BIOLOGY OF REPRODUCTION **64,** 359–367 (2001)

Feed Restriction and Insulin Treatment Affect Subsequent Luteal Function in the Immediate Postovulatory Period in Pigs: Progesterone Production In Vitro and Messenger Ribonucleic Acid Expression for Key Steroidogenic Enzymes[1]

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ABSTRACT

Progesterone production and release in vitro, and mRNA expression for key steroidogenic enzymes, were studied in luteal tissue recovered in the immediate postovulatory period from cyclic gilts allocated to one of three treatments: moderate feed restriction during the first (RH) or second week of the estrous cycle, with (HR+I) or without (HR) concomitant injections of long-acting insulin. Time of feed restriction affected neither progesterone production or release, nor mRNA expression for several key steroidogenic enzymes. However, luteal tissue from RH but not from HR gilts responded to LH stimulation by increasing progesterone production and release ($P < 0.05$). Insulin treatment increased progesterone production and release, restored luteal tissue responsiveness to LH, up-regulated steroidogenic enzyme mRNA expression, and down-regulated the tissue inhibitor of metalloproteinase-I mRNA expression in HR+I compared with HR gilts ($P < 0.05$). In vitro progesterone production and gene expression were affected by time of tissue collection after ovulation in RH and HR gilts but not in HR+I gilts, and were correlated with temporal changes in oviductal and peripheral plasma progesterone concentrations. Inherent differences in luteal function therefore appear to mediate latent effects of nutrition and insulin treatment on circulating progesterone concentrations in the critical postovulatory period in gilts.

corpus luteum, corpus luteum function, insulin, ovary, pregnancy, progesterone

INTRODUCTION

Suboptimal luteal function has been suggested as a major cause of infertility and studies in pigs and cattle suggest that differences in progesterone production in early pregnancy mediate nutritional effects on subsequent fertility [1–3]. An earlier rise in plasma progesterone concentrations after the LH surge has also been observed in more prolific Chinese Meishan gilts than in Large White gilts [4] and may play a key role in the superior embryo survival reported in the Meishan breed. Furthermore, exogenous progesterone treatment in early pregnancy enhanced embryonic survival at Day 30 of pregnancy in gilts [5, 6], providing direct evidence for progesterone-mediated effects on fertility. Previous studies in the gilt and in the lactating and weaned sow suggest that the time of feed restriction during the early period of follicular development may be a critical factor in determining subsequent fertility [1]. In a previous experiment in our laboratory, which formed the basis for the present study, we showed that feed restriction in gilts during the second week of the estrous cycle, depressed circulating progesterone concentrations at 48 and 72 h after onset of estrus, and decreased embryonic survival rate at Day 28 of pregnancy, compared with nonrestricted gilts or gilts that were feed-restricted during the first week of the estrous cycle [7]. Using an extension of this experimental model, we again showed negative effects of feed restriction during the second week compared with the first week of the cycle on plasma progesterone concentrations in early pregnancy; furthermore, insulin treatment during feed restriction in the second week counteracted the negative effects on plasma progesterone [8]. No differences in endocrine status and, by implication, metabolic state, were apparent the day after the end of treatment (Day 16 of the cycle) in this study; however, differences in estrogen, LH, and progesterone secretion in the periovulatory period were apparent, indicating that the previous period of feed restriction and insulin treatment exerted their effects by altering the pattern of follicular maturation in the late luteal phase of the cycle. Furthermore, nutritionally induced differences in metabolism and clearance of progesterone in early pregnancy, which are believed to occur in other experimental models [1], will not contribute to differences in plasma progesterone concentrations in the present experimental paradigm, in which all gilts receive the same level of feed intake from Day 16 of the cycle. Therefore, we hypothesize that differences in progesterone concentrations are due to differences in luteal function that originate from previous treatment effects on the preovulatory and postovulatory development of the follicle.

Early corpus luteum development in the pig is characterized by low numbers of low affinity LH receptors on luteal cells, compared with either granulosa cells before ovulation or luteal cells in the mid-luteal phase [9]. Hence, low progesterone concentrations and a delayed rise in progesterone in feed-restricted gilts we reported previously [7, 8] may be a consequence of low sensitivity of the corpus luteum to LH. Because steroidogenic acute regulatory (StAR) protein, and the P450 cholesterol side-chain cleavage ($P450_{scc}$) and 3β-hydroxysteroid-dehydrogenase (3β-HSD) enzymes are essential to normal luteal function [10, 11], and because StAR protein is a key regulator of progesterone synthesis [12], differences in luteal function may also be related to differences in StAR protein, $P450_{scc}$, or 3β-HSD mRNA expression. Finally, intraovarian growth factors such as insulin-like growth factor I (IGF-I), fibroblast growth factor (FGF), and transforming growth factor β (TGFβ) modulate the response of the corpus luteum to circulating gonadotropins [13] and may exert an additional

[1]This research was supported by the Natural Sciences and Engineering Research Council of Canada, the Alberta Agriculture Research Institute, and the Alberta Pork Producers Development Corporation.
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Received: 22 March 2000.
First decision: 4 May 2000.
Accepted: 31 August 2000.

ISSN: 0006-3363. http://www.biolreprod.org

level of control on luteal function. In turn, the availability of such growth factors is influenced by the activity of matrix metalloproteinases (MMPs) [14–17]. Given that progesterone appears to increase earlier in Chinese Meishan pigs than it does Large White pigs, the report of higher gelatinase activity (MMP-2 and MMP-9) and lower tissue inhibitors of metalloproteinase (TIMP-1 and TIMP-2) in the luteinizing follicles of prolific Meishan females [18] suggests that differences in TIMP-MMP-growth factor activity may also regulate progesterone production. In all cases, our hypothesis implies that any observed differences in these regulators of ovarian function would be a lasting consequence of earlier treatments, and not related to ongoing differences in nutrition or metabolic state.

Insulin has been identified as an important modulator of reproductive function, and treatment-induced differences in insulin status in the present collaborative study [8] may have mediated effects on subsequent follicular maturation and luteal function. In earlier studies from other laboratories, insulin treatment increased follicular fluid steroids [19], the number of large follicles [20], and farrowing rate and litter size [21] in weaned sows. In addition, when insulin was given to cyclic gilts during the preovulatory period it increased ovulation rate [22] and decreased follicular atresia [23, 24]. Exogenous insulin restores follicular growth in nutritionally anestrous gilts [25], and additional dietary energy to their food increased ovulation rate and insulin concentrations [26, 27].

Therefore, as one component of a major collaborative experiment to determine endocrine and other mechanisms that mediate nutritional and insulin-mediated effects on subsequent fertility in cyclic gilts [8], the objectives of the present experiment were to investigate luteal function in the same animals using tissue recovered in the immediate postovulatory period by determining 1) progesterone production and release by minced luteal tissue and by dispersed luteal cells in vitro; 2) responsiveness of luteal tissue and dispersed luteal cells to LH stimulation; 3) mRNA expression for StAR protein, $P450_{scc}$, and 3β-HSD enzymes; 4) mRNA expression for MMP-2, MMP-9, and their inhibitors, TIMP-1, and TIMP-2 in luteal tissue; and 5) associations among these parameters and the relationship between these characteristics and oviductal and peripheral plasma progesterone concentrations. The experimental paradigm we used allowed us to provide some of the first evidence for effects of previous nutritional and metabolic state on subsequent luteal function.

MATERIALS AND METHODS

Animals and Treatments

The research protocol was performed in accordance with the Canadian Council on Animal Care Guidelines and with the approval of the University Animal Care and Use Committee. Details of animals and treatments and endocrine and metabolic status, including plasma insulin concentrations, have been described in detail elsewhere [8]. Briefly, littermate Camborough × Canabrid gilts (Pig Improvement Canada, Ltd., Acme, AB, Canada) were selected from 15 litters and allocated within litter to one of three treatments. Because we had previously shown no difference in fertility between continually high-plane feeding and feed restriction of gilts in the first week of the estrous cycle before breeding, but feed restriction in the second week of the previous cycle decreased embryonic survival to Day 30 of gestation [7], the following experimental groups were included in the present collaborative study: restricted-high (RH) gilts (n = 14) were initially feed-restricted at approximately 75% of ad libitum intake (2.1 × maintenance [M] requirements) from Day 1 to Day 7 of the estrous cycle and then fed approximately 95% of ad libitum intake (2.8 × M) from Day 8 of the cycle until estrus. The pattern of feed restriction was reversed to high-restricted (HR) in the other littermates, which were fed 2.8 × M from Day 1 to Day 7, restricted to 2.1 × M from Day 8 to Day 15, with (HR+I, n = 15) or without (HR, n = 15) concomitant treatment with long-acting insulin injection twice daily at 0.4 IU/kg (Lente Iletin II; Eli Lilly, Indianapolis, IN), and then returned to 2.8 × M until estrus. In the insulin-treated animals, 30 ml of corn syrup was provided in their feed to prevent serious hypoglycemia. All animals were checked for estrus every 6 h from Day 18 of the treatment cycle and were inseminated 12 and 24 h after onset of estrus. From 24 h after onset of estrus, ovulation was monitored every 6 h by real-time ultrasound (Pie Medical Scanner 200; Can Medical, Kingston, ON, Canada). Twelve to 24 h after ovulation, the ovary with the most corpora lutea was surgically removed by hemiovariectomy and immediately transported on ice to the laboratory in 20 ml of Hanks' balanced salt solution (HBSS; Sigma Chemical Company, St. Louis, MO) without Ca^{++} and Mg^{++} at pH 7.4. Corpora lutea were dissected from the ovary and 250 to 300 mg of luteal tissue, pooled from all corpora lutea, was snap-frozen in liquid nitrogen and stored at −80°C for later mRNA extraction and analysis. The remaining luteal tissue was prepared for tissue/cell culture. The interval between surgery and dissection was always less than 20 min.

Incubations

Luteal tissue fragments. The pooled luteal tissue obtained from each gilt was finely minced on ice with two scalpel blades and washed twice in 50 ml HBSS without Ca^{++} and Mg^{++}. Based on the method described by Hunter [28], known amounts of minced tissue (20 ± 2 mg) were placed into 24 culture tubes. Samples in four tubes were immediately frozen for assay of tissue progesterone content before culture (T0 P4). Minced tissue in the remaining tubes was cultured in 2 ml of Eagles minimum essential medium (MEM; Sigma) containing a range of LH concentrations (0, 0.1, 1, 10, and 100 ng pLH/ml), which was previously shown in our laboratory to be effective in assessing luteal cell responsiveness to LH stimulation [29], with four replicates per dose. Luteinizing hormone activity in the media were confirmed by radioimmunoassay (RIA). After enriching the medium with 95% (v/v) O_2/5% (v/v) CO_2 for 10 sec, the tubes were capped and incubated in a shaking water bath for 3 h at 37°C. At the end of incubation, tissue and media were separated by centrifugation at 500 × *g* for 5 min and the tissue was subsequently homogenized (Polytron; Janke & Kunkel IKA-Labortechnik, Germany) in absolute alcohol (HPLC grade; Fisher Scientific Ltd., Nepean, ON, Canada). The ethanol extract and the medium were assayed for progesterone as described below.

Dispersed cells. The remaining luteal tissue was washed and subjected to enzyme dissociation as described by Gadsby et al. [30]. One hundred thousand viable cells were incubated in 1 ml of MEM per tube in quadruplicate at the various LH concentrations described above. Viability, estimated by the 0.2% (w/v) Trypan blue (Sigma) exclusion test [28], was greater than 80% before incubation and greater than 70% after the 3-h incubation. Four aliquots of the

cell suspension were immediately frozen for subsequent estimation of the steroid in the medium before incubation. All steps were carried out at 4°C unless otherwise indicated.

Progesterone Radioimmunoassay

At surgery, peripheral blood samples were collected by jugular venipuncture and oviductal blood samples were taken by venipuncture of a vein draining the mid-section of the oviductal vasculature. Plasma progesterone was analyzed as described previously [31]. Assay sensitivity, defined as 91.4% of total binding, was 0.009 ng/tube and intraassay and interassay coefficients of variation were 10% and 10.4%, respectively.

Progesterone concentrations in culture media were determined using the same direct assay, with intraassay and interassay coefficients of variation of 2.1% and 15.1%, respectively. Tissue progesterone RIA was based on the method described by Hunter et al. [32]. Aliquots (100 μl) of the ethanol extract were evaporated to dryness and redissolved in 1 ml of assay buffer for direct assay of tissue progesterone content. Various volumes of extracted samples exhibited parallelism to the standard curve, and ethanol and buffer blanks were below the limit of sensitivity. The mean intra- and interassay coefficients of variation of these assays were 2.2% and 6.6%, respectively.

Messenger RNA Expression Studies

For the purposes of evaluating mRNA expression for key steroidogenic enzymes as part of the current study, subsets of 10 animals from each treatment group were identified for detailed study on the basis of ovulation-to-surgery interval, peripheral plasma progesterone concentrations, and embryo developmental stage at surgery. Total RNA was extracted from luteal tissue using TRIzol Reagent according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD). The RNA was quantified by spectrophotometric absorbance at 260 nm, then aliquoted and stored at −80°C. RNA integrity was assessed by observing the 28S and 18S ribosomal RNA bands after electrophoresis of one aliquot on a 1% (w/v) formaldehyde agarose gel followed by ethidium bromide staining. Northern blots were prepared by subjecting 20 μg of total RNA to electrophoresis in 1% (w/v) agarose gels in duplicate under denaturing conditions with formaldehyde [33], then transferred to Hybond-XL nylon membrane (Amersham Pharmacia Biotech, Baie d'Urfé, PQ, Canada) overnight and baked at 80°C for 2 h under vacuum. Standard RNA size markers (Life Technologies) were used to estimate the size of mRNA transcripts detected on Northern blots. Blots were prehybridized for a minimum of 3 h at 60°C in 5× SCC (single-strength SCC contains 150 mM sodium chloride, 15 mM citric acid), 100 μg/ml of sheared salmon sperm DNA, and 5× Denhardts solution (100-strength Denhardts contains 2% [w/v] BSA, 2% [w/v] Ficoll, and 2% [w/v] polyvinylpyrrolidone). Blots were then hybridized overnight in fresh prehybridization solution using porcine cDNA probes for StAR protein, $P450_{scc}$ (obtained from Drs. David Silversides and Bruce Murphy, CRRA, Université de Montreal, Canada), and 3β-HSD (obtained from Dr. Fernand Labrie, CHUL Research Centre, Laval University, Canada) to assess steady state mRNA levels. The cDNA fragments were labeled with [^{32}P]dATP (Amersham Pharmacia Biotech) using a Random Primed DNA labeling kit according to the manufacturer's instructions (Life Technologies). Unincorporated nucleotides were removed using G-50 Sephadex Nick Columns (Amersham Pharmacia Biotech). After hybridization, the blots were washed twice (15 min per wash) with 2× saline sodium citrate (SSC)/0.1% (w/v) SDS at 35°C, followed by two washes (15 min per wash) with 1× SSC/0.1% (w/v) SDS, and finally with one 15-min wash in 0.2× SSC/0.1% (w/v) SDS at 60°C. Blots were exposed to BioMAX MS film (Eastman Kodak, Rochester, NY) for 96, 48, and 27 h for StAR, 3β-HSD, and $P450_{scc}$, respectively, at −80°C with two intensifying screens. All RNA data were normalized for loading and are expressed as the following ratios: StAR/28S, 3β-HSD/28S, and $P450_{scc}$/28S.

Reverse transcription-polymerase chain reaction. MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNA expressions were quantified by RT-PCR. Specific primers to amplify part of MMP-2, TIMP-1, and TIMP-2 mRNA were kindly provided by Dr. Denis Balcerzak (AFNS, University of Alberta). The

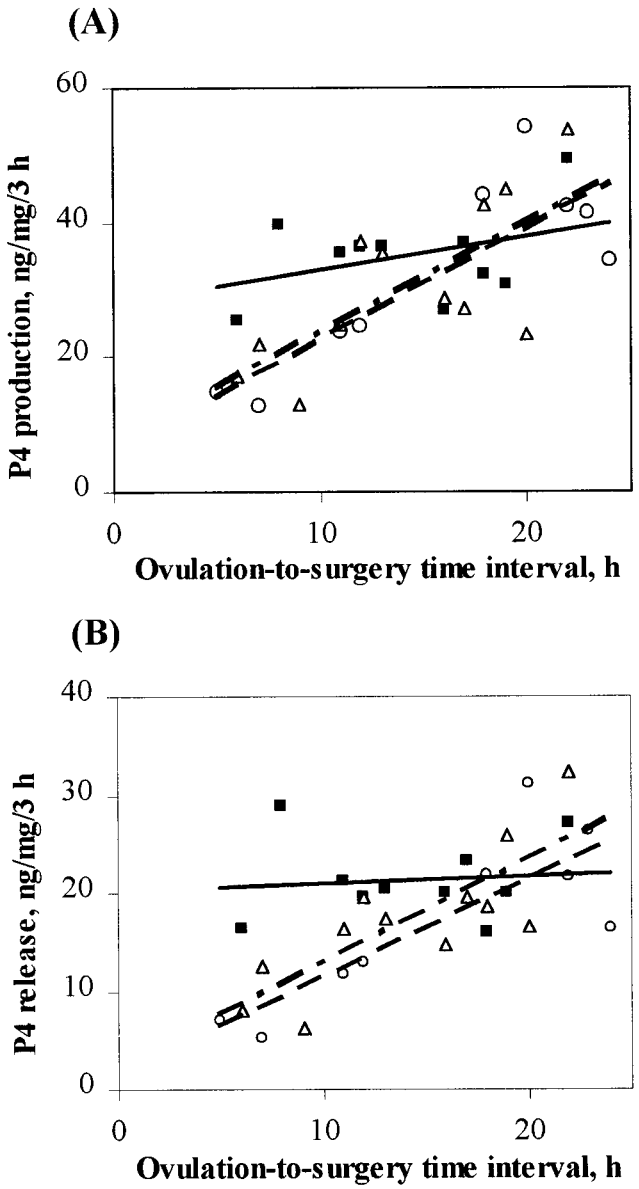

FIG. 1. Correlation between progesterone (P4) production (**A**) and release (**B**) by minced luteal tissue in vitro (ng/mg per 3 h) and ovulation-to-surgery time interval in HR (open circles, dashed lines), HR+I (squares, solid lines) and RH (triangles, broken lines) gilts. Because the plots of progesterone data over time were similar at five different LH levels, data plotted in this figure were based on pooled means for each time point.

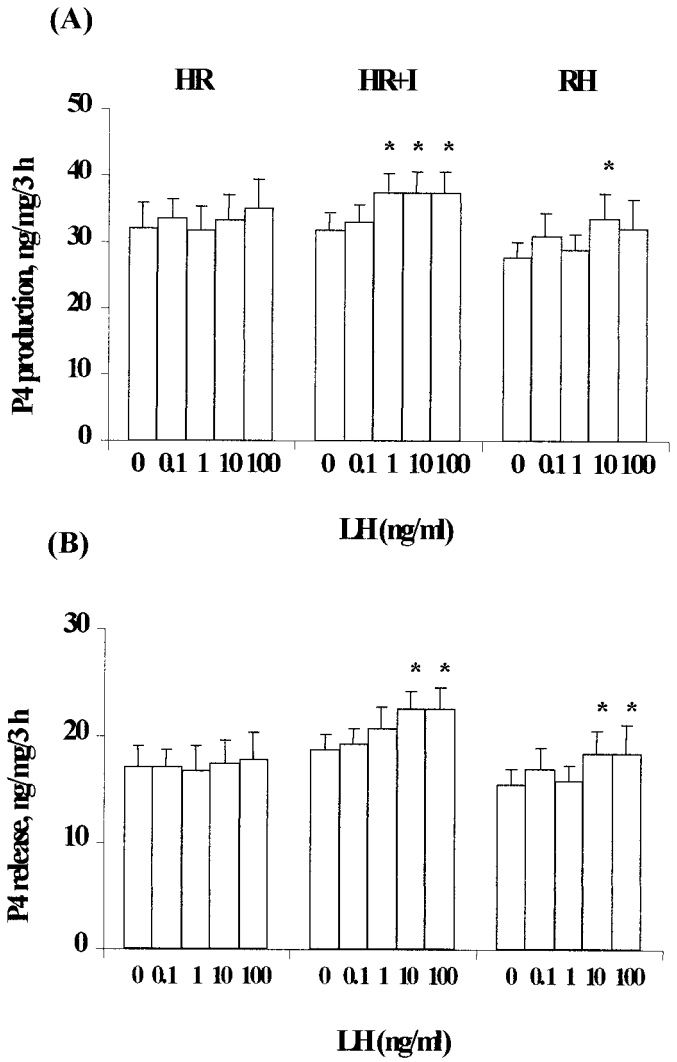

FIG. 2. Progesterone (P4) production (**A**) and release (**B**) in vitro by luteal tissue from HR, HR+I, and RH gilts in response to LH stimulation. The bars represent LS-means ± SEM. *Significant difference within treatment ($P < 0.05$) compared with the 0 ng/ml control.

sequences of MMP-2 (510 base pair [bp]) primers were 5′-CTTCCCCCGCCAGCCCAAGTGGG-3′ (sense) and 5′-CGGGGGTACTTCGGGACAAGTGG-3′ (antisense); TIMP-1 (504 bp) primers 5′-GTACCTGCGTCCCACCCCACC-3′ (sense) and 5′-GGCGGTGGACCGGACGGACGG-3′ (antisense); TIMP-2 (643 bp) primers 5′-CCTCCTGCTGCTGGGGACGCTGC-3′ (sense) and 5′-GGGCATTCGTCCGGTGGTCCTGA-3′ (antisense). Primers for MMP-9 (357 bp) were synthesized according to the method described by Menino et al. [34].

Before running the samples, the amount of total RNA, annealing temperature, and cycle number of PCR were empirically optimized for each of the target genes. Reverse transcription was carried out with M-MLV reverse transcriptase (64 U; Life Technologies) at 37°C for 1 h in a total reaction volume of 5 μl containing 1 μM primer; 0.25 mM each of dATP, dCTP, dGTP, and dTTP; 50 mM Tris-HCL pH 8.3; 70 mM KCl; 3 mM $MgCl_2$; 10 mM DTT; and 2.5 U RNase inhibitor (Life Technologies). A blank was included with RT reactions in which sterile water was substituted for RNA. Polymerase chain reactions were also performed without RT to confirm the absence of DNA contamination. All PCR reactions were carried out in a Perkin Elmer GenAmp 2400 Thermocycler in a reaction volume of 50 μl containing 2 U *Taq* polymerase (Life Technologies), 0.6 μM primers, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, and 0.2 mM dNTP mixture. For each gene, 30 samples were run at the same time and the PCR amplification of target genes for all samples was therefore assumed to be carried out under the same conditions. The identities of the amplified products were confirmed by diagnostic restriction enzyme analysis and DNA sequence analysis. RT-PCR amplification of sample RNA without adding RT did not yield products with the predicted sizes for any of the target genes. A positive control using the Jag-1 porcine trophoblast cell line [35] RNA was run with the unknown sample for each gene. Aliquots of each PCR reaction (10 μl) were electrophoresed through a 2% (w/v) agarose gel stained with 0.1 μg/ml ethidium bromide. Gels were visualized on an ultraviolet transilluminator and photographed. The images were quantified using molecular analyst software (version 6.0; BioRad, Mississauga, ON, Canada). The G3PDH gene [36] was used as an internal RT-PCR control to normalize the target gene expression. Messenger RNA expression for all these genes is expressed as the ratio, target gene/G3PDH.

Zymography

Analysis of TIMP-1 mRNA expression revealed differences between treatment groups. To confirm this finding, gelatinase activity (MMP-2) was directly assessed by zymography [18] using available tissue.

Statistical Analysis

Progesterone content in luteal tissue before culture (T0 P4), and progesterone production and release by minced luteal tissue in vitro, were analyzed based on the data from 44 animals and expressed as ng/mg tissue per 3 h. The amount of progesterone secreted by dispersed luteal cells was expressed as ng/10^5 cells per 3 h. Progesterone production was calculated by subtracting the progesterone content in tissue before incubation from the total progesterone in the medium (progesterone release) and in the tissue after incubation.

All dependent variables were analyzed for normality using the Wilk-Shapiro test [37]. Data for the dependent variables of T0 P4, progesterone production and release by luteal tissue, and dispersed luteal cell progesterone production were analyzed using the general linear model (GLM) procedure of Statistical Analysis Systems (SAS) [37]. Because there was no difference in time interval from LH surge to ovulation among the three groups, progesterone content before culture was analyzed with littermate and treatment as main effects and with ovulation-to-surgery time interval (time) as a covariate. For in vitro progesterone production, progesterone release by tissue, and luteal cell progesterone production, sources of variation were littermate, treatment, hormone (LH), and hormone × treatment interaction, with time as a covariate. If a time × treatment interaction was established, then heterogenous slopes were used to analyze the effect of time with treatment. A multiple comparison procedure was used to analyze hormone effects within treatment [37]. Analysis of the dependent variables of StAR protein, $P450_{scc}$, 3β-HSD, MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNA expression was based on data from 10 animals per treatment as discussed earlier. Treatment effects were tested, fitting time as a covariate, by the GLM procedure of SAS [37]. Again, when a

TABLE 1. Progesterone (P_4) content in pooled luteal tissue (CL) before (T0 P_4) and after culture, and progesterone production and release during culture by luteal tissue, and P_4 production by dispersed luteal cells, recovered from HR, HR + I, and RH gilts.

Item	Treatment		
	HR	HR + I	RH
Number	15	15	14
T0 P_4 content in CL (ng/mg)	10.6 ± 1.2	13.5 ± 1.9	11.8 ± 1.5
Minced luteal tissue			
P_4 content in CL after culture (ng/mg)	26.1 ± 0.8	28.0 ± 0.8	26.6 ± 0.8
P_4 production (ng per mg per 3 h)	32.4 ± 1.0[a]	35.2 ± 1.0[b]	32.3 ± 1.1[a]
P_4 release into medium (ng per mg per 3 h)	16.9 ± 0.6[a]	20.7 ± 0.6[b]	17.8 ± 0.7[a]
Luteal cells			
P_4 production (ng per 100 000 cells per 3 h)	9.9 ± 0.7[a]	12.2 ± 0.6[b]	10.7 ± 0.7[a,b]

[a,b] Means lacking common superscripts differ ($P < 0.05$).

time × treatment interaction was detected, heterogeneous slopes were used for further analysis.

Linear regression analyses [37] were performed to determine the associations among progesterone production, progesterone release by tissue, and oviductal and peripheral plasma progesterone concentrations, and the associations between StAR protein, $P450_{scc}$, 3β-HSD mRNA expression, and in vitro progesterone production by tissue. Oviductal and peripheral plasma progesterone concentrations were logarithmically and square root-transformed, respectively, to approach a normal distribution for regression analysis. All data are expressed as least squares means ± the standard error of the least squares means (LS-means ± SEM).

RESULTS

Progesterone Production and Release by Luteal Tissue, and by Dispersed Luteal Cells

Overall (Table 1), treatment affected progesterone production and release during in vitro culture by both minced tissue ($P < 0.001$) and by dispersed luteal cells ($P < 0.01$). Progesterone production and release by minced luteal tissue from HR+I gilts was higher than from HR and RH gilts ($P < 0.05$), with no difference between HR and RH groups ($P > 0.05$). Progesterone production by dispersed luteal cells from HR+I gilts was also higher than that from HR gilts ($P < 0.02$), with no difference between HR and RH or between RH and HR+I gilts ($P > 0.05$). A litter effect was established for progesterone production and release by minced luteal tissue in vitro ($P < 0.001$).

Ovulation-to-surgery time interval (time) interacted with treatment ($P < 0.01$) to affect progesterone production and release by luteal tissue (Fig. 1, A and B, respectively). Both progesterone production and release in vitro by luteal tissue from HR and RH gilts increased as ovulation-to-surgery time interval increased (progesterone production: $r = 0.84$ and 0.71; progesterone release: $r = 0.79$ and 0.82 for HR and RH gilts, respectively; $P < 0.01$). In contrast, progesterone production and release by luteal tissue from HR+I gilts were consistently high regardless of when the tissue was collected ($r = 0.28$ and 0.1 for progesterone production and release, respectively; $P > 0.05$).

Treatment-dependent effects on LH-induced progesterone production and release were observed (Fig. 2, A and B), whereas treatment did not affect LH-induced progesterone production by dispersed luteal cells ($P > 0.05$).

Correlation Between Progesterone Production and Release In Vitro by Luteal Tissue and Oviductal, and Peripheral Plasma Progesterone Concentrations

Oviductal and peripheral plasma progesterone concentrations were variable and were not normally distributed. However, after logarithmic and root square transformations

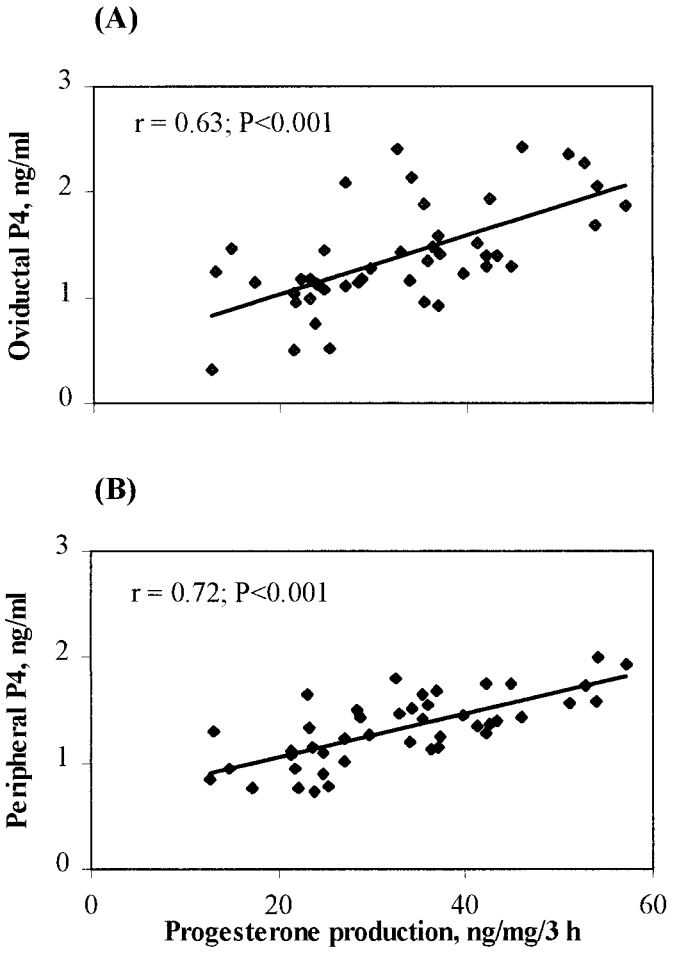

FIG. 3. Correlation between progesterone production by minced luteal tissue in vitro and oviductal vein plasma progesterone (**A**; Oviductal P_4), and peripheral vein plasma progesterone (**B**; Peripheral P_4) concentrations at the time of surgery (n = 44). Oviductal and peripheral vein plasma progesterone concentration data were logarithmically and root square transformed, respectively. The distribution was similar among HR, HR+I, and RH groups.

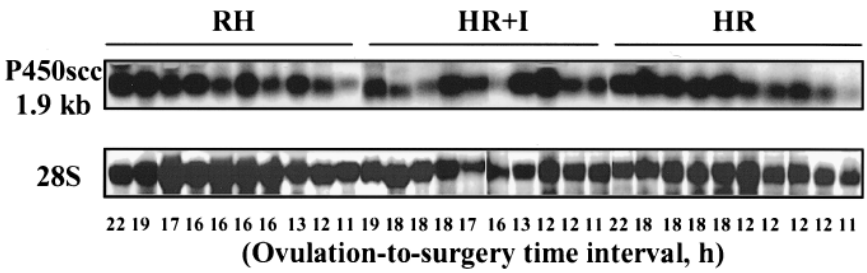

FIG. 4. Representative $P450_{scc}$ transcript expression and loaded total RNA from one gel. Samples were organized in time order from shortest to longest interval from right to left within each treatment.

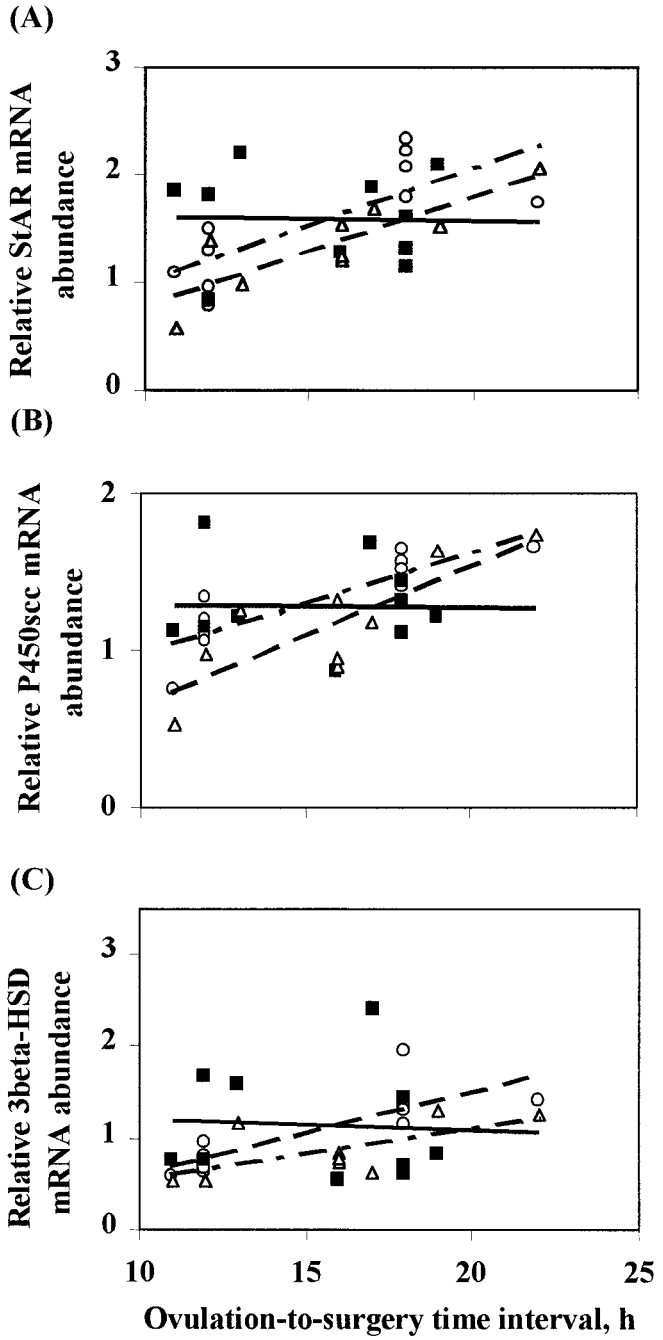

FIG. 5. StAR protein (**A**), $P450_{scc}$ (**B**), and 3β-HSD (**C**) mRNA expression in luteal tissue relative to ovulation-to-surgery time interval in HR (circles, dashed lines), HR+I (squares, solid lines) and RH (triangles, broken lines) gilts. As the interval increases, mRNA expression for these three key enzyme increased in HR and RH gilts ($P < 0.01$), but was consistently high in HR+I pigs ($P > 0.05$).

of oviductal and plasma progesterone data, respectively, strong positive correlations were established between progesterone production in vitro by luteal tissue and oviductal (Fig. 3A) and peripheral (Fig. 3B) vein plasma progesterone concentrations, and between progesterone release by luteal tissue and oviductal ($r = 0.53$; $P < 0.001$) and peripheral ($r = 0.59$; $P < 0.001$) vein plasma progesterone concentrations. Progesterone content in luteal tissue before culture was also associated with oviductal ($r = 0.58$, $P < 0.001$) and peripheral ($r = 0.64$, $P < 0.001$) vein plasma progesterone concentrations.

StAR Protein, $P450_{scc}$, and 3β-HSD mRNA Expression in Luteal Tissue

Time × treatment interactions also affected expression of the key steroidogenic genes studied ($P < 0.001$). $P450_{scc}$ (Figs. 4 and 5B), StAR protein (Fig. 5A), and 3β-HSD (Fig. 5C) mRNA expression increased in HR and RH groups as time interval from ovulation to luteal tissue collection increased ($P < 0.01$), but was consistently high and not affected by time in HR+I gilts ($P > 0.05$). Furthermore, $P450_{scc}$ (Fig. 6B), StAR protein (Fig. 6A), and 3β-HSD (Fig. 6C) mRNA expression in luteal tissue were positively correlated with de novo progesterone production by minced luteal tissue in vitro ($r = 0.74$, 0.72, and 0.64, respectively; $P < 0.001$).

MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNA Expression and MMP-2 Activity in Luteal Tissue

Treatment did not affect MMP-2, MMP-9, and TIMP-2 mRNA expression in luteal tissue ($P > 0.05$, data not shown); however, treatment did affect TIMP-1 mRNA expression ($P < 0.01$), which was lower in luteal tissue from HR+I gilts than from HR and RH gilts (3.6 ± 0.1 vs. 4.1 ± 0.1 and 4.0 ± 0.1 arbitrary units; $P < 0.01$). Time interval did not affect MMP-2, MMP-9, TIMP-1, or TIMP-2 mRNA expression ($P > 0.05$). MMP-2 activity, as assessed by zymography and expressed as arbitrary units, was 2.7 ± 0.3 (n = 7), 3.4 ± 0.3 (n = 7), and 2.6 ± 0.6 (n = 2) for HR, HR+I, and RH gilts, respectively ($P < 0.08$).

DISCUSSION

The method of in vitro culture of luteal tissue appeared to be critical for evaluating luteal function. Although progesterone production and release by minced tissue were different between HR+I and RH groups, these differences were not apparent using cell culture. Furthermore, minced luteal tissue from RH and HR+I groups responded to LH, but dispersed luteal cells from all groups did not. This lack of sensitivity in dispersed cells may be related to the dispersion process itself, because Ascoli and Segaloff [38] re-

ported that after digestion with collagenase, LH receptors may be degraded and cannot be regenerated during short-term culture. Therefore, minced luteal tissue cultured in serum-free medium in vitro appears to provide the most physiologically meaningful assessment of luteal function.

The results of the present study provide convincing evidence for the dynamic changes that occur in porcine luteal function in the immediate postovulatory period. Consistent with the more limited data of Ricke et al. [39] on progesterone content and 3β-HSD protein expression, we observed a rapid increase in progesterone production, progesterone release, and mRNA expression for StAR protein, $P450_{scc}$, and 3β-HSD in HR and RH gilts in the 12- to 24-h period after ovulation. Our observation that progesterone content in luteal tissue was highly correlated with both oviductal and peripheral plasma progesterone concentrations further extends the data of Ricke et al. [39] and suggest that because of the activity of the subovarian countercurrent transfer system in pigs [40], oviductal progesterone concentrations will reflect changes in early luteal function and exert regulatory effects on the oviduct [41]. Although Ricke et al. [39] indicated that luteal progesterone content provides a good index of normal luteal function, our data suggest that progesterone production and release by luteal tissue in vitro are better measures of luteal status. For example, progesterone production and release in tissue recovered from the HR+I group was higher than in HR and RH groups, whereas luteal tissue progesterone content did not differ among the three treatments. Evidence for litter effects on progesterone production and release in vitro indicates the advantage of using littermates as part of well-controlled experiments to study nutritional effects on reproductive function in litter bearing mammals.

Using the same experimental paradigm as in the present study, we have observed lower plasma progesterone concentrations at 48 and 72 h after onset of estrus in HR compared with RH gilts [7]. Although a similar treatment effect was not apparent for the subset of gilts from which we recovered luteal tissue, sequential blood sampling in another subset of cannulated gilts allowed us to describe a more rapid increase in plasma progesterone in both RH and HR+I than in HR gilts [8]. Collectively, these data support the suggestion that differences in plasma progesterone in the immediate postovulatory period may be an important mediator of effects of nutrition and metabolic state on subsequent fertility in the pig [1, 4]. The comprehensive data presented by our laboratory [8] suggest that during feed restriction in HR gilts, factors affecting follicular maturation at the intraovarian level trigger different patterns of steroidogenic activity over the subsequent periovulatory period. From the perspective of the present assessment of luteal function, evidence that peak follicular phase estradiol concentrations and the size of the preovulatory LH surge were higher in RH than in HR gilts was intriguing because evidence from studies in cattle suggests that the degree of LH stimulation during the surge period is quantitatively linked to subsequent luteal activity [42]. As physiological doses of LH increased progesterone production and release by luteal tissue from RH, but not from HR gilts, we suggest that both the higher LH surge in RH gilts and a greater sensitivity to LH stimulation contribute to more rapid luteinization of granulosa cells and the quicker rise in peripheral progesterone concentrations [8].

The lack of any difference in luteal progesterone production in vitro or in expression of mRNA for StAR protein, $P450_{scc}$, 3β-HSD in luteal tissue from HR and RH gilts

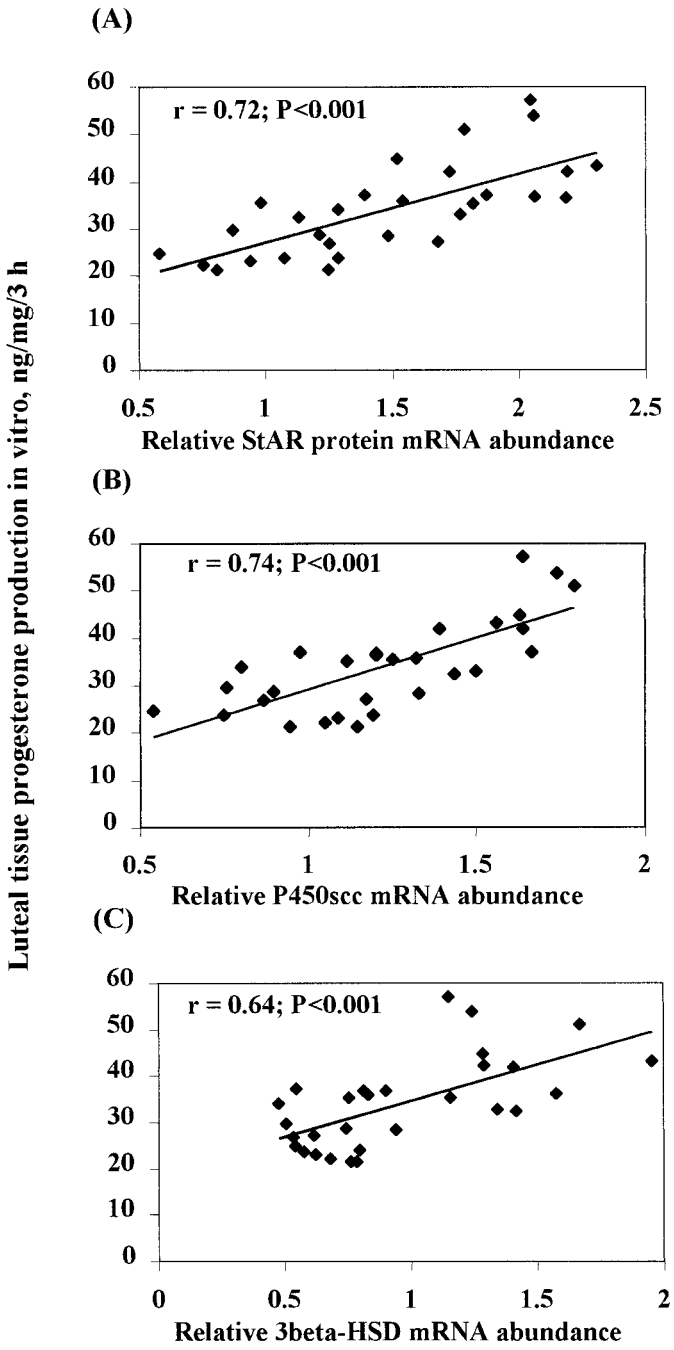

FIG. 6. Correlation between StAR protein (**A**), $P450_{scc}$ (**B**), and 3β-HSD (**C**) mRNA expression in luteal tissue and progesterone production by luteal tissue in vitro (n = 29).

may also relate to the difference in sensitivity to LH, as Yuan et al. [33] demonstrated that administration of recombinant porcine somatotropin decreased luteal function by affecting LH receptor gene expression, but did not affect the mRNA expression for 3β-HSD or $P450_{scc}$. However, from a functional perspective, there was a close association in the present study between progesterone production and mRNA expression for three of the steroidogenic enzymes over the different ovulation-to-surgery time intervals in HR and RH gilts, and progesterone production in vitro was strongly correlated with circulating plasma progesterone concentrations in the gilts used for recovery of luteal tissue. These associations suggest that regulation of gene expres-

sion in differentiating luteal tissue could still be a key mechanism mediating the difference in the rate of increase in plasma progesterone between HR and RH gilts. Our inability to demonstrate treatment-induced differences in gene expression may relate to the relatively small subsample of gilts used for the gene expression studies and the need to impose even stricter timing on the recovery of luteal tissue to accommodate the dynamic changes in luteal function that are occurring in the immediate postovulatory period in pigs.

The current experiment demonstrated key mechanisms that mediate effects of exogenous insulin treatment during follicular maturation on subsequent luteal function, as evidenced by increased progesterone production and release by luteal tissue in vitro, enhanced StAR protein, $P450_{scc}$, and 3β-HSD mRNA expression, and decreased TIMP-1 mRNA expression in luteal tissue from HR+I gilts. Insulin could support follicular development by reducing follicular atresia [23, 24, 43, 44] and by suppressing follicular apoptosis [45]. Other in vivo studies also demonstrated that exogenous insulin treatment for 4 days in gonadotropin-treated prepubertal gilts increased the number of follicles less than 3 mm in size [24], and maintained the 4- to 6-mm size follicle population in cyclic gilts when insulin treatment began on Day 15 of the cycle [23]. In primiparous sows, insulin treatment for 3 days after weaning increased the number of large follicles [19, 20]. Conversely, withdrawal of insulin in streptozotocin-induced-diabetic pigs reduced follicle diameter and estradiol production, and increased atresia [46, 47]. Endocrine data from the same animals used in the present study showed that although postprandial insulin concentrations on Day 15 of the cycle were higher in HR+I gilts than in RH and HR animals, there were no differences in insulin concentrations after Day 16 among the three treatment groups. These data taken together suggest that insulin treatment from Day 8 to Day 15 of the cycle in HR+I gilts most likely enhances follicular development. In turn, this is reflected in the up-regulation of gene expression for key steroidogenic enzymes and enhanced progesterone production in vitro, as reported here, and the increase in ovulation rate reported elsewhere [8]. As combined insulin and FSH treatment acted synergistically to increase the number of LH receptors on porcine granulosa cells in vitro [48], this would explain the high sensitivity of luteal tissue from HR+I gilts to LH stimulation and provide an additional mechanism mediating positive effects of previous insulin treatment on subsequent luteal function. Finally, we believe our data are the first to demonstrate that previous insulin treatment produces subsequent effects on TIMP-1 mRNA expression and possibly MMP-2 activity in luteal tissue. An increase in MMP-2 activity could, in turn, increase availability of IGF-I following IGF binding protein proteolysis [49] and thus enhance progesterone production in HR+I gilts [14]. Very similar mechanisms have been suggested by Driancourt et al. [18] as possible mediators of enhanced progesterone production during luteinization in the high prolific Chinese Meishan pigs.

ACKNOWLEDGMENTS

We thank Dr. F. Labrie of Laval University, Drs. D. Silversides and B. Murphy of Université de Montreal for the gifts of porcine 3β-HSD, StAR, and $P450_{scc}$ probes; NIDDK's National Hormone and Pituitary Program and Dr. A.F. Parlow from Harbor-UCLA Medical Center for the gift of pLH; Dr. D. Balcerzak of the University of Alberta for the gift of MMP-2, TIMP-1, and TIMP-2 primers and technical assistance with RT-PCR and zymography; and Dr. M.G. Hunter of University of Nottingham for the tissue/cell culture protocol. We thank Shirley Shostak and Rose O'Donoghue for technical support with RIA and in vitro culture; and Dr. P. Blenis for assistance with statistical analysis of data. We also acknowledge the University of Alberta Swine Unit staff for taking care of the animals and Metabolic Unit staff for assistance with surgery.

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