Influence of Nitric Oxide on the Secretory Function of the Bovine Corpus Luteum: Dependence on Cell Composition and Cell-to-Cell Communication

JERZY J. JAROSZEWSKI,*'§'[1] DARIUSZ J. SKARZYNSKI,† ROBERT M. BLAIR,‡'§ AND WILLIAM HANSEL§

Department of Pharmacology, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, 10-718 Olsztyn, Poland; †Division of Reproductive Endocrinology and Pathophysiology, Institute of Animal Reproduction and Food Research, Polish Academy of Science, Olsztyn 10-747 Olsztyn, Poland; ‡Department of Pathology/Comparative Medicine, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157; and §Department of Reproductive Biotechnology, Pennington Biomedical Research Center, Louisiana State University, Baton Rouge, Louisiana 70808

The objective of the present study was to investigate the role of cell-to-cell contact in the influence of nitric oxide (NO) on the secretory function of the bovine corpus luteum (CL). In Experiment 1, separate small luteal cells (SLC) or large (LLC) luteal cells were perfused with 100 μM spermineNONOate, a NO donor, or with 100 μM Nω-nitro-L-arginine methyl ester (L-NAME), a NO synthase (NOS) inhibitor; in Experiment 2, a mixture of LLC and SLC and endothelial cells was cultured and incubated with spermineNONOate or L-NAME; in Experiment 3, spermineNONOate was perfused into the CL (100 mg/4 hr) by a microdialysis system *in vivo*. Perfusion of isolated SLC and LLC with the NO donor or NOS inhibitor (Experiment 1) did not affect ($P > 0.05$) secretion of progesterone (P_4) or oxytocin (OT). L-NAME perfusion increased ($P < 0.05$) leukotriene C_4 (LTC_4) secretion by both SLC and LLC cells. Treatment of mixtures of luteal cells with an NO donor (Experiment 2) significantly decreased ($P < 0.001$) secretion of P_4 and OT and increased ($P < 0.001$) production of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and LTC_4. L-NAME stimulated ($P < 0.001$) P_4 secretion, but did not influence ($P > 0.05$) OT, $PGF_{2\alpha}$ or LTC_4 production. Intraluteal administration (Experiment 3) of spermineNONOate increased ($P < 0.001$) LTC_4 and $PGF_{2\alpha}$, decreased OT, but did not change P_4 levels in perfusate samples. These data indicate that cell-to-cell contact and cell composition play important roles in the response of bovine CL to treatment with NO donors or NOS inhibitors, and that paracrine mechanisms are required for the full secretory response of the CL in NO action. Endothelial cells appear to be required for the full secretory response of the CL to NO. Exp Biol Med 228:741–748, 2003

Key words: nitric oxide; progesterone; oxytocin; prostaglandin $F_{2\alpha}$; leukotriene C_4; bovine

During the last decade, many studies have shown that nitric oxide (NO), a highly reactive free radical, generated from L-arginine by NO synthase (NOS), acts as an important regulator of many physiological events (1, 2), including regulation of bovine ovarian functions (3). Although recent data from our laboratory indicate that both inducible and endothelial isoforms of NOS (iNOS and eNOS) activities are present in the bovine corpus luteum (CL) (4), the role of NO in the regulation of steroidogenesis in bovine CL remains unclear. We showed that perfusion of bovine CL with Nω-nitro-L-arginine methyl ester (L-NAME), an NOS inhibitor, stimulated progesterone (P_4) and oxytocin (OT) secretion in both mid- and late luteal phases and prolonged the duration of the estrous cycle after administration in the late luteal phase (5). Skarzynski and Okuda (6) showed that NOS inhibitors stimulated P_4 secretion, but did not affect the production of prostaglandin E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) in cultured dispersed bovine luteal cells. Moreover, an NO donor inhibited P_4 production and stimulated both PGE_2 and $PGF_{2\alpha}$ secretion (6).

It has been postulated that NO is negatively involved in the regulation of steroidogenesis because it exhibits two distinct activities on cytochrome P450 activity (7). A negative effect of NO on steroidogenesis was indeed evident as

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[1] To whom requests for reprints should be addressed at Department of Pharmacology, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, Oczapowskiego 13, 10-718 Olsztyn, Poland. E-mail: jerzyj@uwm.edu.pl

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Van Voorhis *et al.* (8) showed that addition of NO donors caused a dose-dependent decrease in both estradiol (E_2) and P_4 secretion by cultured human granulosa-luteal cells. A similar inhibitory effect of NO on secretion of ovarian steroids was observed in cells from luteinized rat ovaries (9) and cultured human luteal cells (10–12). However, Friden *et al.* (13) showed that spermine NONOate reduced P_4 production in dispersed human CL cells from the late, but not mid-luteal phase. On the other hand, Dong *et al.* (14) demonstrated a dose-dependent increase in P_4 production in response to a NO donor in equine chorionic gonadotrophin (eCG)-treated immature rats. More recently, Motta *et al.* (15) showed that endogenous NO increased both glutathione and P_4 production in rat ovaries from mid-stage CL. It is suggested that NO could have a dual action, protective or pro-oxidant, in development of CL (15). The discrepancies between the above findings might be due to the stage of the estrous cycle at which the luteal tissue was harvested (6, 14, 15). However, we suggest that differences result from the presence or absence of different subpopulations of steroidogenic and nonsteroidogenic cells, and in cell-to-cell contact, both of which may affect the action of NO in the synthesis of steroid hormones in the CL. It is postulated that cell-to-cell contact and communication among luteal and nonluteal cells are essential for the development (16), maintenance (17, 18), and regression of bovine CL (19, 20). Therefore, in the present study, we examined the roles of cell composition and cell-to-cell communication in the influence of NO on secretory function of bovine CL, using different experimental models.

The aim of the first experiment was to investigate the influence of an NO donor or NOS inhibitor on dynamic changes in secretory function of pure, separate small luteal cells (SLC) and large luteal cells (LLC) using a perfusion system. The objective of the second experiment was to study how an NO donor or NOS inhibitor affect a mixture of all types of the luteal cells cultured with structural contact in conglomerates/aggregates (21). Finally, in the third experiment, the influence of NO on bovine CL was studied *in vivo* using a microdialysis system (MDS).

Materials and Methods

Collection of CL for *In Vitro* Experiments. Normally, cycling Holstein heifers (n = 8) were injected (i.m.) with 25 mg of $PGF_{2\alpha}$ (Lutalyse; Pharmacia & UpJohn, Bridgewater, NJ) to synchronize estrus. CL were removed on Days 10 to 12 of the subsequent estrous cycle. For CL collection, heifers were treated with 5.0 ml of a 2% lidocaine hydrochloride solution (Lidocaine; Butler, Columbus, OH) via an epidural injection to achieve a local plane of anesthesia. An incision was made through the anterior vaginal wall and the CL was extirpated manually from the ovary (Experiment 1), or the entire ovary was collected transvaginally (Experiment 2). The tissues were placed immediately on ice for transportation to the laboratory for dissociation and cell separation as described below.

Experiment 1: Isolation and Perfusion of Pure Populations of SLC and LLC. The CL (n = 4) were thoroughly washed in cold (4°C) Dulbecco's modified Eagle's medium (DMEM)/Nutrient Mixture F-12 with 15 mM HEPES buffer and L-glutamine (DMEM/F-12; GIBCO Laboratories, Grand Island, NY) and were supplemented with penicillin-streptomycin (100 units/ml penicillin and 100 μg/ml streptomycin; GIBCO Laboratories). The connective tissue was stripped away and the CL were sliced into thin pieces. Luteal cells were dispersed by incubation at 35°C with collagenase (2,500 units/g luteal tissue; C-0130; Sigma, St. Louis, MO) in media (10 ml/g luteal tissue) as described by Alila and co-workers (22). LLC and SLC were separated by unit gravity sedimentation as described by Koos and Hansel (23) providing a highly enriched population of SLC (>90% pure) and an impure LLC fraction. Three groups of 100 to 150 LLC each were aspirated from the LLC fraction using small diameter Pasteur pipettes with the aid of an inverted microscope. No accessory cells, which consist mainly of endothelial cells, were included in either the SLC or the LLC preparations. The viability of the cells was higher than 89% as assessed by trypan blue exclusion, and there were no significant changes in this parameter before and at the end of the experiment.

After separation, LLC and SLC were incubated in an *in vitro* perfusion culture system (Endotronics, Minneapolis, MN) for 6 hr. Luteal cells were cultured in DMEM/F-12 medium with 15 mM HEPES and L-glutamine and were supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). During the incubation, the medium was gassed continuously with a 95% O_2:5% CO_2 mixture and the temperature was maintained at 37°C. Luteal cells were placed in 1.5-ml culture chambers (130,000-150,000 SLC/chamber and 100-150 LLC/chamber) and were perfused at a rate of 100 μl/min. The initial 2 hr of culture were used to establish baseline hormone profiles. At the end of the 2nd hr, luteal cells received a bolus treatment of either control medium, 100 μ*M* spermineNONOate (Cayman Chemical Co., Ann Arbor, MI), or 100 μ*M* L-NAME (Cayman Chemical Co.), an NOS inhibitor, and were cultured for another 4 hr. The doses of reagents were established in preliminary experiments (data not shown). Culture media were collected simultaneously from each culture chamber at 30-min intervals with the aid of a fraction collector. Culture media samples were stored immediately at –80°C until they were assayed for P_4, OT, $PGF_{2\alpha}$, and leukotriene C_4 (LTC_4). Based on a time-course study using dye, perfusion at a flow rate of 100 μl/min resulted in treatments initially reaching the culture chamber within 12 to 15 min of bolus injection.

Experiment 2: Isolation and Culture of Dispersed Luteal Cell Mixtures. The enzymatic dissociation of the luteal tissue (n = 4; each experiment made in triplicate) and the co-culture of the luteal cells in glass tubes in a shaking water bath were performed as previously described (24, 25). The cell suspension contained approxi-

mately 10% of endothelial cells, 20% of large and 70% of SLC, and no erythrocytes or fibroblasts. Cell viability was higher than 95% as assessed by trypan blue exclusion. Finally, the cells were adjusted to 150,000 viable cells/ml culture medium (DMEM/F-12 medium with 15 mM HEPES and L-glutamine supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin) and were cultured in glass tubes in a shaking water bath (24, 25). During the incubation, the medium was gassed continuously with a 95% O_2:5% CO_2 mixture and the temperature was maintained at 37°C. After a 2-hr preincubation, when the cells started to form the conglomerates/aggregates (21, 24), the cells were stimulated simultaneously with the same regimen of reagents as in Experiment 1: control medium, 100 μ*M* spermineNONOate, or 100 μ*M* L-NAME. After an additional 4 hr of incubation, the media samples were collected and immediately stored at –80°C until they were assayed for P_4, OT, $PGF_{2\alpha}$, and LTC_4.

Experiment 3: Perfusion of Bovine CL *In Vivo*. Mature Holstein heifers (n = 4) were injected (i.m.) with 25 mg of $PGF_{2\alpha}$ (Lutalyse) during the luteal phase to induce luteolysis and estrus. On Day 11 of the subsequent estrous cycle, an intraluteal MDS was implanted into the CL as described by Blair *et al.* (26). The animals were premedicated with xylazine at a dose of 50 mg/animal i.m. (Rompun, Miles, Shawnee Mission, KS) and local anesthesia (epidural and infiltration at the site of incision) using 2% lidocaine hydrochloride (Lidocaine 2% injectable; Butler) was induced before surgery. The MDS was threaded through the CL such that the dialysis tubing (Fresenius SPS 960; Frankfurt, Germany; MW cutoff = 1,000,000 Daltons; o.d. = 500 μm; i.d. = 340 μm) was localized within CL. After surgery, heifers were kept in individual stalls and CL were perfused with Ringer's solution. On Day 12 of the cycle, CL were perfused for 4 hr with 100 mg of spermineNONOate. SpermineNONOate was diluted in 800 μl of 0.01 *M* NaOH. The syringes containing 50 mg of spermineNONOate, 400 μl of 0.01 *M* NaOH, and 5.6 ml of 0.1 *M* phosphate-buffered saline (PBS) for a final concentration of 8.33 mg/ml NO donor were prepared immediately before perfusion of the drug and were changed every 2 hr. Four hours before and after drug treatment, 11.2 ml of 0.1 *M* PBS solution with 800 μl of 0.1 *M* NaOH was infused. The perfusate samples were collected every 30 min before, during, and after spermineNONOate infusion. Additionally, eight perfusate and eight plasma samples were collected during infusion of Ringer's solution (4 hr before start of experiment) to determine if there were any differences between Ringer's and PBS solutions on secretory function of the bovine CL. All perfusions were at a flow rate of 3 ml/hr. Peripheral blood samples were collected at 30-min intervals through a catheter implanted into the jugular vein.

The recovery rates of hormones across the MDS were measured as previously described (27) and amounted to approximately 0.1% for OT and LTC_4, 0.3% for $PGF_{2\alpha}$, and 1.0% for P_4.

All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee.

Hormone Determination. ***Enzyme Immunoassays.*** Culture media and perfusate samples were analyzed for $PGF_2\alpha$ and LTC_4 using commercially available enzyme immunoassay (EIA) kits (Cayman Chemical Co.) according to the manufacturer's instructions. Assay sensitivities were 9.8 and 9.4 pg/ml for $PGF_2\alpha$ and LTC_4, respectively. The intra- and inter-assay coefficients of variation (CV_s) were less than 10% for all EIA kits.

Progesterone Radioimmunoassay (RIA). P_4 concentrations in culture media, perfusate, and plasma samples were determined by RIA as previously described (28). The detection limit of the assay was 0.15 ng/ml, and the intra- and inter-assay CV_s were 5.3% and 9.44%, respectively.

Oxytocin RIA. OT concentrations in culture media, perfusate, and plasma samples were determined by RIA as previously described (29). The detection limit of the assay was 0.34 pg/ml, and the intra- and inter-assay CV_s were 4.6% and 9.5%, respectively. The efficiency of extraction was 87%. The relationship between the real (x) and determined (y) amounts of five different concentrations of OT was illustrated by the linear regression equation ($y = 0.98x + 0.12$).

Luteinizing Hormone (LH) RIA. LH concentration in plasma samples was determined by RIA as described by Thompson *et al.* (30). The assay sensitivity was 0.44 ng/ml, and intra- and inter-assay CV_s were 3.7% and 9.6%, respectively. The precision of the method for the four different concentrations of LH was illustrated by the linear regression equation ($y = 1.03x + 0.19$).

Statistical Analysis. Experimental data are shown as means ± SEM. Hormone concentrations and characteristics of hormone profiles from Experiment 1 were analyzed by analysis of variance (ANOVA) for repeated measures using the General Linear Models (GLM) from the SAS Institute (31). Mean hormone concentrations were analyzed for treatment, time, and cell type differences. Characteristics of mean hormone profiles (concentration at treatment, peak concentration, time to peak concentration, and percentage increase in concentration between treatment and peak levels) were analyzed for treatment and cell type differences. For Experiments 2 and 3, differences among concentrations of hormones before, during, and after treatment were assessed by one-way ANOVA followed by Bonferroni's multiple comparison test (GraphPad Prism; GraphPad Software, San Diego, CA). Differences with a $P < 0.05$ were considered statistically significant.

Results

Experiment 1. In SLC cultures, there were no significant treatment effects on P_4 (Fig. 1a), $PGF_2\alpha$ (Fig. 1b), or OT (data not shown) secretion. LTC_4 production by SLC (Fig. 1c) was increased ($P < 0.05$) by treatment with L-NAME compared with controls at 270, 300, and 330 min of culture, which coincides with 150, 180, and 210 min after

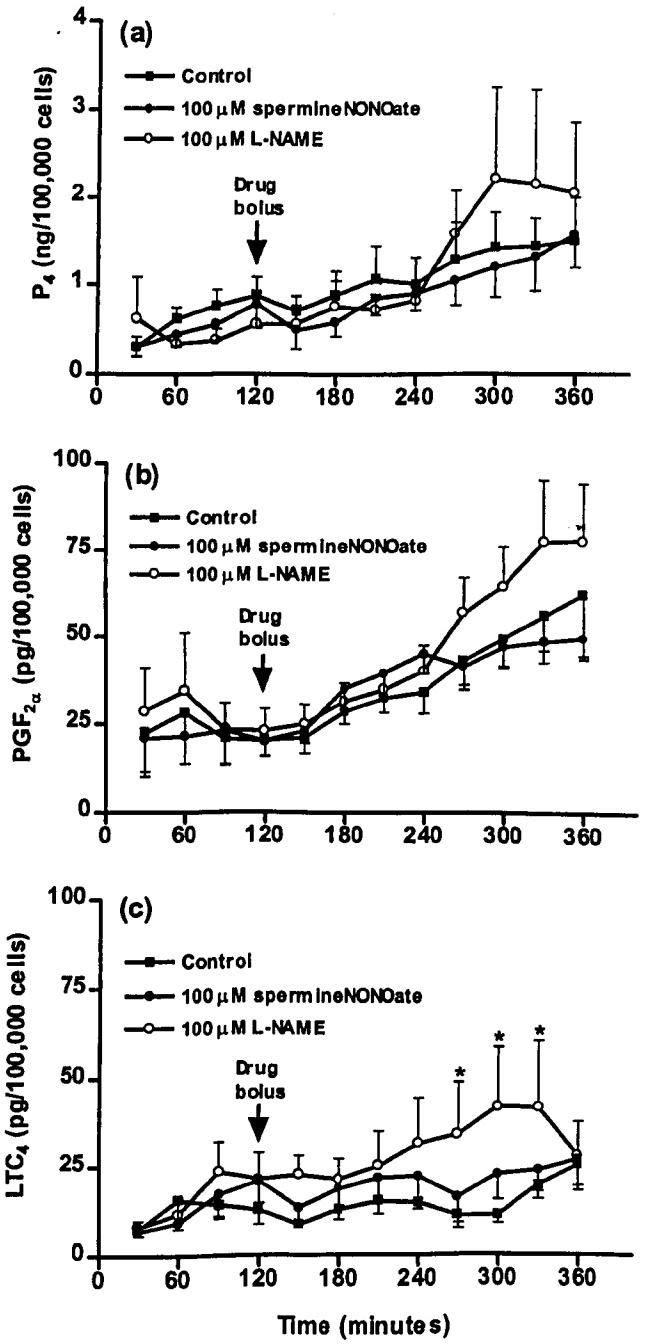

Figure 1. Production (mean ± SEM from four separate experiments) of progesterone (P_4; a), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; b), and leukotriene C_4 ($LT\ C_4$; c) by bovine small luteal cells during *in vitro* perfusion. Cells were cultured for 2 hr before treatment to establish baseline concentrations. Cells were treated with either control media, 100 μ*M* spermineNONOate (an NO donor), or 100 μ*M* L-NAME (NOS inhibitor), and cultures continued for another 4 hr. ↓ Indicates introduction of treatment into the perfusion culture system. * Indicate significant differences ($P < 0.05$) compared with control.

treatment. The main effect of time was significant ($P < 0.05$) for each secretory product measured, indicating that SLC production of P_4, $PGF_2\alpha$, and LTC_4 increased with time in culture when treatment effects were not considered.

Production of P_4 by LLC was constant for the duration of the culture period and no treatment effects were evident (Fig. 2a). Significant differences ($P < 0.05$) in $PGF_2\alpha$ concentrations occurred at 120 and 240 min of culture between control and L-NAME-treated cells (Fig. 2b). In LLC treated with L-NAME, LTC_4 production was increased ($P < 0.05$) compared with controls at 330 and 360 min of culture, or 210 and 240 min after treatment (Fig. 2c). Differences ($P <$

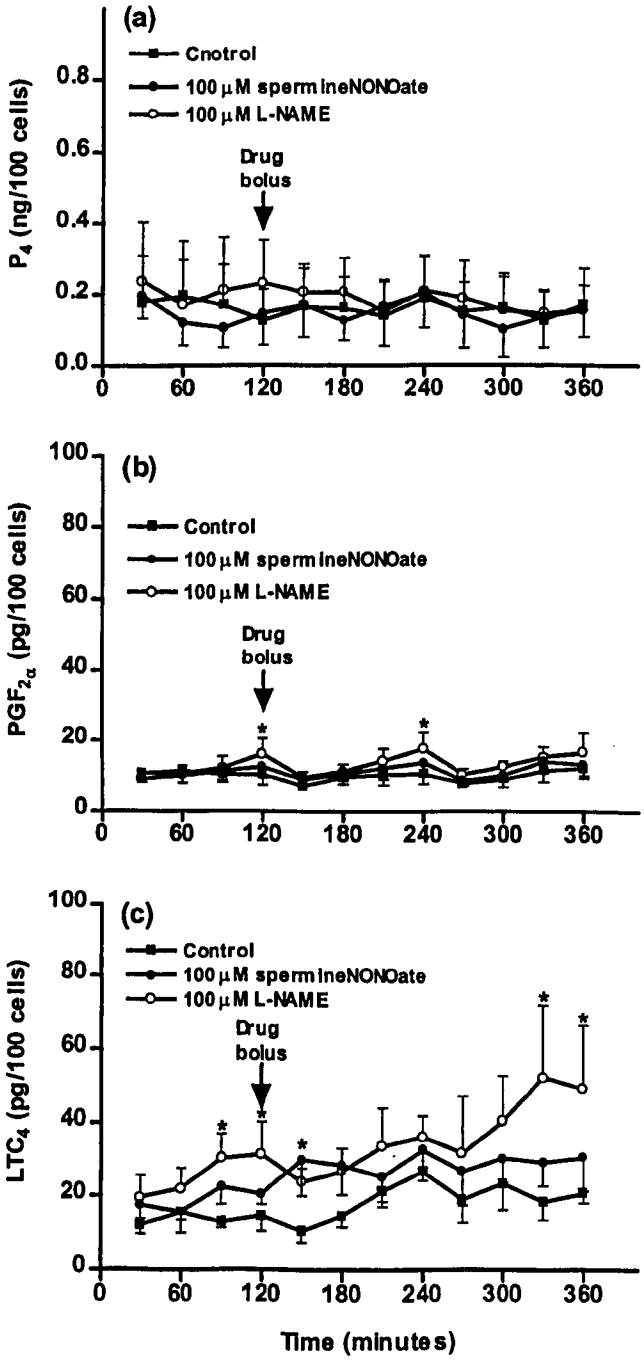

Figure 2. Production (mean ± SEM from four separate experiments) of P_4 (a), $PGF_{2\alpha}$ (b), and $LT\ C_4$ (c) by bovine large luteal cells during *in vitro* perfusion. Cells were cultured for 2 hr before treatment to establish baseline concentrations. Cells were treated with either control media, 100 μ*M* spermineNONOate (an NO donor), or 100 μ*M* L-NAME (NOS inhibitor), and cultures continued for another 4 hr. ↓ Indicates introduction of treatment into the perfusion culture system. * Indicate significant differences ($P < 0.05$) compared with control.

0.05) in LTC_4 production between L-NAME and control-treated cells were also observed at 90 and 120 min of culture; however, this was before administration of treatments. Administration of spermineNONOate to LLC resulted in an increase in LTC_4 production 30 min after treatment or 150 min of culture (Fig. 2c). Regardless of treatment, LLC produced significantly greater ($P < 0.05$) amounts of P_4 (ng/100 cells), $PGF_{2\alpha}$ (pg/100 cells), and LTC_4 (pg/100 cells) compared with SLC (data not shown). OT production by LLC was not affected by treatment (data not shown).

Analysis of the mean hormone profiles demonstrated that the percentage increase in P_4 production by SLC between basal P_4 concentration at the time of treatment and peak P_4 concentration was greater ($P < 0.05$) for L-NAME compared with controls. All other hormone profile characteristics were similar between treatment groups.

Experiment 2. The changes of P_4, OT, $PGF_{2\alpha}$, and LTC_4 secretion by the co-cultured cells of bovine CL observed in response to spermineNONOate and L-NAME treatments are shown in Figure 3. SpermineNONOate significantly inhibited P_4 and OT secretion ($P < 0.001$), whereas L-NAME stimulated the release of P_4 ($P < 0.001$) and did not significantly affect OT secretion ($P > 0.05$) compared with controls. On the other hand, secretion of LTC_4 and $PGF_{2\alpha}$ from the cells was strongly stimulated by spermineNONOate (both $P < 0.001$). Secretion of $PGF_{2\alpha}$ and LTC_4 was not significantly ($P > 0.05$) influenced by L-NAME treatment during the period of incubation.

Experiment 3. No significant changes in the production of P_4, OT, $PGF_{2\alpha}$, and LTC_4 (Fig. 4) between saline and PBS were observed. Concentrations of OT in the perfusate samples were very low and decreased ($P < 0.05$) to nondetectable levels during spermineNONOate administration (Fig. 4a). Furthermore, large increases ($P < 0.001$) of both $PGF_{2\alpha}$ and LTC_4 concentrations occurred in the perfusate samples (Fig. 4b). The increases were observed in the first samples collected during drug infusion and the concentrations remained high until the end of spermineNONOate administration. The levels of LTC_4 were approximately 5-fold higher than $PGF_{2\alpha}$. Perfusion of CL with NO donor did not change ($P > 0.05$) the concentration of P_4 in perfusate samples (Fig. 4a). In this experiment, spermine NONOate perfusion did not influence ($P > 0.05$) P_4, LH, or OT concentrations in jugular plasma samples (Fig. 5).

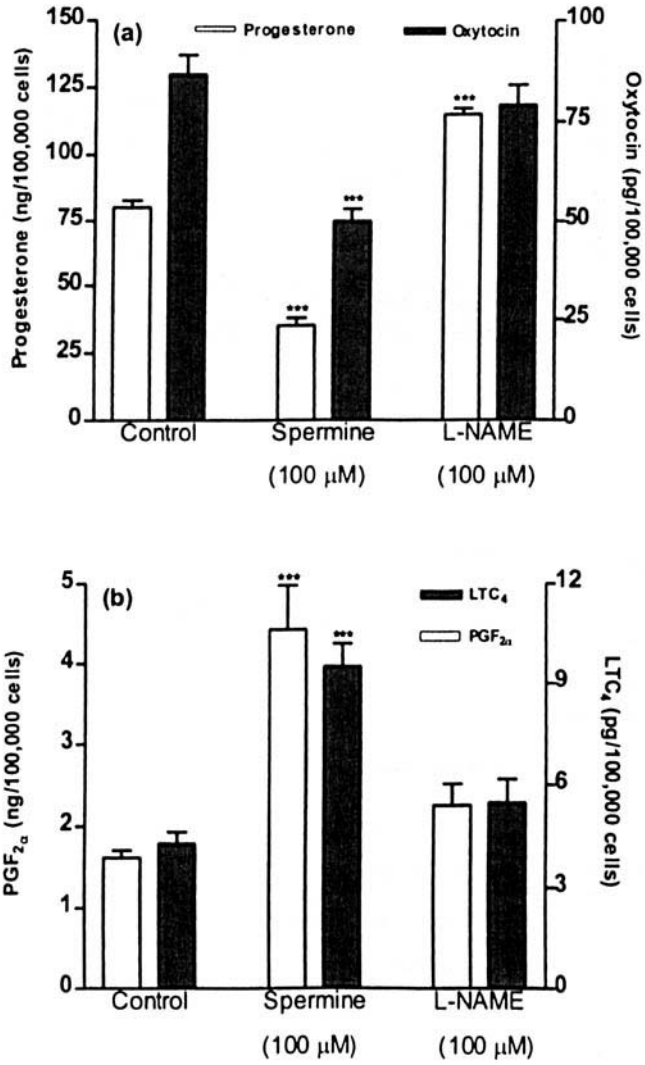

Figure 3. Secretion of P_4 and OT (a), and $PGF_{2\alpha}$ and LT C_4 (b) by co-cultured bovine luteal cells obtained from the mid-luteal stage of the estrous cycle. The cells were exposed to spermineNONOate (spermine, 100 μ*M*), an NO donor or an inhibitor of NOS (L-NAME, 100 μ*M*) for the last 4 hr of total 6-hr incubations in glass tubes in a shaking water bath. Data show means ± SEM (four experiments, each in triplicate). * Indicate significant differences (***$P < 0.001$) compared with controls as determined by analysis of variance followed by Bonferroni's multiple comparison test.

Discussion

The divergent results of Experiment 1, in which neither a NO donor (spermineNONOate) nor a NOS inhibitor (L-NAME) had marked effects on pure cultures of SLC and LLC, and Experiment 2, in which the NO donor decreased P_4 and OT and increased $PGF_{2\alpha}$ and LTC_4, whereas the NOS inhibitor clearly increased P_4 and, to a lesser extent, $PGF_{2\alpha}$ and LTC_4 in the cell mixtures, suggest the importance of endothelial cells (absent in Experiment 1 and present in the cell mixtures used in Experiment 2) in mediating the effects of NO donors and NOS inhibitors. These results also point to the potential importance of cell-to-cell communication (absent in Experiment 1 and possible in Experiment 2) in mediating the NO effects. The relative importance of these two factors remains to be determined. It has been shown that endothelin-1 (ET-1), which is produced by endothelial cells, decreases P_4 production in bovine CL (19, 32–34).

The results of Experiment 3, in which the effects of the NO donor were tested *in vivo* after intraluteal administration by MDS, resemble the *in vitro* results obtained with cell mixtures in Experiment 2. The NO donor decreased OT, but not P_4. The NOS inhibitor (L-NAME) administered intraluteally was previously shown (5) to cause increased P_4 and OT secretions and to prolong the functional life of the CL. Thus, NO donors are, for the most part, luteolytic, whereas

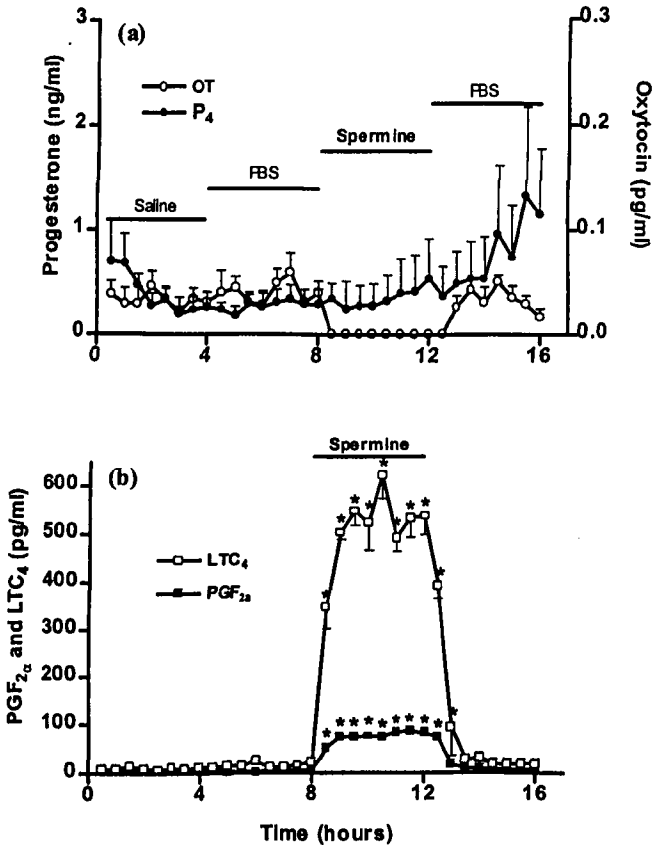

Figure 4. Concentrations (mean ± SEM from four separate experiments) of P_4 and OT (a), and $PGF_{2\alpha}$ and LTC_4 (b) in perfusate samples during intraluteal spermineNONOate (spermine; 100 mg/4 hr), an NO donor infusion (horizontal line) on Day 12 of the estrous cycle. * Indicate significant differences ($P < 0.01$) compared with PBS treatment.

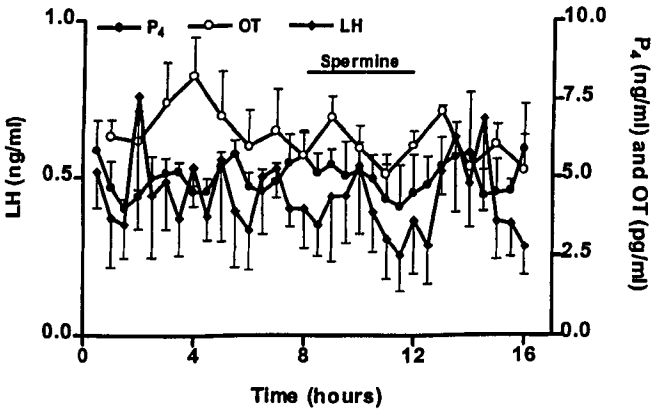

Figure 5. Concentrations (mean ± SEM from four separate experiments) of P_4, OT, and LH in plasma samples collected during perfusion of bovine corpus luteum with spermineNONOate (spermine; 100 mg/4 hr) on Day 12 of the estrous cycle.

NOS inhibitors have luteotrophic effects on mid-cycle bovine CLs *in vivo* and *in vitro* when all cell types (SLC, LLC, and accessory cells including endothelial cells) are present in the culture medium. It is possible that the inhibition of P_4 secretion observed in the *in vitro* culture of mixed SLC and LLC after spermineNONOate administration, in contrast to lack of inhibition after spermineNONOate administration *in vivo*, may be due, in part, to the high level of oxygen used in the incubation. High levels of oxygen promote generation of reactive oxygen species, such as superoxide and hydrogen peroxide, which in turn generate peroxynitrite radicals that may damage luteal cells, resulting in lowered P_4 production. However, significant changes in P_4 secretion by separated LLC or SLC were not observed after NO donor treatment when the same gas mixture was used.

In the present study, we showed that intraluteal administration of a NO donor *in vivo* caused immediate large increases of LTC_4 and $PGF_{2\alpha}$, a result consistent with the data found when mixed cells cultured in glass tubes were treated with spermineNONOate, and significantly increased LTC_4 and $PGF_{2\alpha}$ secretion. In perfused luteal cells, LTC_4 secretion was elevated by a NO donor at 30 min after drug treatment in LLC, but no effect was observed in SLC. Secretion of LTC_4 was also elevated in both types of cells treated with L-NAME. However, this effect seems to be nonspecific, because an increase in LTC_4 (compared with control group) was also observed in LLC before drug administration. $PGF_{2\alpha}$ secretion was not significantly augmented by either treatment in perfused SLC and LLC.

Administration of NO donor by microdialysis *in vivo* caused the disappearance of OT from the perfusate, but did not affect P_4 concentrations. However, spermineNONOate decreased both P_4 and OT secretion by the mixed cells—a result that extends our previous results obtained from cultured bovine CL cells in which NO inhibited P_4 production (6, 25, 35) and augmented $PGF_{2\alpha}$-induced OT secretion (25). The lack of inhibition of P_4 production observed in our present *in vivo* study compared with the decrease in secretion observed in the mixed CL cells may be a consequence of differences in cell composition. Lei *et al.* (36) showed that accessory cells (endothelial, immune cells, and fibroblasts) constitute about 70% to 80% of total cells of the intact bovine CL. In our culture (Experiment 2), the cell population consisted of 90% steroidogenic luteal cells and about 10% endothelial cells (24). Thus, the direct inhibitory effect of NO on P_4 secretion from luteal cells during the mid-luteal phase *in vivo* may be compensated by actions of such luteotrophic factors as arachidonic acid metabolites (prostaglandins, HETE acids, etc.) released by NO from different accessory cells. Moreover, NO is known to play a role not only as a modulator of steroidogenesis, but also as a survival/cell death inducer factor (37). The dual effects of NO seems to be dose dependent (38) and cell-type specific (39). At high concentrations, NO inhibits DNA fragmentation in the bovine granulosa cells, and in low concentration, it stimulates cellular apoptosis in granulosa cells from large follicles (38). Moreover, our previous *in vitro* data showed that spermineNONOate (an NO donor) strongly reduced viability of the late luteal cells, whereas L-NAME (an NOS inhibitor) had opposite effects in a long-term (3 days) culture (Korzekwa A, Okuda K, Jaroszewski JJ and Skarzynski DJ, unpublished data).

Differences in the effects of NO may depend on the stage of the estrous cycle. An NO donor stimulated PGE_2 secretion in luteal cells from early and mid-cycle CL, whereas $PGF_{2\alpha}$ production was stimulated only in luteal cells from mid- and late luteal phase CL (6). Moreover, spermineNONOate increased both PGE_2 and $PGF_{2\alpha}$ production in hCG-stimulated human dispersed luteal cells from the late luteal phase (13). These observations may explain differences in NO effects on the bovine CL during the estrous cycle (5, 6, 15). The NO donor infusion in the present experiment *in vivo* was carried out on Day 12 of the estrous cycle and resulted in a relatively small increase in $PGF_{2\alpha}$ secretion compared with LTC_4 output. Recently, we showed that both NADPH-diaphorase (a marker for NOS) and iNOS and eNOS activities are the highest in the bovine CL during the late luteal phase (4), confirming the suggestion that the influence of NO on secretory function is dependent on phase of estrous cycle.

Changes in P_4 concentrations in the *in vivo* experiment may have been influenced by an NO-dependent increase in blood and lymph flow in the CL that may have compensated for the local effect of NO on steroidogenic cells. It is well documented that NO binds to the heme-prosthetic group of soluble guanylate cyclase and increases intracellular cGMP concentrations, which have been associated with vascular smooth muscle relaxation (1). Therefore, it is possible that NO produced locally in the CL or administered exogenously, as in our *in vivo* study, may act in a paracrine fashion to cause local vasodilatation and increased blood flow that indirectly influences steroid release. Our recent study (40) showed that NADPH-D activity in the ovarian artery was higher during the luteal than the follicular stage of the estrous cycle, as well as in ovarian arteries ipsilateral to the CL as compared with the contralateral side.

Immune cells are known to be involved in the regulation of CL function (20, 41). However, these cells were absent in the cell mixtures used in Experiment 2. Interleukin-1β (IL-1β) was shown to increase NO production in dispersed ovarian cell cultures (42). Recently, Tobai and Nishiya (43) showed that IL-1β stimulated NO production and inhibited the production of E_2 in cultured human granulosa-luteal cells, suggesting that IL-1β stimulated NO production, and NO inhibited the production of E_2. Tumor necrosis factor-α (TNFα) was also shown to stimulate the NO-guanosine monophosphate pathway in bovine theca cells, resulting in an increase in iNOS production (44). Although TNFα was shown to be luteotropic *in vitro* (45), it effects *in vivo* depend on the dose (Woclawek I and Skarzynski DJ, unpublished data); low doses had a luteolytic effect, whereas high doses increased CL activity and prolonged the estrous cycle in cattle.

NO appears to act through multiple mechanisms to exert its effects on luteal cell function *in vivo*. Despite the failure of spermineNONOate to cause a significant reduction in P_4 secretion after intraluteal infusion *in vivo*, the bulk of evidence in these experiments and others indicates that NO plays a luteolytic role and NOS inhibitors, such as L-NAME, are luteotropic in mid- and late cycle bovine CL. These effects could not be demonstrated in pure cultures of either SLC or LLC perfused with an NO donor or NOS inhibitor. Therefore, it is suggested that the presence of endothelial cells and/or cell-to-cell communication are required for the full secretory response of the CL in NO action.

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