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Effects of luteinizing hormone and growth hormone on luteal development in hypophysectomized ewes

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To test the hypothesis that growth hormone (GH) as well as luteinizing hormone (LH) is required for normal luteal growth and function, 16 western range ewes were hypophysectomized (HPX) on day 5 of the estrous cycle. Ewes were randomly assigned to receive saline (S), LH, GH, or LH + GH ($n = 4$ per group) from the time of HPX until collection of corpora lutea 7 days after HPX (day 12). Corpora lutea were also collected from pituitary-intact ewes on days 5 (day 5 control, $n = 4$) and 12 (day 12 control, $n = 4$) of the estrous cycle. To assess luteal function, concentrations of progesterone in sera, luteal weights and luteal concentrations of mRNA encoding cytochrome P450 side-chain cleavage enzyme ($P450_{scc}$) and 3β-hydroxysteroid dehydrogenase/Δ5,Δ4 isomerase (3β-HSD) were determined. Concentrations of progesterone in sera and luteal weights increased between days 5 and 12 of the estrous cycle in control ewes, but not in HPX + S ewes. In HPX ewes treated with LH, concentrations of progesterone in sera and luteal mRNA for $P450_{scc}$ and 3β-HSD increased but luteal weights were unaffected. Treatment with GH increased luteal weight and luteal concentrations of mRNA encoding $P450_{scc}$ but did not increase concentrations of mRNA encoding 3β-HSD compared to HPX + S ewes. Concentrations of progesterone in sera of GH-treated, HPX ewes were similar to those of day 12 control ewes but not significantly different from those in HPX + S ewes. Treatment of HPX ewes with LH + GH increased all parameters of luteal function measured to values similar to those in day 12 controls. In conclusion, both GH and LH are necessary for normal luteal development in the ewe.

Keywords: Luteal development; growth hormone; luteinizing hormone; sheep

Introduction

Maintenance of pregnancy in most mammals is initially dependent upon production of progesterone by the corpus luteum. Initiation of luteal function occurs when granulosal and thecal cells differentiate in response to a preovulatory surge of luteinizing hormone (LH). Following ovulation, differentiation and growth of these cells continues until a fully functional corpus luteum is formed. Increased secretion of progesterone from the corpus luteum is due to many factors including increased quantity of steroidogenic tissue and increased activity of cytochrome P450 side chain cleavage enzyme ($P450_{scc}$) and 3β-hydroxysteroid dehydrogenase/Δ5, Δ4 isomerase (3β-HSD), two key enzymes in the biosynthesis of progesterone (recently reviewed in Smith *et al.*, 1994). In the ewe, weight of the corpus luteum and concentrations of progesterone in sera increased 2-3-fold between days 4 and 12 of the estrous cycle (Diekman *et al.*, 1978). This was correlated with an increase in total number of receptors for LH (Diekman *et al.*, 1978). More recently, concentrations of mRNA encoding $P450_{scc}$, but not 3β-HSD, were found to increase between days 3 and 9 of the estrous cycle (Juengel *et al.*, 1994).

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Hypophysectomy (HPX) on day 5 of the estrous cycle prevented the increases in concentrations of progesterone in sera and luteal weight that normally occur between day 5 and 12 (Farin *et al.*, 1990). Treatment of HPX ewes with pharmacological doses of LH restored normal luteal growth and function; however, treatment of HPX ewes with a physiological regime of LH did not result in normal luteal growth (Farin *et al.*, 1990). In contrast to the cessation of normal luteal development seen in HPX ewes (Farin *et al.*, 1990), ewes subjected to hypothalamic-pituitary stalk-disconnection (HPD) maintained normal secretion of progesterone although luteal weight did not increase (Niswender *et al.*, 1986). Since serum concentrations of growth hormone (GH) and prolactin were not decreased following HPD (Hamernick & Nett, 1988), either of these hormones could be involved in luteal development. However, since suppression of prolactin with α-ergocryptine in ewes following HPD did not affect luteal development (Niswender *et al.*, 1986) it does not appear likely that prolactin is directly involved in luteal development. Bovine corpora lutea contain receptors for GH (Lucy *et al.*, 1993) and GH increases secretion of insulin-like growth factor I (IGF-I) from a number of target tissues. This may be important since addition of IGF-1 to luteal cell cultures increased progesterone production (Einspanier *et al.*, 1990; Parmer *et al.*, 1991). Therefore, the purpose of this study was to test the hypothesis that both GH and LH are required for normal luteal development in the ewe.

Results

Following HPX on day 5 of the estrous cycle concentrations of prolactin in sera were reduced >98% and concentrations of cortisol in sera had decreased to non-detectable levels in all HPX ewes on day 12 of the estrous cycle confirming completeness of HPX. Concentrations of GH in sera of LH + GH treated HPX ewes, although approximately 2-fold higher, were not different ($P = 0.17$) from those in intact control ewes (7.3 ± 0.8 *vs* 3.4 ± 2.7 ng/ml). Hypophysectomized ewes treated with LH ± GH had mean pulse amplitudes of LH of 1.3 ± 0.1 ng/ml following each injection of LH. No additional pulses of LH were detected in these animals. Amplitudes of pulses of LH in the two control ewes which had statistically significant pulses during the 8.5 h frequent bleeding period averaged 0.47 ± 0.17 ng/ml.

Concentrations of progesterone in sera and luteal weights doubled ($P < 0.05$) between days 5 and 12 of the estrous cycle in pituitary-intact ewes (Figure 1). While concentrations of $P450_{scc}$ and 3β-HSD mRNA tended to be higher on day 12 than on day 5 of the estrous cycle in pituitary-intact ewes, these increases were not significant ($P = 0.12$ and $P = 0.10$ respectively, Figure 2). Mean concentrations of progesterone in sera, luteal weights, and concentrations of $P450_{scc}$ and 3β-HSD mRNA in HPX + S ewes were similar ($P > 0.44$) to values obtained from day 5 control ewes but less ($P < 0.05$) than values in day 12 control ewes (Figures 1 and 2). In addition, concentrations of progesterone in daily sera samples did not increase following HPX in HPX + saline (S) ewes (mean progesterone ranged from 94-112% of pre-surgery values).

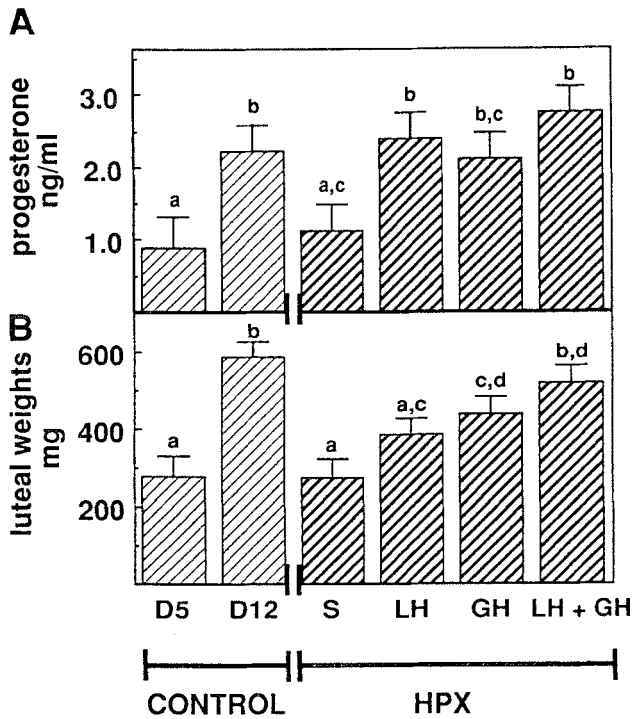

Figure 1 Least squares means ± SEM of concentrations of progesterone in serum at time of luteal collection (**A**) and luteal weights (**B**) in control and hypophysectomized (HPX) ewes. See text for details on day 5 and 12 control ewes and hormonal replacement [saline (S), luteinizing hormone (LH), growth hormone (GH) and luteinizing hormone and growth hormone (LH + GH)] of HPX ewes. Means without common letters were different ($P<0.05$) within each measurement.

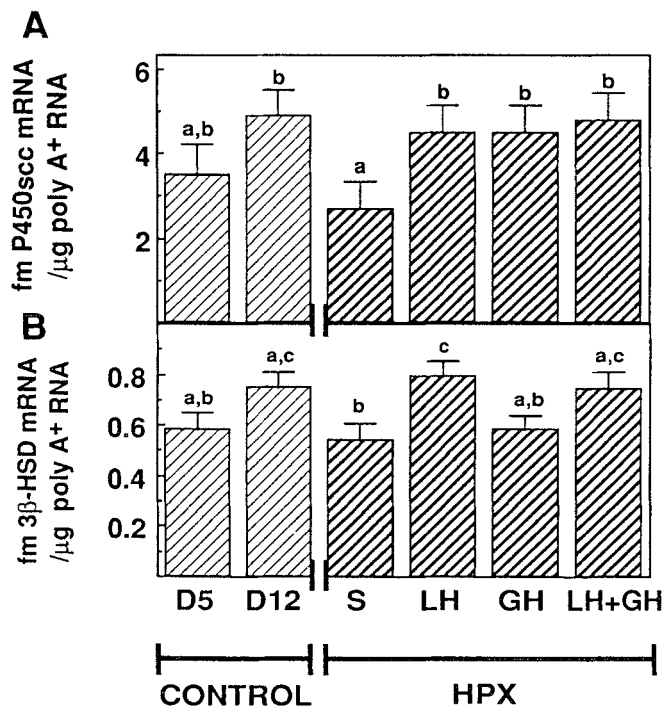

Figure 2 Least squares means ± SEM of luteal concentrations of $P450_{SCC}$ (**A**) and 3β-HSD (**B**) in control and hypophysectomized (HPX) ewes. See text for details on day 5 and 12 control ewes and hormonal replacement [saline (S), luteinizing hormone (LH), growth hormone (GH) and luteinizing hormone and growth hormone (LH + GH)] of HPX ewes. Means without common letters were different ($P<0.06$) within each measurement.

Treatment of HPX ewes with LH increased ($P<0.05$ compared to HPX + S group) concentrations of progesterone in sera and mRNA encoding $p450_{SCC}$ and 3β-HSD to values similar ($P>0.61$) to those in day 12 control ewes (Figures 1A and 2). Luteal weights in HPX + LH ewes tended to be greater ($P=0.09$) than those of HPX + S ewes, but did not reach the weights obtained in day 12 control ewes ($P<0.01$; Figure 1B).

Mean concentration of progesterone in sera of HPX + GH ewes tended to be greater ($P=0.08$) than that in HPX + S ewes and was not different ($P=0.82$) from the value in day 12 control ewes (Figure 1A). Luteal weights in HPX + GH ewes were intermediate between values obtained in HPX + S ewes ($P<0.05$) and day 12 control ewes ($P<0.05$; Figure 1B). Concentrations of mRNA encoding $P450_{SCC}$ increased ($P<0.06$ *vs* HPX + S) in HPX + GH ewes to values similar ($P=0.61$) to those in day 12 control ewes (Figure 2A). The mean concentration of mRNA encoding 3β-HSD in HPX + GH ewes was similar ($P=0.71$) to that in HPX + S ewes and tended ($P=0.08$) to be less than that in day 12 control ewes (Figure 2B).

Treatment of HPX ewes with a combination of LH and GH resulted in increased ($P<0.05$) concentrations of progesterone in sera, luteal weights and concentrations of $P450_{SCC}$ mRNA and 3β-HSD mRNA when compared to those in HPX + S ewes (Figures 1 and 2). Mean number of receptors for LH did not differ among treatment groups (range 1.0 ± 0.3 to 1.8 ± 0.3 fmoles/mg tissue). Values for all parameters of luteal function measured were similar in HPX + LH + GH and day 12 control ewes (Figures 1 and 2).

Discussion

Hypophysectomy prevented the normal increase in concentrations of progesterone in sera and luteal weight that occurs during luteal development in ewes. Farin *et al.* (1990) previously reported that HPX prevented an increase in luteal weight and secretion of progesterone.

In HPX ewes, replacement of LH enhanced the capacity of the corpus luteum to biosynthesize and secrete progesterone, as measured by concentrations of progesterone in sera and luteal concentrations of mRNA encoding $P450_{SCC}$ and 3β-HSD. However, LH replacement did not support normal increases in luteal weight (compared to day 12 controls). These results are similar to data published by Farin *et al.* (1990) in which HPX ewes treated with a physiological regime of LH had similar amounts of progesterone in serum but smaller corpora lutea than day 12 pituitary-intact ewes. The increased luteal concentrations of both $P450_{SCC}$ and 3β-HSD mRNA in LH-treated, HPX ewes may provide insight to one of the underlying mechanisms by which LH maintains normal luteal synthesis and secretion of progesterone. However, since LH was unable to restore luteal weight in HPX ewes, normal luteal growth appears to require a pituitary factor(s) other than LH.

Growth hormone replacement increased luteal weight in HPX ewes. This is consistent with the findings of Lucy *et al.* (1992; 1994) who reported that injection of cows with GH increased size of the corpus luteum. Besides increasing luteal weight in the present study, GH also increased luteal concentration of mRNA encoding $P450_{SCC}$. Thus GH appears to be important for normal luteal growth.

The mechanism(s) by which GH supports luteal growth and function could not be ascertained from this experiment. Bovine corpora lutea contain receptors for GH (Lucy *et al.*, 1993), thus a direct action of GH on luteal cells is possible. Growth hormone could also influence luteal function through the IGF-I system since GH increases concentrations of IGF-I in sera of HPX ewes (Eckery *et al.*, 1994). Corpora lutea secrete IGF-I (Einspanier *et al.*, 1990, Amselgruber *et al.*, 1994, bovine; Parmer *et al.*, 1991, murine); contain receptors

for this growth factor (Parmer *et al.*, 1991); and IGF-I increases luteal secretion of progesterone *in vitro* (Einspanier *et al.*, 1990; Parmer *et al.*, 1991). In murine corpora lutea, IGF-I is thought to act in an autocrine-paracrine manner in large luteal cells to support secretion of progesterone (Parmer *et al.*, 1991). In follicular cells IGF-I may increase sensitivity to gonadotrophins by affecting interaction of the gonadotrophin receptor with adenylate cyclase (Adashi *et al.*, 1988). Whether IGF-I affects the interaction between LH-receptor and adenylate cyclase in luteal cells in a similar manner is not known.

Treatment of ewes HPX on day 5 of the estrous cycle with a combination of LH and GH supported normal luteal growth and function through day 12. Either hormone could affect expression or function of receptors for LH or GH on luteal cells. However, since GH or LH alone were able to support partial luteal development, they must be acting, at least in part, independently of each other. In addition, number of receptors for LH/mg luteal tissue was not different in any group suggesting this was not a factor limiting luteal development. Farin *et al.* (1990) previously reported that concentration of LH receptor in luteal tissue was not different between HPX and control ewes, although total number of receptors per corpus luteum decreased.

In summary, LH supports synthesis and secretion of progesterone in the corpus luteum. Growth hormone is important for luteal growth. However, only when both GH and LH were replaced in HPX ewes was normal luteal growth and development restored.

Materials and methods

Experimental design

The experimental protocol described below was approved by CSU/ACUC. Western range ewes displaying signs of behavioral estrus (naturally occurring or synchronized with two injections of prostaglandin $F_2\alpha$; 5 mg each 4 h apart; Upjohn Company, Kalamazoo, MI) in the presence of vasectomized rams were randomly assigned to one of six groups. Only multiple ovulating ewes (2–3 corpora lutea as determined by laparotomy) were utilized to ensure sufficient quantities of luteal tissue for biochemical analyses. On day 5 of the estrous cycle, (estrus = day 0) 16 ewes were HPX using the transnasal, transphenoidal approach (Clarke *et al.*, 1983; Farin *et al.*, 1990). Hypophysectomized ewes were randomly assigned to receive saline, LH, GH or LH and GH. Luteinizing hormone and GH replacement regimes were designed to approximate physiological profiles. Ewes receiving LH (HPX + LH or HPX + LH + GH; $n = 4$ ewes/group) were injected with 4.0 μg oLH (NIAMDD-oLH-24) in 1 ml of saline containing 1% BSA (saline-BSA) at 4 h intervals through an indwelling jugular cannula. The injection cannula was flushed with 0.3 ml of saline-BSA immediately following each injection. Ewes which did not receive LH (HPX + S or HPX + GH) were injected with 1.3 ml saline-BSA at 4 h intervals. Ewes treated with GH (HPX + GH or HPX + LH + GH; $n = 4$ ewes/group) received 1.5 mg recombinant bovine GH (Sometribove, donated by Monsanto, St Louis, MO) subcutaneously in 0.3 ml of saline every 12 h. Ewes not receiving GH (HPX + S or HPX + LH) were administered 0.3 ml saline every 12 h. Ewes were treated from time of HPX until collection of luteal tissue on day 12 of the estrous cycle. Luteal tissue from one ewe in the HPX + LH + GH group was collected on day 11 of the estrous cycle. In addition, four intact ewes (day 12 control) were laparotomized on day 5 of the estrous cycle and received saline injections on the same schedule as the HPX + S control group until luteal collection on day 12 of the estrous cycle. Corpora lutea were collected from 4 additional intact ewes (day 5 control) on day 5 of the estrous cycle. After collection, corpora lutea were decapsulated, weighed, sliced with a hand microtome, frozen in liquid nitrogen and stored at −70°C until isolation of RNA.

Jugular blood samples were obtained daily from each ewe at 8 am beginning on day 5 (prior to laparotomy) and continuing until collection of luteal tissue and concentrations of progesterone were determined (Niswender, 1973). To assess completeness of HPX, concentrations of prolactin (Davis *et al.*, 1971) and cortisol (Reimers *et al.*, 1983) were determined in sera collected prior to surgery and 7 days following surgery. In addition, to determine if replacement regimens of LH and GH approximated the normal profiles of these hormones in pituitary intact ewes, multiple samples were obtained from ewes assigned to day 12 control or HPX + LH + GH on one day of treatment. Concentrations of LH (Niswender *et al.*, 1969) were determined in blood samples collected every 15 min for 8.5 h. Concentrations of GH (Davis, 1972) were determined in blood samples collected every 4 h for 24 h. Samples for each hormone were analysed in a single radioimmunoassay. Intra-assay coefficients of variation were 16%, 6%, 2%, 5% and 6% for progesterone, prolactin, cortisol, LH and GH, respectively. Sensitivities of the assays were 68 pg/ml, 1.4 ng NIH-P-S11/ml, 1.5 ng/ml, 176 pg NIH-oLH-24/ml and 273 pg NIDDK-oGH-I-4/ml for progesterone, prolactin, cortisol, LH and GH, respectively.

Determination of luteal concentration of mRNA encoding $P450_{SCC}$ and 3β-HSD

A plasmid containing the cDNA encoding bovine $P450_{SCC}$ (John *et al.*, 1984) was provided by Dr M. Waterman, Department of Biochemistry, Vanderbilt University, Nashville TN. A plasmid containing the cDNA encoding human placental 3β-HSD (Lorence *et al.*, 1990) was provided by Dr J.I. Mason, University of Texas Southwestern Center, Dallas TX. Bases 345–1182 of the $P450_{SCC}$ cDNA and bases 659–1064 of the 3β-HSD cDNA were labeled by the random primer method (Feinberg & Vogelstein, 1983) to a specific activity of $0.9–1.0 \times 10^9$ d.p.m./ μg DNA with α[^{32}P]dCTP (3000 Ci mmol; Amersham; Arlington Heights, IL).

Corpora lutea were homogenized in lysis buffer (0.2 M NaCl, 0.2 M Tris, 1.5 mM $MgCl_2$, 2% SDS, and 0.4 mg/ml proteinase K) and poly(A)$^+$ RNA isolated by binding to oligo dT cellulose (Collaborative Biomedical Products; Bedford MA; Badley *et al.*, 1988). Amount of RNA was quantified by absorption at 260 nm. Northern analysis (Sambrook *et al.*, 1989) was used to confirm integrity of mRNA. Fifty ng poly (A)$^+$ were separated on a 1.5% agarose-formaldehyde-3-[N-Morpholino]propanesulfonic acid gel, transferred to a nylon filter (Hybond, Amersham) by capillary action, and cross-linked to filters by ultraviolet light. As expected, ^{32}P-labeled $P450_{SCC}$ cDNA hybridized to mRNA in a single band at approximately 2.0 kb in experimental samples (Juengel *et al.*, 1994). There was no indication of degradation of RNA in any sample. To quantitate each specific mRNA, 50 ng ($P450_{SCC}$) or 75 ng (3β-HSD) poly(A)$^+$ RNA isolated from each sample were applied in duplicate onto a nylon filter using a slot blot apparatus. Varying quantities (5, 10, 30, 50, 100 and 300 pg) of appropriate sense mRNAs, positive control RNA (5, 10, 30, 50 and 100 ng poly(A)$^+$ RNA isolated from corpora lutea collected on day 10 of the estrous cycle) and negative control RNA (100 ng poly(A)$^+$ RNA isolated from ovine heart) were also cross-linked to the nylon filters by ultraviolet light. Conditions for prehybridization, hybridization, and washing of filters were as previously described (Juengel *et al.*, 1994). Amount of specific mRNA was determined by comparing hybridization of radiolabeled cDNA to samples and standards (Juengel *et al.*, 1994). Coefficients of variation were 9% for determination of both $P450_{SCC}$ and 3β-HSD mRNAs. Sensitivity was 0.15 and 0.18 fmoles/μg poly(A)$^+$ RNA for $P450_{SCC}$ and 3β-HSD mRNAs, respectively.

To determine if equal amounts of RNA were loaded on each slot, filters were hybridized to ^{32}P-end-labeled

dT(18mer), generated with T4 polynucleotide kinase (New England Biolabs; Beverly, MA) and [^{32}P]γATP (3000 ci/mmol; Amersham) as previously described (Juengel *et al.*, 1994). There was a high correlation between amount of RNA applied to the two filters used in this study and densitometric reading following autoradiography ($r = 0.95 \pm 0.04$). Densitometric values after hybridization to the end-labeled dT averaged 744 ± 25 and 1435 ± 38 units for all samples on the filters used to determine amount of mRNA encoding $P450_{SCC}$ and 3β-HSD mRNAs, respectively. Therefore, no corrections were made for differences in loading.

Determination of number of receptors for LH

Number of receptors for LH was determined by the standard curve method (Braden *et al.*, 1986). Briefly, number of receptors in a standard membrane preparation made from corpora lutea collected from superovulated ewes on day 9–11 of the estrous cycle was determined by Scatchard analysis (Scatchard, 1949). Number of receptors in experimental samples was determined in duplicate in a single assay. Membranes from experimental samples (1 and 4 mg wet weight equivalent) and varying concentrations of the standard membrane preparation (0.1–50 mg wet weight equivalents) were incubated with 0.6 ng [^{125}I-] human chorionic gonadotrophin (24.3 μCi/μg; Diekman *et al.*, 1978) in the presence or absence of 200 ng non-radioactive human chorionic gonadotrophin for 16 h. Bound human chorionic gonadotrophin was separated from free human chorionic gonadotrophin by centrifugation. The number of receptors was determined by comparing radioactivity bound to sample membranes to that bound to the varying concentrations of the standard membrane preparation.

Statistical analysis

To ascertain the number and amplitude of pulses of LH a pulse was identified when two sequential samples had non-overlapping 95% confidence intervals (Duddleson, 1972). All statistical analyses were performed using the general linear model procedures of SAS (SAS, 1987). Because concentrations of progesterone in sera were different between groups on day 5, the model included concentrations of progesterone in the blood sample collected on day 5 as a covariate. Differences between least squares means were evaluated by least significant differences (SAS, 1987).

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