOS inhibitor, L-NAME at either mid- or late phases of the estrous cycle increased the secretion of both P_4 and OT in the perfusate samples, and L-NAME administered on Days 17–18 of the cycle prolonged the functional life of the CL. Moreover, L-NAME perfusion marginally increased LTC_4 in both phases and $PGF_{2\alpha}$ in the late luteal phase. The levels of OT, LH, and P_4 in the plasma samples, for the most part, were unaffected by the intraluteal treatments of L-NAME. These data are the first to show that NO acts as a significant auto/paracrine factor in the mid- and late luteal phases and plays an important local role in the initiation of luteal regression in a ruminant.

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