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Luteal Expression of Steroidogenic Factor-1 mRNA During the Estrous Cycle and in Response to Luteotropic and Luteolytic Stimuli in Ewes

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Steroidogenic factor-1 (SF-1) is a transcription factor involved in regulating basal and/or cAMP-induced increases in expression of several components of the steroidogenic pathway, including cytochrome P450 side-chain cleavage (P450scc), steroidogenic acute regulatory protein (StAR), and 3β-hydroxysteroid dehydrogenase/Δ^5, Δ^4 isomerase (3β-HSD). In experiment 1, on days 3, 6, 9, 12, and 15 of the estrous cycle, steady-state concentrations (fmol/μg poly A^+ RNA) of SF-1 mRNA in luteal tissue were 0.09 ± 0.01, 0.17 ± 0.01, 0.24 ± 0.03, 0.30 ± 0.09, and 0.20 ± 0.05, respectively (estrus = day 0; *n* = 4/d). Concentrations of SF-1 mRNA increased ($p < 0.05$) between days 3 and 12, but were not different among the other days of the estrous cycle. Luteal concentrations of SF-1 mRNA and concentrations of progesterone in sera were highly correlated ($p < 0.01$; $r = 0.72$). In experiment 2, ewes on days 11 or 12 of the estrous cycle were injected with 25 mg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) into the jugular vein followed by an injection of 10 mg $PGF_{2\alpha}$ im 2 h later. Corpora lutea were collected 4, 12, and 24 h after the first injection of $PGF_{2\alpha}$ (*n* = 4–5 ewes/time). Control luteal tissue was collected from ewes on days 11–13 of the estrous cycle, which had not been injected (*n* = 4) or had been injected with saline 24 h previously (*n* = 4). Steady-state concentrations of SF-1 mRNA had decreased ($p < 0.05$) to 48% of control values by 4 h after injection, and remained low at 12 and 24 h. In experiment 3, ewes on days 9–12 of the estrous cycle were administered $PGF_{2\alpha}$ (1 μmol), phorbol 12-myristate 13-acetate (PMA; 2 μmol), luteinizing hormone (LH; 20 μg), forskolin (50 μg), or vehicle (1 mL saline) directly into the ovarian artery. Corpora lutea were collected 0 (noninfused) 4, 12, or 24 h later (*n* = 3–4 animals/treatment/time) for quantification of SF-1 mRNA. Steady-state concentrations of mRNA encoding SF-1 were not affected by infusion of $PGF_{2\alpha}$ or PMA, although concentrations of mRNA encoding StAR and 3β-HSD were decreased ($p < 0.05$) by these treatments. Concentrations of mRNA encoding SF-1 were increased ($p < 0.05$) to 157 and 149% of control values by LH and forskolin, respectively, 12 h following infusion and returned to control values by 24 h following either treatment. In contrast, infusion of LH or forskolin did not change concentrations of mRNA encoding StAR, P450scc, or 3β-HSD. In summary, during the estrous cycle, the pattern of expression of SF-1 mRNA was similar to the pattern of concentrations of progesterone in serum and expression of mRNA encoding P450scc, but differed from that previously shown for 3β-HSD and StAR mRNA. The effects of administration of $PGF_{2\alpha}$ on concentrations of SF-1 mRNA appeared to be dose-dependent. However, acute effects of $PGF_{2\alpha}$ on mRNA encoding 3β-HSD and StAR were observed when concentrations of mRNA encoding SF-1 were not influenced. In addition, although LH or forskolin increased luteal SF-1 mRNA 12 h following infusion, no increases in mRNA encoding StAR, P450scc, or 3β-HSD were observed. Thus, during the midluteal phase of the estrous cycle, neither luteotropic nor luteolytic hormones appear to coordinately regulate mRNA encoding SF-1 and mRNA encoding StAR, P450scc, or 3β-HSD.

Key Words: Steroidogenic factor-1; mRNA; ovine; corpus luteum.

Introduction

Continued secretion of progesterone from the corpus luteum is necessary for initial maintenance of pregnancy in mammals. However, if fertilization has not occurred, the corpus luteum must stop producing progesterone before another ovulation, and another chance for pregnancy can occur. Thus, understanding how hormones control secretion of progesterone from the corpus luteum in a positive or

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negative manner is essential to understanding the regulation of reproductive cycles. Luteinizing hormone (LH) is a primary positive (luteotropic) regulator of progesterone secretion in many mammals, whereas prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is the primary negative (luteolytic) regulator of progesterone secretion *(1)*. The tropic actions of LH are mediated through activation of protein kinase A (PKA), whereas the antisteroidogenic effects of $PGF_{2\alpha}$ are caused by activation of protein kinase C (PKC; *1*).

Synthesis of progesterone in the corpus luteum requires enzymatic conversion of cholesterol to pregnenolone by cytochrome P450 side-chain cleavage (P450scc; *2*) and conversion of pregnenolone to progesterone by 3β-hydroxysteroid dehydrogenase /Δ^5, Δ^4 isomerase (3β-HSD; *3*). Because P450scc is located on the inner mitochondrial membrane, transport of cholesterol across the outer and inner mitochondrial membranes is also essential for progesterone production *(4)* and appears to involve steroidogenic acute regulatory protein (StAR; *5*). Therefore, transcription factors that control synthesis of mRNA encoding P450scc, 3β-HSD, and StAR are likely to play a key role in the regulation of progesterone production.

The transcription factor steroidogenic factor-1 (SF-1; also termed AdBP-4) was identified in adrenal tissue as a protein that bound to one of the cAMP-responsive regions of the cytochrome P450 (11β) promoter *(6)*. In further studies, SF-1 was shown to be important in basal or cAMP regulation of several genes in the cytochrome P450 family *(7)*. Recently, this transcription factor has also been shown to be essential for expression of the genes encoding several other proteins important in regulation of reproduction and/or steroidogenesis *(8)*. It has also been shown to be involved in basal or cAMP-induced regulation of genes encoding StAR *(9,10)*, P450scc *(11,12)*, and 3β-HSD *(13)*. Therefore, the objectives of these experiments were to:

1. Determine steady-state concentrations of mRNA encoding SF-1 in ovine luteal tissue on selected days of the estrous cycle.
2. Determine the effects of $PGF_{2\alpha}$ and LH on concentrations of mRNA encoding SF-1.
3. Determine the relationship between mRNAs encoding SF-1 and those encoding StAR, P450scc, and 3β-HSD in ovine luteal tissue following treatment with $PGF_{2\alpha}$ and LH.

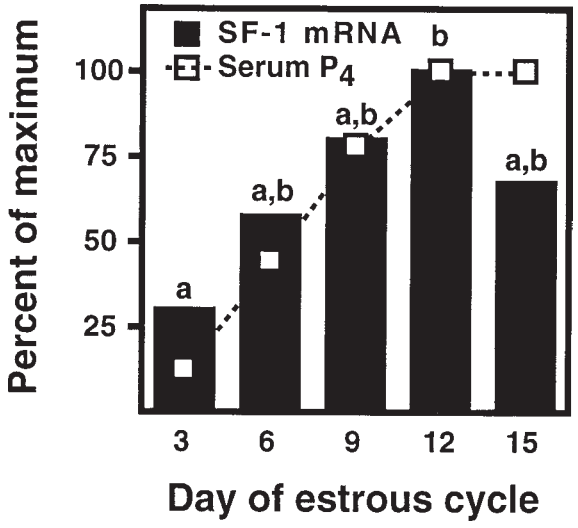

Fig. 1. Pattern of expression of steady-state concentrations of mRNA encoding SF-1 during the estrous cycle. Values from days that do not share a common letter were different ($p < 0.05$). Concentrations of progesterone in sera also increased between days 3 and 12 of the estrous cycle *(14)* and these two parameters were highly correlated ($r = 0.72$; $p < 0.01$).

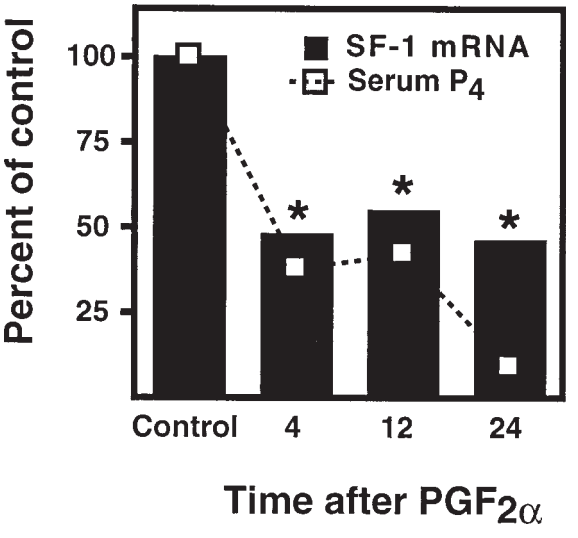

Fig. 2. Effects of $PGF_{2\alpha}$ on expression of mRNA encoding SF-1. Values with asterisks were different than values in the control group ($p < 0.01$). Complete luteal regression was induced by $PGF_{2\alpha}$ as assessed by the rapid and sustained reduction in concentrations of progesterone (P4) in sera *(15)*.

Results

In experiment 1, steady-state concentrations of mRNA encoding SF-1 increased between days 3 and 12 of the estrous cycle (Fig. 1). Concentrations of progesterone in sera also increased between days 3 and 12 of the estrous cycle *(14)*, and these parameters were highly correlated ($p < 0.01$; $r = 0.72$). In experiment 2, ewes on days 11–12 of the estrous cycle were injected with 25 mg $PGF_{2\alpha}$ into the jugular vein followed by 10 mg $PGF_{2\alpha}$ im 2 h later. The initial dose of $PGF_{2\alpha}$ was administered directly into the jugular vein to time the initiation of luteolysis precisely. The second im injection of $PGF_{2\alpha}$ was given to ensure that complete luteal regression would occur. Injections of $PGF_{2\alpha}$ induced luteolysis as indicated by a rapid and sustained decline in concentrations of progesterone in sera and decreased luteal weights (Fig. 2; *15*). Following injection of $PGF_{2\alpha}$, luteal concentrations of mRNA encoding SF-1 decreased ($p < 0.01$) to 48% of control values within 4 h and

Table 1
Steady-State Concentrations (x + SEM) of mRNA Encoding SF-1, StAR, P450scc, and 3β-HSD in Ovine Luteal Tissue 0 (Control; n = 16), 4, 12, and 24 h After Administration of LH, Forskolin, $PGF_{2\alpha}$, or PMA (n = 3–4/time/trt)

	fmol/μg poly A^+ RNA			
Treatment	SF-1 mRNA	StAR mRNA[a]	P450scc mRNA	3β-HSD mRNA
Control	0.37 ± 0.04	84.04 ± 3.20	12.55 ± 0.74	25.70 ± 1.51
LH				
4 h	0.34 ± 0.04	73.72 ± 4.46	14.13 ± 1.12	20.19 ± 4.09
12 h	0.58 ± 0.09[b]	74.17 ± 8.82	14.21 ± 1.39	29.54 ± 1.33
24 h	0.44 ± 0.09	71.85 ± 4.54	11.83 ± 1.11	29.46 ± 3.80
Forskolin				
4 h	0.41 ± 0.06	84.78 ± 4.39	13.72 ± 0.95	27.60 ± 3.37
12 h	0.56 ± 0.09[b]	91.32 ± 12.41	13.69 ± 2.74	33.88 ± 5.75
24 h	0.44 ± 0.06	92.22 ± 9.46	12.74 ± 0.55	33.96 ± 4.57
$PGF_{2\alpha}$				
4 h	0.26 ± 0.04	70.55 ± 3.49	10.80 ± 1.24	14.22 ± 3.15[b]
12 h	0.46 ± 0.06	30.14 ± 2.54[b]	10.51 ± 1.61	13.93 ± 4.68[b]
24 h	0.31 ± 0.03	21.57 ± 5.78[b]	8.63 ± 1.51	15.43 ± 2.96[b]
PMA				
4 h	0.35 ± 0.07	62.84 ± 4.93[b]	12.64 ± 1.33	20.82 ± 0.91
12 h	0.24 ± 0.07	41.65 ± 9.51[b]	8.66 ± 1.56	10.86 ± 1.02[b]
24 h	0.36 ± 0.11	55.96 ± 20.81	10.70 ± 3.14	24.68 ± 6.22

[a]A portion of these data has been published *(15)*.
[b]Within each parameter, these values are different from control values ($p < 0.05$).

remained low through 24 h (Fig. 2). Experiment 3 was designed to:

1. Determine if LH or $PGF_{2\alpha}$ acutely regulate mRNA encoding SF-1.
2. Determine if the effects of LH or $PGF_{2\alpha}$ on SF-1 mRNA could be mimicked by activation of PKA or PKC, the second messenger systems that are activated by LH and PGF2a, respectively *(1)*.
3. Determine if changes in mRNA encoding SF-1 preceded or occurred in tandem with changes in mRNA encoding StAR, P450scc, or 3β-HSD.

Therefore, ewes on days 9–12 of the estrous cycle were administered $PGF_{2\alpha}$ (1 μmol), phorbol 12-myristate 13-acetate (PMA) (activates PKC; 2 μmol), LH (20 μg), forskolin (activates PKA; 50 μg), or vehicle (1 mL saline) directly into the ovarian artery as described *(16)*. Infusion of LH or forskolin transiently increased concentrations of mRNA encoding SF-1 12 h later; however, mRNAs encoding StAR, P450scc, or 3β-HSD were not affected by either LH or forskolin (Table 1). Infusion of $PGF_{2\alpha}$ or PMA did not change luteal concentrations of SF-1 mRNA (Table 1), although concentrations of mRNA encoding StAR and 3β-HSD were decreased by both $PGF_{2\alpha}$ and PMA (Table 1).

Discussion

SF-1 is critical for development of steroidogenic tissue *(17)*. In addition, DNA sequences that are responsive to SF-1 appear crucial for basal and/or cAMP-induced expression of mRNAs encoding StAR *(9,10)*, P450scc *(11,12)*, and 3β-HSD *(13)*, proteins essential for synthesis of progesterone. Steady-state concentrations of mRNA encoding SF-1 and concentrations of progesterone in sera increased during luteal development and were highly correlated. Apparent coordinate regulation of mRNA encoding SF-1 and SF-1 protein has been observed previously *(18,19)*. The pattern of expression of SF-1 mRNA during the estrous cycle was also similar to that observed for P450scc mRNA *(20,21)*. However, concentrations of mRNAs encoding StAR and 3β-HSD, two other proteins crucial for synthesis of progesterone, peak very early in the estrous cycle, and expression of these mRNAs remains high until luteolysis occurs *(15,20,22)*. This pattern of expression is very different from that observed for mRNA encoding SF-1. Since both 3β-HSD and StAR were maximally expressed when concentrations of SF-1 mRNA were low, it is possible that expression of these mRNAs is dependent on a basal level of SF-1 as would be suggested from data obtained from gene knockout *(10,17)* and promoter analysis experiments *(9,10)*. However, expression of mRNA encoding StAR and 3β-HSD does not appear to be tightly linked to the total amount of SF-1 mRNA present.

Both LH and $PGF_{2\alpha}$ regulated concentrations of SF-1 mRNA. Prostaglandin $F_{2\alpha}$ decreased mRNA encoding SF-1 in experiment 2, and the downregulation of this mRNA was similar to the decreases seen with mRNAs

encoding StAR, P450scc, and 3β-HSD (*15*, *23*, unpublished observations). Thus, one could speculate that decreases in SF-1 mRNA, and presumably protein, would lead to decreased transcription of these genes and thereby lower concentrations of mRNA encoding StAR, P450scc, and 3β-HSD. However, in the third experiment, neither $PGF_{2\alpha}$ nor activation of PKC decreased concentrations of mRNA encoding SF-1. However, both PMA and $PGF_{2\alpha}$ decreased ($p < 0.05$) concentrations of progesterone in sera at 4, 12, and 24 h following infusion *(24)*, and $PGF_{2\alpha}$, but not PMA, decreased ($p < 0.05$) luteal weight at 24 h. Thus, infusion of $PGF_{2\alpha}$ into the ovarian artery was effective in inducing luteolysis, whereas infusion of PMA mimicked the antisteroidogenic, but not lytic effects of $PGF_{2\alpha}$ as previously described *(16)*. Why $PGF_{2\alpha}$ decreased concentrations of SF-1 mRNA in the second, but not the third experiment appears to be a function of dose and method of administration. Ewes in the second experiment were administered more $PGF_{2\alpha}$ and, with the prolonged release of $PGF_{2\alpha}$ from the two injections, were exposed to $PGF_{2\alpha}$ for a longer period of time. That the effects of $PGF_{2\alpha}$ on SF-1 mRNA were dose-dependent was confirmed in a further experiment in which increasing doses of $PGF_{2\alpha}$ caused dose-dependent decreases in luteal concentrations of mRNA encoding SF-1 (86, 78, and 61% of control values 24 h after ewes received an intrajugular injection of 3, 10, or 30 mg $PGF_{2\alpha}$/60 kg body wt, respectively). However, acute exposure to $PGF_{2\alpha}$ did not decrease concentrations of mRNA encoding SF-1 in the same tissues where concentrations of mRNA encoding StAR and 3β-HSD were decreased. Thus, a decrease in SF-1 mRNA was not obligatory for decreased mRNA encoding StAR or 3β-HSD. However, it is possible that $PGF_{2\alpha}$ treatment modifies SF-1 protein in some manner, rendering it inactive in promoting transcription. Indeed, Young and McPhaul *(25)* have shown a correlation between the amount of intact SF-1 protein and the basal rate of steroidogenesis in tumor cells lines, and have postulated that a specific nuclear protease inactivates SF-1. Whether a similar enzyme could be induced following treatment with $PGF_{2\alpha}$ in luteal tissue is not known.

In experiment 3, concentrations of progesterone in sera of ewes administered LH via the ovarian artery were increased ($p < 0.05$) 1–2 h following injection, but then returned to control values *(24)*. Forskolin did not alter concentrations of progesterone in sera, and luteal weights were not affected by treatment with either LH or forskolin *(24)*. LH, or treatment with forskolin to activate PKA, increased concentrations of mRNA encoding SF-1 when administered during the midluteal phase. This is in direct contrast to results obtained with rat granulosal and mouse testicular tumor (MA-10) cells where forskolin induced mRNA encoding P450scc, but not SF-1 *(11,26)*. Similarly, hCG also decreased the amount of SF-1 mRNA in rat granulosal cells while increasing mRNA encoding P450scc *(27)*. Whether this difference is due to the use of different species or different cell types is not known. However, the observed increase in SF-1 mRNA in the present experiment was not associated with upregulation of mRNA encoding StAR, P450scc, or 3β-HSD. The promoter regions of all three of these genes have response elements that bind SF-1 *(9–13)*. In addition, basal or cAMP responsiveness of the these promoters appears dependent, at least in part, on the presence of the SF-1 binding element *(9–13)*. Thus, although the importance of SF-1 in development of steroidogenic tissue is clearly demonstrated by the lack of adrenal and gonadal tissue in SF-1 knockout mice *(17)*, in the mature corpus luteum, it may be acting as a permissive rather than an acute regulatory factor in controlling expression of mRNA encoding StAR, P450scc, and 3β-HSD.

In conclusion, increasing amounts of SF-1 mRNA, and presumably protein, may underlie the increases in mRNA encoding P450scc, since patterns of expression of these mRNAs are similar during luteal development. However, maximal expression of StAR and 3β-HSD mRNA does not appear to be dependent on maximal expression of SF-1 mRNA. In addition, once the corpus luteum is fully functional, regulation of mRNA encoding StAR, P450scc, and 3β-HSD does not appear to be associated with regulation of SF-1 mRNA.

Materials and Methods

Western range ewes exhibiting normal estrous cycles (17 ± 1 d) were used in all experiments. Ewes were observed for estrus ($d = 0$) with the aid of vasectomized rams. All experiments were approved by the Colorado State University animal care and use committee.

Experiment 1

Corpora lutea were collected from ewes on days 3, 6, 9, 12, and 15 of the estrous cycle, decapsulated, weighed, and pooled ($n = 4$ pools/d; 1–5 ewes/pool). An aliquot of this tissue was frozen at –70° until isolation of polyadenylated (poly A^+) RNA. Portions of this tissue have been used in other experiments, and details concerning average concentrations of progesterone in serum and weight of corpora lutea have been published *(14)*.

Experiment 2

Ewes on days 11–12 of the estrous cycle were injected with 25 mg $PGF_{2\alpha}$ into the jugular vein followed by 10 mg $PGF_{2\alpha}$ im 2 h later. Corpora lutea were collected 4, 12, or 24 h after the iv injection ($n = 4$–5 ewes/time). Control luteal tissue was collected from ewes on days 11–13 of the estrous cycle that had not been treated ($n = 4$) or had been treated with saline ($n = 4$) 24 h prior to tissue collection. After collection, corpora lutea were decapsulated, weighed, frozen in liquid nitrogen, and stored at –70°C until isolation of poly A^+ RNA. Concentrations of progesterone in serum and average luteal weight in these ewes have been published *(15)*.

Experiment 3

In experiment 3, ewes on days 9–12 of the estrous cycle were administered $PGF_{2\alpha}$ (1 μmol), PMA (activates PKC; 2 μmol), LH (20 μg), forskolin (activates PKA; 50 μg), or vehicle (1 mL saline) directly into the ovarian artery as described *(16)*. Luteal tissue was collected 0 (untreated), 4, 12, or 24 h following injection (n = 3–4/trt group/time) and processed as described for experiment 2. Data concerning concentration of progesterone in serum and luteal weight have been published *(24)*.

Quantification of mRNAs Encoding SF-1, StAR, P450scc, and 3β-HSD

Unless otherwise specified, all materials used were purchased from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific (Denver, CO). Polyadenylated RNA (Poly A^+ RNA) was isolated using oligodeoxythymidine (dT) cellulose as previously described *(28,29)*. Concentrations of mRNA in individual samples were determined by absorbance at 260 nm.

Concentrations of SF-1 mRNA in luteal samples were determined by slot-blot analysis using a cDNA that encodes approx 450 bases of the coding region of ovine SF-1 *(30)*. This cDNA has previously been used in Northern and slot-blot analysis of ovine tissue and was specific for SF-1 mRNA *(30)*. Polyadenylated RNA (300–1000 ng) isolated from individual luteal samples was applied in duplicate to a nylon filter with a slot-blot apparatus. In addition, a negative control sample (liver 300–1000 ng) and a positive control sample (pool of luteal RNA, three different concentrations ranging from 200 to 1000 ng) were applied in duplicate to each filter. Standards for ovine SF-1 mRNA were synthesized by transcribing the *Nco*I linearized plasmid with SP6 RNA polymerase. Filters containing samples and standards (10–500 pg) were prehybridized overnight at 42°C in hybridization buffer (5-strength SSC: 750 m*M* NaCl and 75 m*M* sodium citrate; 1X Denharts: 0.1% [wt/vol] Ficoll 400, 0.1% [wt/vol] polyvinylpyrrolidone, 0.1% [wt/vol] BSA [U.S. Biochemicals, Cleveland, OH]; 0.1% SDS; 50% deionized formamide; 50 m*M* sodium phosphate, and heat-denatured salmon sperm DNA [100 μg/mL]). Filters were then hybridized for 24 h with 2×10^6 cpm/mL of ^{32}P-labeled cDNA encoding ovine SF-1 (*31*; generated with the random primer method; specific activity 0.5×10^9 dpm/μg DNA). For experiments 1 and 2, concentrations of SF-1 mRNA were determined from a single filter. Correlation (r^2) between amount of standard applied and densitometric reading was 0.97 and 0.96 for experiment 1 and 2, respectively. Coefficients of variation, calculated from the values obtained from the positive control samples, were 11 and 20% for experiment 1 and 2, respectively. Sensitivity for the assay was 0.06 fmol/μg poly A^+ RNA in both experiments 1 and 2. Concentrations of SF-1 mRNA in experiment 3 were measured in four assays. Correlation (r^2) between amount of standard applied and densitometric reading was 0.99. Intra-assay coefficient of variation averaged 11%, and interassay coefficient of variation was 12%. Sensitivity of the assay was 0.05 fmol/μg poly A^+ RNA.

In the third experiment, concentrations of mRNAs encoding StAR *(15)*, P450scc *(20)*, and 3β-HSD *(23)* were determined in four assays by slot-blot analysis. Radioactive cDNAs were generated with the random primer method and had specific activities ranging from 0.7 to 0.9×10^9 dpm/μg DNA. Standard curves were linear with correlations (r^2) between amount of standard added and the resulting densitometric readings ranging from 0.97 to 0.99. Intra-assay CV averaged 10, 11, and 13% for mRNAs encoding StAR, P450scc, and 3β-HSD, respectively. Interassay CV was 19, 12, and 6% for mRNAs encoding StAR, P450scc, and 3β-HSD. Sensitivity of the assay, as determined by the lowest detectable point on the standard curve, was 6, 1.4 and 5.1 fmol/μg poly A^+ RNA for mRNAs encoding StAR, P450scc, and 3β-HSD.

To determine whether equal amounts of RNA were loaded onto each slot, following hybridization to a specific cDNA, all filters were hybridized with ^{32}P-end-labeled dT (18 mer) as previously described *(20)*. Correlation *(r)* between the amount of positive pool luteal poly A^+ RNA applied to the slot and densitometric reading averaged 0.95 ± 0.01, 0.93 ± 0.01, 0.96 ± 0.01, and 0.95 ± 0.01 for mRNAs encoding SF-1, StAR, P450scc, and 3β-HSD, respectively. Coefficient of variation averaged 13, 12, 14, and 12% for mRNAs encoding SF-1, StAR, P450scc, and 3β-HSD, respectively. Therefore, no corrections were made for loading differences.

Statistical Analysis

All statistical analyses were performed with the general linear model procedure of SAS *(32)*. Concentrations of mRNA encoding SF-1 in experiments 1 and 2 and 3β-HSD in experiment 3 were not normally distributed or had heterogeneous variance, and were transformed prior to final statistical analysis. However, the mean ± SEM of raw data is presented. There were no differences between noninjected and saline-injected controls in experiments 2 and 3; therefore, these groups were combined for final statistical analysis. In experiment 1, differences among means were determined with Tukey's test. In experiments 2 and 3, differences between least-squares means in treated groups and the control group were determined by least-significant differences. Differences between means were considered significant when $p < 0.05$. Correlation coefficients between parameters were determined with Pearson's test.

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