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Temporal Release, Paracrine and Endocrine Actions of Ovine Conceptus-Derived Interferon-Tau During Early Pregnancy[1]

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ABSTRACT

The antiviral activity of interferon (IFN) increases in uterine vein serum (UVS) during early pregnancy in sheep. This antiviral activity in UVS collected on Day 15 of pregnancy is blocked by anti-IFN-tau (anti-IFNT) antibodies. Conceptus-derived IFNT was hypothesized to induce IFN-stimulated gene (ISG) expression in endometrium and extrauterine tissues during pregnancy. To test this hypothesis, blood was collected from ewes on Days 12–16 of the estrous cycle or pregnancy. Serum progesterone was >1.7 ng/ml in pregnant (P) and nonpregnant (NP) ewes until Day 13, then declined to <0.6 ng/ml by Day 15 in NP ewes. A validated IFNT radioimmunoassay detected IFNT in uterine flushings (UFs) on Days 13–16 and in UVS on Days 15–16 of pregnancy. IFNT detection in UF correlated with paracrine induction of ISGs in the endometrium and occurred prior to the inhibition of estrogen receptor 1 and oxytocin receptor expression in uterine epithelia on Day 14 of pregnancy. Induction of ISG mRNAs in corpus luteum (CL) and liver tissue occurred by Day 14 and in peripheral blood mononuclear cells by Day 15 in P ewes. Expression of mRNAs for IFN signal transducers and ISGs were greater in the CL of P than that of NP ewes on Day 14. It is concluded that: 1) paracrine actions of IFNT coincide with detection of IFNT in UF; 2) endocrine action of IFNT ensues through induction of ISGs in peripheral tissues; and 3) IFNT can be detected in UVS, but not until Days 15–16 of pregnancy, which may be limited by the sensitivity of the IFNT radioimmunoassay.

conceptus, endocrine, interferon, pregnancy, radioimmunoassay

INTRODUCTION

Maternal recognition of pregnancy in ruminants requires elongation of the conceptus and coincides with production of interferon-tau (IFNT) [1–3]. The ovine conceptus secretes IFNT from Day 10 to Days 21–25, with the greatest release occurring on Days 14–16 of pregnancy (reviewed in Roberts et al. [4]). Herein, we determined the temporal pattern of IFNT and IFN-stimulated gene (ISG) expression during consecutive days in various tissue types, demonstrating the paracrine and endocrine actions of IFNT. IFNT is a major product of ovine and bovine concepti during the peri-implantation period of pregnancy. Paracrine action of IFNT on the endometrial luminal and superficial glandular epithelia inhibits the upregulation of estrogen receptor 1 (ESR1) and oxytocin receptor (OXTR), thereby preventing the oxytocin-mediated pulsatile release of luteolytic prostaglandin F2 alpha (PGF) and lysis of the corpus luteum (CL) [5]. In addition, IFNT has recently been reported to function through endocrine action on the ovine CL [6].

IFNT binds to type 1 interferon receptors (IFNR1 and IFNR2) and activates the Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway [7]. The JAK/STAT pathway includes downstream mediators, such as the STATS (1 and 2), interferon regulatory factors (IRFs), and ISGs. The mechanism by which IFNT mediates maternal recognition of pregnancy is hypothesized to occur through the increased expression of several ISGs in the uterus [8–10], such as ISG15 [11], interferon induced with Helicase C Domain (IFIH1; also known as MDA5) [12], and DEAD (Asp-Glu-Ala-Asp) Box Polypeptide-58 (*DDX58*; formerly known as *RIGI*) [13], and through expression of IRF2, a potent transcriptional repressor that silences expression of ESR1 and, in turn, OXTR [3, 6]. Additionally, pregnancy factors in sheep induce expression of ISGs in several extrauterine tissues, such as the CL [6, 14]. ISG15, first named ubiquitin cross-reactive protein because of its cross-reactivity with antibody against ubiquitin [15, 16], is increased in mouse [17] and human [18] endometria in response to pregnancy. ISG15 is induced by type I IFN and becomes conjugated to intracellular proteins [19] in a mechanism parallel to, but different from, that described for ubiquitin [20].

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In a recent study, we evaluated expression of ISGs in extrauterine tissues during early pregnancy in sheep [6]. Expression of ISG15 was greater in large luteal cells (LLCs) from Day 15 pregnant (P) compared with LLCs from Day 15 nonpregnant (NP) sheep using semiquantitative real-time PCR, Western blot analysis, and immunohistochemistry. An antiviral assay detected more type I IFN bioactivity in the serum from Day 15 P compared with NP ewes [6]. Uterine vein serum (UVS) samples from Day 15 of pregnancy, which had been preadsorbed with anti-IFNT antibody, had diminished antiviral activity, confirming that IFNT was the type I IFN released into the uterine vein on Day 15 of pregnancy [14]. Thus, endocrine IFNT signaling occurred as early as Day 14 of pregnancy in sheep. To test this hypothesis, a sensitive and specific radioimmunoassay was developed and used to examine concentrations of IFNT in uterine flushing (UF) and UVS.

Because IFNT was detected in UF and UVS, these studies also examined temporal events following detection of IFNT in UF in the context of the regulation of gene expression in endometria, CL, and liver. Some of the well-characterized endometrial responses to IFNT, such as preventing upregulation and transcription of *ESR1* and *OXTR* mRNA, were also examined in the CL [21]. Previously published experiments have focused on temporal relationships between paracrine effects of the conceptus on the endometrium, development of antiluteolytic responses, and endocrine induction of genes in the CL on Days 12, 13, 14, and 15, the critical period of maternal recognition of pregnancy in the ewe [22–24]; however, these studies have not examined UF and uterine vein concentrations of IFNT during consecutive days of pregnancy. The aims of this study were: 1) to develop a specific and sensitive radioimmunoassay (RIA) for IFNT in order to detect IFNT in UF and UVS, and 2) examine the temporal relationships between concentrations of IFNT in blood; expression of ISG15, ESR1, and OXTR in the endometrium; and IFNT signaling in the CL.

MATERIALS AND METHODS

Experimental Design: Day of Estrous Cycle and Early Pregnancy

The Colorado State University Animal Care and Use Committee approved all experimental procedures with animals. Mature crossbred ewes were observed daily for estrus using a caudoepididectomized ram. On the day of standing estrus (Day 0), half of the ewes were bred by an intact ram. The NP ewes were exposed to a caudoepididectomized ram. Groups were assigned according to pregnancy status (NP and P) and days after detection of estrus. On Days 12, 13, 14, and 15 of either the estrous cycle or pregnancy (12NP = 5; 12P = 4; 13NP = 5; 13P = 5; 14NP = 5; 14P = 3; 15NP = 6; 15P = 4; and 16P = 4), three to six ewes were anesthetized, and blood samples were taken from the jugular vein and a uterine vein to provide serum. Serum from UVS was collected, and then ewes were killed to allow for the collection of lymph nodes (iliac and submandibular), CL, endometrium, uterine vein (tissue), and liver. Uterine fluid was collected by flushing the uterus with physiological saline after killing. Tissues were snap frozen in liquid nitrogen and stored at −80°C for later processing. Pregnancy was confirmed by the presence of a morphologically normal conceptus (normalcy: embryos that were translucent, long, and not collapsible).

Progesterone Assay

Concentrations of progesterone in serum were determined by RIA as previously described [25] in two separate assays. The average sensitivity of the assays was 14.7 pg/ml; the intra-assay coefficient of variation was 5.83% for assay one and 3.67% for assay two. The interassay coefficient of variation between the two assays was 3.86%.

IFNT Radioimmunoassay

Radioiodination of recombinant ovine (ro) IFNT with ^{125}I was completed using the chloramine T procedure and purified using column chromatography (Sephadex G25; GE Health Care). These methods have been described previously by Niswender et al. [26]. Briefly, UF samples were diluted 1:50 in 0.1% PBS gel for analysis in the RIA. If IFNT was undetectable in the diluted samples in the first assay, the undiluted samples were analyzed in a second assay. Anti-roIFNT antibody (1:100 000 dilution; generated by coauthor F.W.W.) was added to 200 μl of UF samples, thoroughly mixed, and incubated at 4°C for 24 h. Radioactive ^{125}I-labeled roIFNT was added (100 μl, 50 000 counts), mixed, and incubated for 24 h at 4°C. This was followed by an incubation at 4°C for 72 h with secondary anti-rabbit antibody (1:25 dilution; generated at Colorado State University). The assay was terminated by the addition of 3 ml of cold PBS and centrifugation at 2800 rpm for 30 min. The supernatant was removed and the radioactivity in the pellet was determined using a gamma counter (Apex automatic gamma counter; ICN Micromedic Systems). Specificity of the assay was evaluated by measuring the cross-reactivity of other closely related ovine interferon molecules (α, β, and γ). Parallelism of IFNT in UF to the standard curve was demonstrated by assaying multiple dilutions of the UF. Sensitivity was optimized by choosing a dilution of antiserum that bound ~20% of the radioiodinated IFNT and by using a nonsequential incubation in which antibody was allowed to react with standard or sample prior to the addition of radioiodinated IFNT. Sensitivity was defined as that amount of IFNT that inhibited binding of radioiodinated IFNT to a degree that was significantly different from B_o. Intra-assay and interassay coefficients of variation were determined by measuring quality control samples that inhibited binding of radioiodinated IFNT by ~20%, 50%, and 75%. This RIA was optimized for the detection of roIFNT in UF samples at a sensitivity of 0.1 ng/ml and a range of detection of 0.1 to 13 ng/ml. The intra-assay coefficient of variation was 6.2% and the interassay coefficient of variation was 4.0%.

RNA Isolation and RT-PCR

Extraction of RNA from tissues (endometrium, uterine vein, lymph nodes, CL, and liver) and blood was performed using TRIZOL and TRI BD reagent (MRC), respectively. After extraction, RNA was treated with DNase I (Qiagen) and total RNA was purified using RNeasy MinElute Cleanup Kit (Qiagen). Single-stranded cDNA was synthesized from 1 μg of total RNA using an iScript cDNA Synthesis Kit (Bio-Rad). Reactions were completed using a final volume of 10 μl per well using iQ Supermix Bio-Rad with cDNA (2 μl) as template. Amplification was performed at 95°C for 30 sec, 62°C for 30 sec, and 72°C for 15 sec for 40 cycles. The primers used in this study for the target genes are listed in Table 1. All primers were designed to have single-product melting curves, as well as consistent amplification efficiencies between 1.8 and 2.2 [27]. All amplicons were verified by sequencing.

Statistical Analysis

All statistical analyses were performed using an SAS software package (SAS Institute Inc.). Data are presented as mean ± SEM, unless otherwise stated. A probability value of $P \leq 0.05$ was considered significant. Main effects of treatment day, pregnancy status, and their interaction were analyzed by two-way ANOVA with a Tukey adjustment using the general linear models in continuous data; pregnancy data that included Day 16P were further analyzed using one-way ANOVA and a Tukey range test to examine the main effect of pregnancy. GAPDH was used as the primary housekeeping gene to normalize each targeted mRNA amplification by using the ΔCT values [27, 28]. *GAPDH*, *POLR2A*, *RPL19*, *RN18s*, and the geometric mean of all four housekeeping genes were tested, which provided similar data. Each targeted CT value was analyzed, and relative expression of RT-PCR products was presented using mean $2^{-\Delta CT}$ values calculated for each group.

RESULTS

Progesterone Profile of Mid-Late Estrous Cycle and Early Pregnancy

Concentrations of progesterone in serum were not affected by pregnancy status on Days 12 and 13. Serum progesterone declined from Day 13 to Day 14, and by Day 15 concentrations were less than 1 ng/ml in NP ewes, whereas in P ewes concentrations of serum progesterone stabilized to >2 ng/ml (Fig. 1).

TABLE 1. Oligonucleotide primer sequences used for semi-quantitative RT-PCR.

Target	Accession no.	Primer sequences[a]
ISG15	NM_174336	F: ggtatccgagctgaagcagtt R: acctccctgctgtcaaggt
ESR1	AY033393	F: gttgcatggctgtagcagaa R: caaatttgaaggggcagtgt
OXTR	AF101724	F: ggcagaatttacggctcaag R: gatgagcttgacgttgctga
GAPDH	AV610889	F: tgacccttcattgaccttc R: cgttctctgccttgactgtg
POLR2A	XM_004013289.1	F: agtccaacatgctgaaggacatga R: agccaagtgccggtaattgacgta
RPL19	XM_004012837.1	F: tcgccggaagggcaggcata R: ggctgtgatacatgtgggggtc
RNA18s	AY753190.1	F: gaggccctgtaattagaatgag R: gcagcaactttaatatacgctattgg
STAT1	NM_001166203.1	F: ccaccgaacttacccagaa R: accaattttgcagctgatcc
STAT2	XM_004006580.1	F: agaaccttgacagcccgtttt R: caagtgctgtcgaacatcca
IRF3	NM_001029845.2	F: ggcttgtgatggtcaaggtt R: tgcaggtcgacagtgttctc
IRF7	NM_001105040.1	F: gcctcctggaaaaccaactt R: ccttatgagggtcggtaggg
IRF9	XM_004010311.1	F: ctgggtggtagagcaagtgg R: gccttctgagttcccctcct
IFIH1	XM_004004655.1	F: cttgccttctccatcgtttg R: ctgcaaccagaagcttgtcc
DDX58	XM_004005323.1	F: cagcaagatcctggaccta R: tttttctcggcctgaatacg
IFNAR1	NM_001009748.1	F: ctcagattggtcccccagac R: cttctgttccaggggggagag
IFNAR2	NM_001009342.1	F: tcccaggataccaagtgaagaa R: atttggggttgatgcctctt

[a] F, forward; R, reverse.

RIA Detection of IFNT in UF Samples and UVS

Specificity of the IFNT RIA was tested against other IFNs, including IFN α, β, and γ. No cross-reactivity of the anti-IFNT antibody with similar (type I) or distinct (type II) IFNs was detected (Fig. 2A). Dilutions of primary antibody resulted in improvement of sensitivity with some loss in range of detection (Fig. 2B). A 1:100 000 dilution of primary antibody was determined as optimal for achieving the greatest sensitivity in the assay (Fig. 2B). The UFs were collected from NP ewes on Days 12, 13, 14, and 15 and served as a negative control for this study. Two standard IFNT dose-response curves were analyzed within each assay. The UFs from Day 16 P ewes (n = 3) were diluted in assay buffer and used to demonstrate that detection of native IFNT in UFs was parallel to detection of roIFNT in the standards (Fig. 2C). IFNT was not detected in UFs or UVS from NP ewes (Fig. 3). However, IFNT was detectable as early as Day 13 of pregnancy in UF (Fig. 3A) and increased in concentration by Day 14, which continued through Day 16 of pregnancy. IFNT also was present in concentrations that could be accurately quantified for detectable in UVS from 7 of 10 ewes on Day 15 or 16 of pregnancy (Fig. 3B). The range of these assays was from 54 pg/ml to 16.5 ng/ml in UF, and 126.5 pg/ml to 12 ng/ml in UVS. The limit of detection was 39 pg/ml in UF and 148 pg/ml in UVS. The intra-assay coefficients of variation for the two assays were 3.7% for UF and 2.6% for UVS.

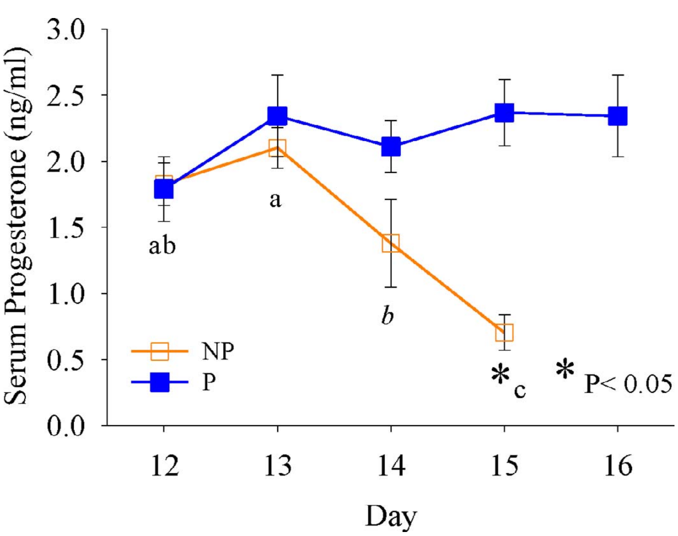

FIG. 1. Concentration of serum progesterone in NP and P ewes during the estrous cycle or early pregnancy. *Means differ between P and NP groups on specific days ($P < 0.05$). Letters indicate difference between days. Italicized letters indicate tendencies between days ($P < 0.1$).

Messenger RNA Profiles of ISG15*,* ESR1*, and* OXTR *in the Endometrium*

Concentrations of *ISG15* mRNA were very low in endometria from NP ewes but were increased in P versus NP ewes by Day 13 and remained greater in P ewes through Day 15 (Fig. 4A). Endometrial expression of *ESR1* (Fig. 4B) and *OXTR* (Fig. 4C) mRNAs were greater in NP compared with P ewes by Day 14. The concentrations of *ESR1* mRNA increased dramatically from Days 13 to 14 and remained greater on Day 15 in NP compared with P ewes. *OXTR*

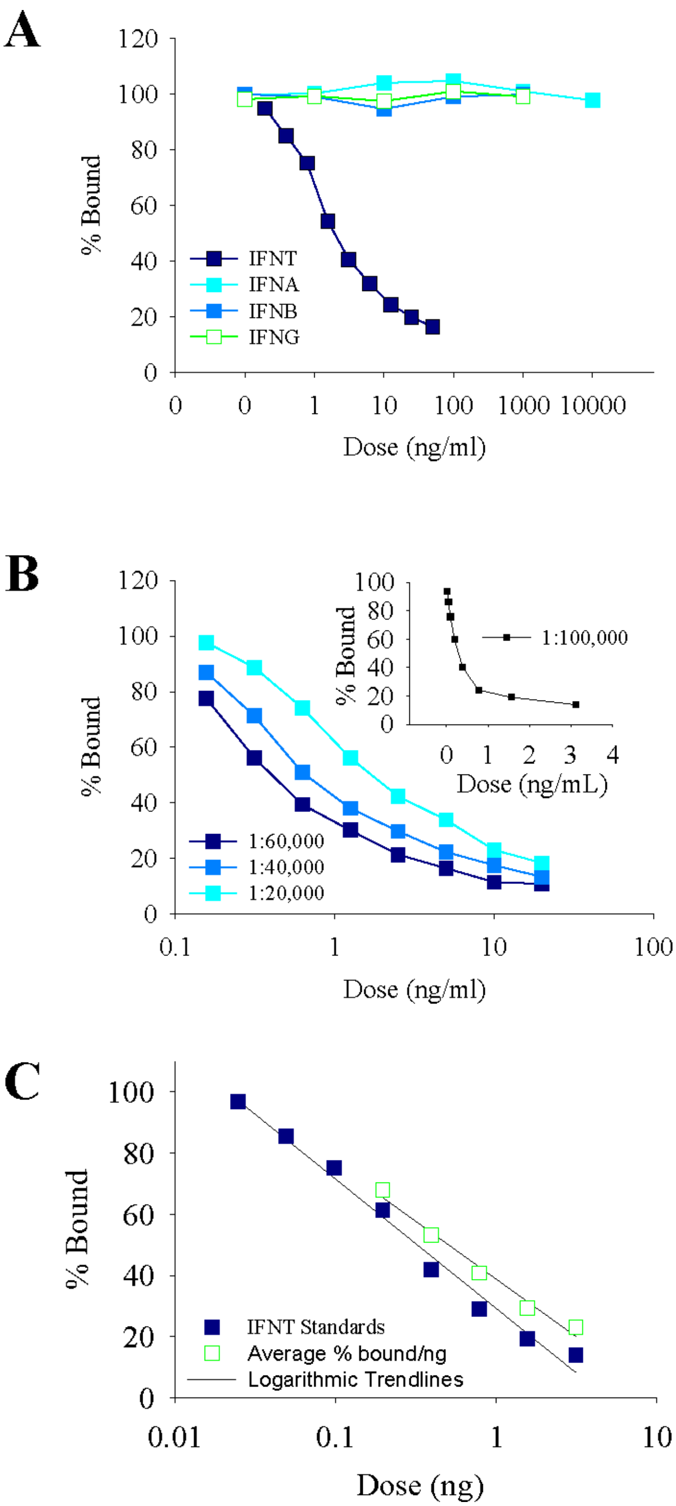

FIG. 2. IFNT RIA. **A**) Specificity: competitive RIA testing IFNT against other type I (α and β) and type II (γ) IFN. **B**) Sensitivity: RIA testing different antibody concentrations. **C**) Parallelism: demonstrating that detection of native IFNT in UFs was parallel to detection of roIFNT in the standards.

mRNA concentration increased in NP compared with P ewes on Day 14, which continued through Day 15 of the estrous cycle. This increase in *OXTR* mRNA concentration in NP ewes was completely blocked in response to pregnancy.

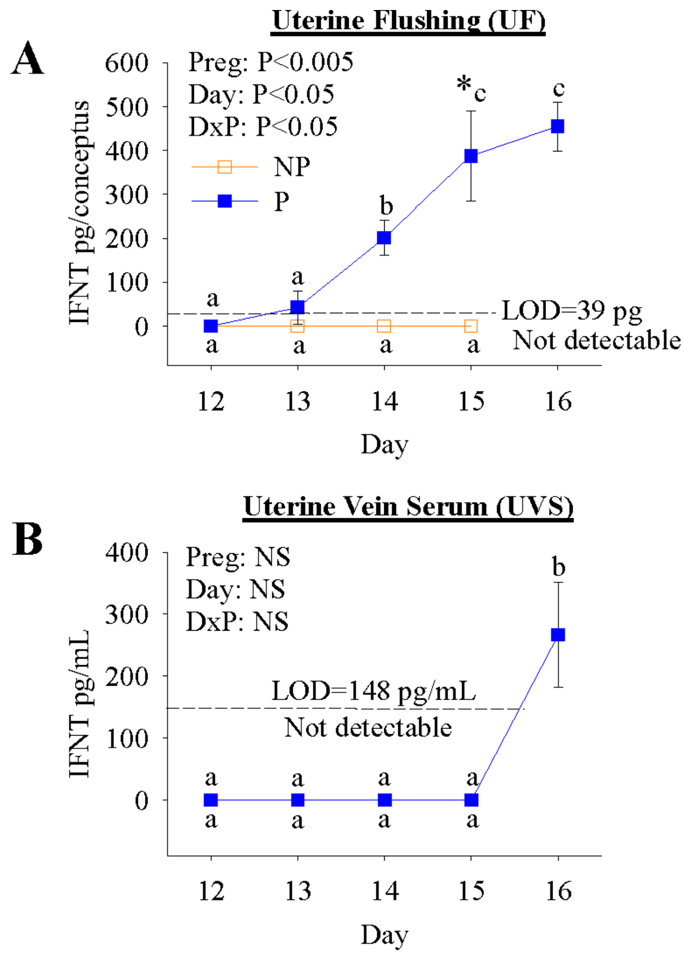

FIG. 3. Detection of IFNT in UF and uterine vein using RIA. **A**) Detection of IFNT in UFs from Days 12–16 NP and P ewes. **B**) Detection of IFNT in UVS from Days 12–15 NP and P ewes. *Means differ between P and NP groups on specific days. Letters indicate difference between days for NP comparison only or P comparison only. NS, not significant.

ISG15 *mRNA Profiles in Tissues Peripheral to Endometrium*

In the uterine vein tissue, there was no main effect of pregnancy status or day on concentration of *ISG15* mRNA (i.e., Day 15 NP: 0.29 ± 0.28; Day 15 P: 0.39 ± 0.33; values are mean ± SEM; $2^{-\Delta CT}$). ISG15 mRNA was present in iliac (0.21 ± 0.09; $2^{-\Delta CT}$) and submandibular (0.13 ± 0.09; $2^{-\Delta CT}$) lymph nodes collected on Day 15, but it did not differ in concentration by pregnancy status or by anatomic location (overall means presented). Concentrations of *ISG15* mRNA in peripheral blood mononuclear cells (PBMCs) from uterine vein (Fig. 5A) and jugular vein (Fig. 5B) blood were detectable but very low in NP ewes. In contrast, uterine vein and jugular vein ISG15 mRNA concentrations increased in PBMCs in response to pregnancy on Day 15. Concentrations of *ISG15* mRNA in liver were similar during the estrous cycle, but they were increased in P compared with NP ewes by Day 14, which continued through Day 15 (day × pregnancy status interaction; $P < 0.05$; Fig. 5C).

Endocrine Actions of IFNT in the CL

Interferon receptor 1 (*IFNAR1*) and Interferon receptor 2 (*IFNAR2*) mRNAs were present in CL during the estrous cycle and early pregnancy (data not shown), but neither of the mRNA-encoding subunits of the IFNARs were affected by

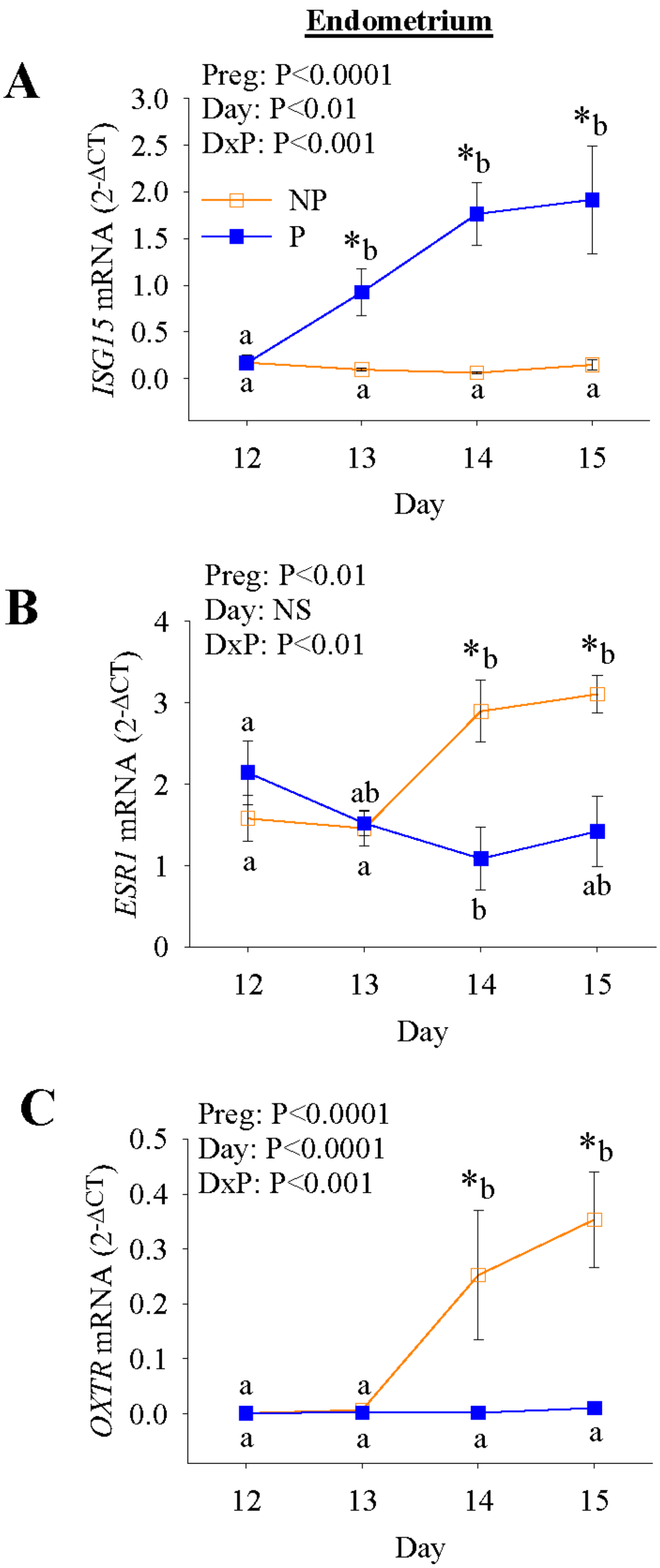

FIG. 4. *ISG15*, *ESR1*, and *OXTR* mRNA in the endometrium during estrous and early pregnancy. Relative expression ($2^{-\Delta CT}$) of mRNA for *ISG15* (**A**), *ESR1* (**B**), and *OXTR* (**C**; amplified inlay of Days 12–14 for better visualization) in the endometrium, on Days 12–14 and Day 15 of the estrous cycle or early pregnancy in sheep relative to the housekeeping gene GAPDH. Data are presented as mean ±SEM. *Means differ between P and NP groups on specific days ($P < 0.05$). Letters indicate difference between days for NP comparison only or P comparison only. NS, not significant.

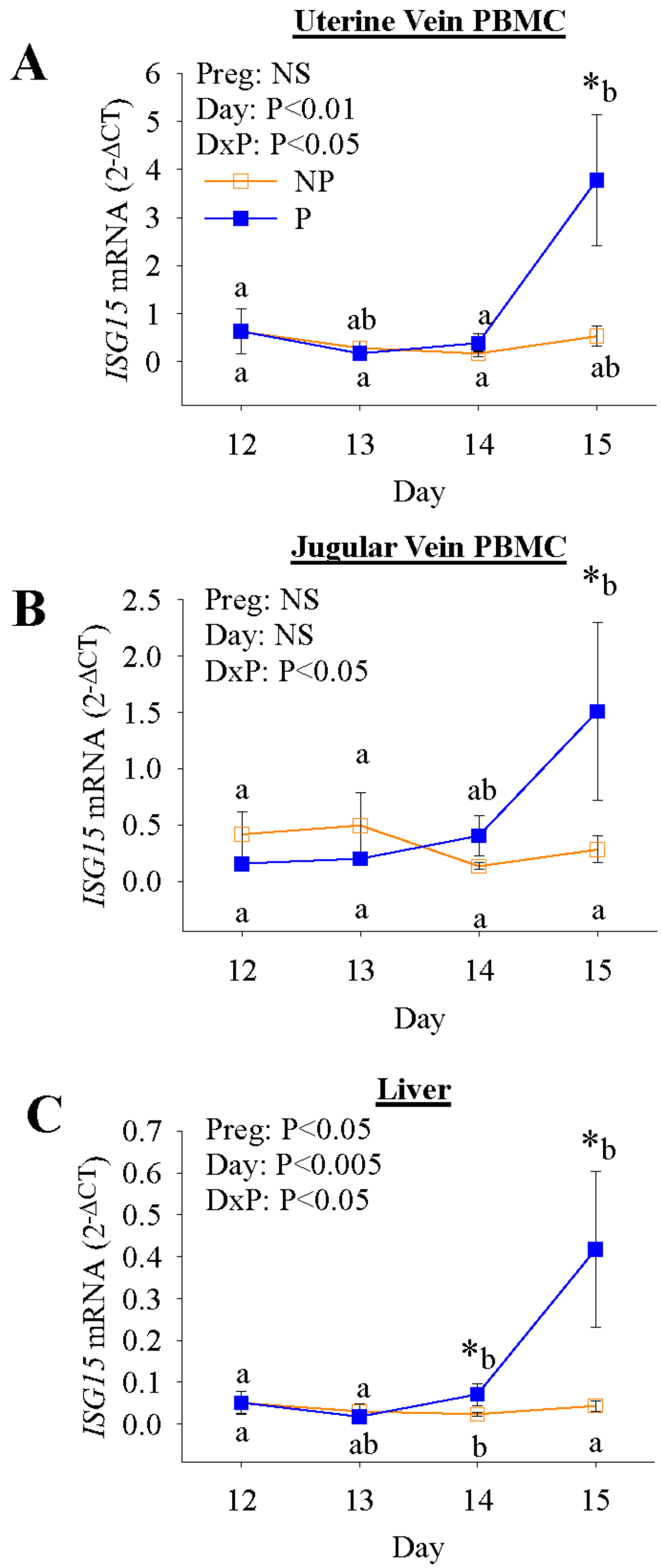

FIG. 5. *ISG15* mRNA in liver and PBMCs isolated from uterine and jugular vein blood. Relative expression ($2^{-\Delta CT}$) of mRNA for *ISG15* in uterine vein blood (**A**), jugular vein blood (**B**), and liver (**C**) on Days 12–14 and Day 15 of the estrous cycle or early pregnancy in sheep relative to the mean of housekeeping gene *GAPDH*. Data are presented as mean ± SEM. *Means differ ($P < 0.05$) between P and NP groups on specific days ($P < 0.05$). Letters indicate difference between days for NP comparison only or P comparison only. NS, not significant.

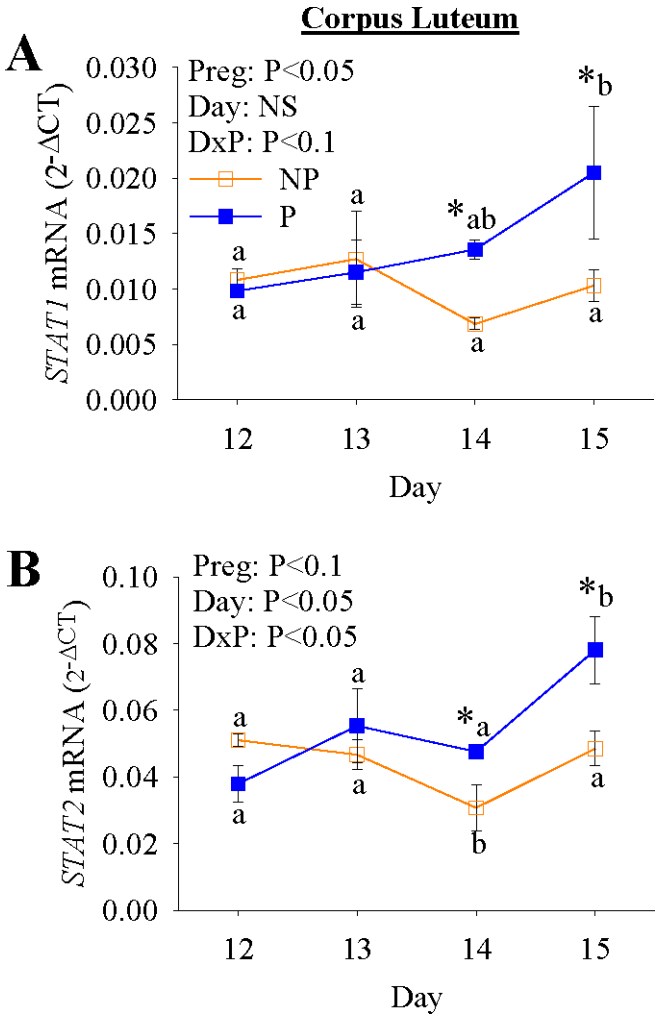

FIG. 6. IFN signaling in the CL. Relative expression to GAPDH mean ($2^{-\Delta CT}$) of mRNA for *STAT1* (**A**) and *STAT2* (**B**) on Days 12–14 and Day 15 of the estrous cycle or early pregnancy in sheep. Data are presented as mean ± SEM. *Means differ ($P < 0.05$) between P and NP groups on specific days ($P < 0.05$). Letters indicate difference between days for NP comparison only or P comparison only. NS, not significant.

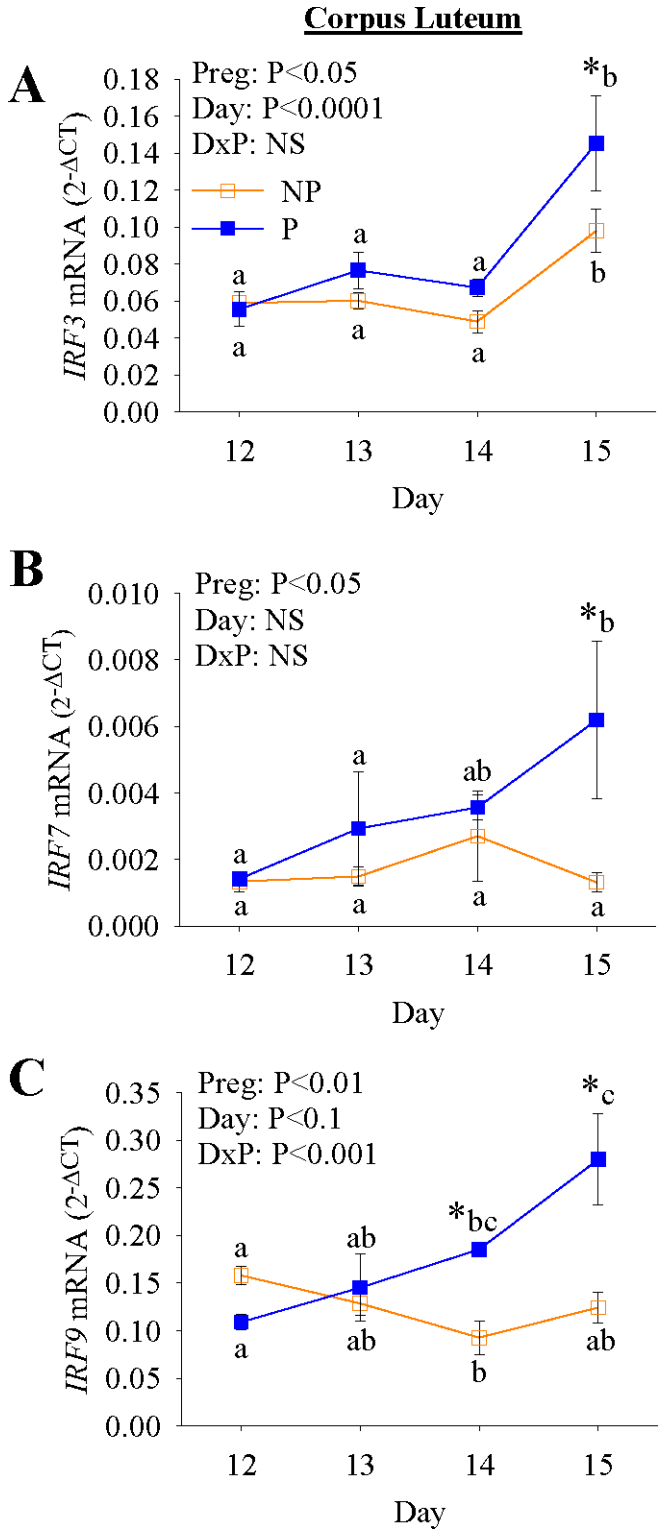

FIG. 7. IFN signaling in the CL. Relative expression to GAPDH mean ($2^{-\Delta CT}$) of mRNA for *IRF3* (**A**), *IRF7* (**B**), and *IRF9* (**C**) on Days 12–14 and Day 15 of the estrous cycle or early pregnancy in sheep. Data are presented as mean ± SEM. *Means differ ($P < 0.05$). *Means differ between P and NP groups on specific days. Letters indicate difference between days for NP comparison only or P comparison only. NS, not significant.

pregnancy status or day. However, signaling via the IFN receptors was upregulated in the CL during pregnancy, as evidenced by increased *STAT1* and *STAT2* mRNA concentrations on Days 14–15 in P compared with NP ewes (Fig. 6). *IRF3* mRNA concentration increased in CL in response to pregnancy and as days progressed from Days 12–14 to Day 15 (Fig. 7A). *IRF7* mRNA concentration was upregulated in CL by Day 15 of pregnancy, and IRF9 mRNA concentrations were upregulated by Day 14 and remained high on Day 15 of pregnancy (Fig. 7, B and C) compared with the estrous cycle. Concentrations of *DDX58* and *IFH1* mRNAs were greater in CL from P compared with NP ewes by Day 14 of pregnancy and remained at these high levels through Day 15 of pregnancy, which coincided with the significant increase in ISG15 mRNA concentrations (Fig. 8).

DISCUSSION

The primary paracrine role of IFNT during early pregnancy is its antiluteolytic effect abrogating the mechanism for pulsatile release of PGF from the uterine epithelia. IFNT is produced by the ruminant conceptus and for the last three decades was thought to have only paracrine functions through binding to receptors in the endometrium [1, 29]. This conclusion was based primarily on a lack of detection of antiviral activity in peripheral blood and lymph node using a

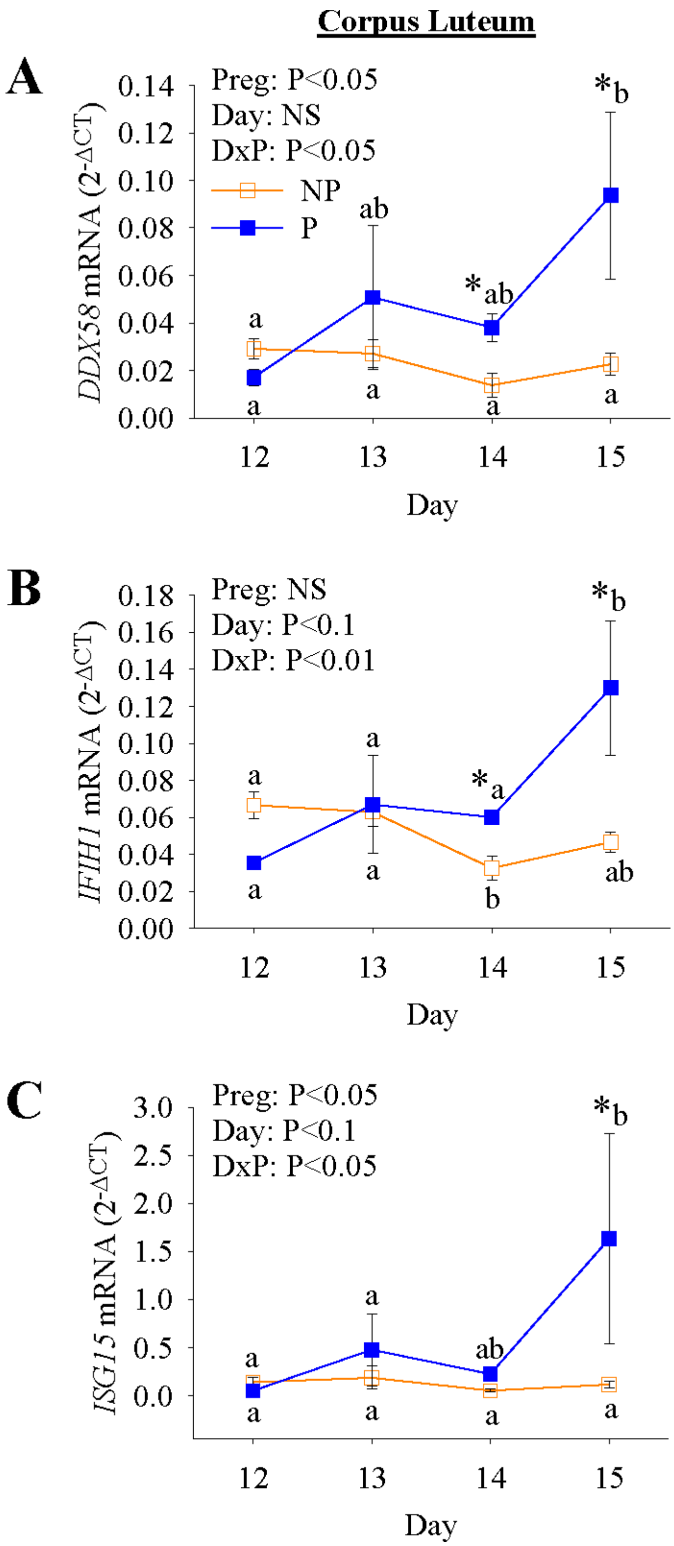

FIG. 8. IFN signaling in the CL. Relative expression to GAPDH mean ($2^{-\Delta CT}$) of mRNA for *DDX58* (**A**), *IFIHI* (**B**), and *ISG15* (**C**) on Days 12–14 and Day 15 of the estrous cycle or early pregnancy in sheep. Data are presented as mean ± SEM. *Means differ ($P < 0.05$) between P and NP groups on specific days. Letters indicate difference between days for NP comparison only or P comparison only. NS, not significant.

bioassay for IFN, and on a lack of detection of IFNT in blood using antibody-based detection methods. Increased antiviral activity during pregnancy has not been observed in systemic blood collected from ruminants during early pregnancy. However, one report in 1991 [30] described an antiviral assay with a sensitivity of ~1 U/ml that could not detect IFNT in jugular vein serum but was efficacious in detecting 58 U/ml in UVS from sheep on Day 15 of pregnancy. The 58 U/ml antiviral activity is equivalent to 72.5 pg/ml based on the 8×10^8 U/mg IFN standard used in that study. The conclusion that IFNT was not detectable in jugular vein serum was based on a lack of detection of IFNT by using either ELISA or RIA. For example, an RIA for IFNT with sensitivity of detecting 6.1–7.8 ng of IFNT per milliliter was developed by Takahashi et al. [31]. The authors reported a sensitivity of 6.1 ng/ml, but the lowest standard used in the assay was 7.8 ng, with binding of ~95%. This group used the RIA to describe detection of IFNT in UF from Day 16 of bovine pregnancy but did not report attempting to detect IFNT in serum from these cows.

A more recent study by our group also found detectable antiviral activity in UVS from Day 15 P sheep, without detection of antiviral activity in systemic blood [6]. We demonstrated that the antiviral activity was due to IFNT and not due to other type I IFNs, because the antiviral activity was blocked by preadsorbing UVS with an antibody against IFNT [14]. The amount of IFNT in UVS on Day 15 of pregnancy was ~500–1000 U/ml, which we estimated to be 5–10 ng/ml using a 1×10^8 U/mg IFN standard [6]; however, this method of estimating IFNT concentration in UVS was dependent on the viral titer, number of cells plated, and range of detection, which can vary based on IFN standard and virus used in the antiviral assay. Consequently, there may be room for improvement for these bioassays in context of specificity and accuracy.

Indirect evidence that IFNT might be released into the uterine vein to exert a systemic-endocrine role during pregnancy was the determination that ISGs were upregulated in PBMCs in response to pregnancy [6, 32, 33]. Based on these studies, it was concluded that IFNT produced by the conceptus during early stages of pregnancy attenuated PGF release from the endometrium, and it was released into the uterine vein in concentrations adequate to have functional and biological effects on peripheral tissues, such as blood cells, the CL, and liver [6, 14, 34]. However, until the experiments described herein were completed, IFNT had never been directly detected in systemic blood during early pregnancy in ruminants.

A double-antibody RIA for IFNT is described herein using roIFNT and anti-roIFNT antibody. The RIA was performed using methods described previously by Niswender et al. [26]. Specificity of the RIA for IFNT was demonstrated by a lack of competitive binding of up to 10 µg/ml related type I and II IFNs. Sensitivity of this IFNT RIA was improved through increasing dilutions of the primary antibody. The amount of ligand required to displace 50% binding decreased from ~1.5 to 0.4 ng with increasing dilutions of primary anti-roIFNT antibody to 1:100 000. Parallelism was confirmed through demonstrating that serial dilutions of roIFNT competed with similar dilutions of ovine UFs containing native IFNT in the RIA.

Although this assay is more sensitive compared with other published IFNT assays [31, 35, 36] and detected IFNT in ovine UFs from Days 13–16 of pregnancy and in UVS from Days 15–16 of pregnancy, it was not able to detect IFNT in jugular vein serum in sheep. The sensitivity of the assays for IFNT in ovine UF was 39 pg/ml, compared with 148 pg/ml in serum. Reduced sensitivity of the IFNT RIA in serum compared with UF may be due to a serum matrix effect. These serum factors

may interact with the antigen or antibodies and impact the dynamics and sensitivity of this assay. The nature of this serum matrix effect is currently under investigation.

IFNT was first reported in 1982 by Godkin et al. [37], who quantified concentrations (50–100 μg) of IFNT derived from the trophoblast cells of Day 14 and Day 16 conceptuses using a Lowry assay. Since this initial discovery, there have been several attempts to develop highly sensitive methods for the detection of IFNT. In 1988, the first RIA for IFNT was validated and was used to quantify IFNT in culture media as well as UF [36]. Using this assay, Davis and collaborators [35] quantified IFNT in UF at concentrations of 117-164 μg per conceptus for Day 16 pregnant ewes. More recently, Takahashi et al. [31] developed a new RIA for IFNT and detected bovine IFNT at concentrations of 11.7–15.7 μg in Day 16 UF. By increasing the primary antibody dilution, we have increased the sensitivity of the RIA to 39 pg/ml in UF and 148 pg/ml in serum. This RIA was used herein to demonstrate the initial day of detection (within the constraints of this assay) of IFNT in the context of temporal paracrine and endocrine induction of downstream ISGs. The detection of IFNT in UF on Day 14 corresponds with changes in pregnancy-associated gene expression.

During early pregnancy, the first response to release of IFNT by a conceptus is the induction of ISGs, specifically ISG15 in the endometrium. This paracrine action of the conceptus occurs between Days 12 and 13 of pregnancy, which is 1 day prior to the decline in serum progesterone between Days 13 and 14 in NP ewes ([38] and reviewed in McCracken et al. [39]). The paracrine action coincides with the initial detection of IFNT by RIA in UF on Day 13 and in UVS of Days 15 and 16 of pregnancy. It also temporally corresponds with the time of upregulation of *ESR1* and *OXTR* mRNAs in the endometrium of NP ewes, which results in luteolytic pulses of PGF. Upregulation of *ISG15* mRNA in response to pregnancy has been described for sheep and cattle [22, 40]. In pregnant cows, Austin et al. [40] reported that endometrial ISG15 upregulation occurs as early as Day 17 and is maintained until Day 45. On Day 11 of pregnancy in sheep, concentrations of *ISG15* mRNA are low, and then there is a significant increase by Day 15. However, no time points were analyzed between Days 11 and 15 to clarify when this response to IFNT occurred [23]. In the present study, we analyzed expression of *ISG15* mRNA daily from Days 12 to 15 and were able to determine greater expression of *ISG15* mRNA in P compared with NP ewes in endometrium between Days 12 and 13 after onset of estrus, which is consistent with the greatest temporal increase in release of IFNT from the ovine conceptus [41].

Endometrial expression of ESR1 is high on Day 1, decreases on Days 2–11, and then increases between Days 11 and 15 of the estrous cycle [24]. In P ewes, upregulation of ESR1 is blocked between Days 11 and 15 [42], which is confirmed in our experiments, where expression of ESR1 did not increase between Days 12 and 15 of pregnancy. Lower concentrations of *ESR1* and *OXTR* in ovine endometrium in Day 15 for P compared with cyclic ewes have been described previously by Spencer and Bazer [5]. Spencer and Bazer [5] also reported that IFNT suppressed transcription of ESR1 and OXTR genes in ovine uterine epithelia, which prevents a pulsatile pattern of PGF, thus abrogating the luteolytic effect of PGF.

Recently, ISGs, such as Mx [43] and ISG15 [32], have been detected in tissues other than the uterus in response to early pregnancy. *Mx* mRNA concentrations were not different on Days 0, 9, and 12, but they increased beginning on Day 15 of pregnancy compared with the estrous cycle [43]. Han and collaborators [32] examined concentrations of *ISG15* mRNA in PBMCs from cows on Days 15–21, 25, and 32 of the estrous cycle or pregnancy. Analysis of *ISG15* mRNA in PBMCs allowed these investigators to predict 100% of NP cows on Day 32, which was confirmed with ultrasound [32]. In the present experiments, expression of *ISG15* mRNA was examined in blood from the jugular and uterine veins of NP and P ewes between Days 12 and 15. ISG15 mRNA concentrations increased in jugular and uterine vein PBMCs on Day 15 of pregnancy compared with the estrous cycle or earlier days of pregnancy.

ISGs are expressed in ovine extrauterine tissues besides PBMCs, such as liver and CL on Days 14 and 15 of pregnancy [6, 14, 34]. In the present experiments, we examined *ISG15* mRNA concentrations in these tissues during several days of the estrous cycle and pregnancy. Uterine vein was examined as a target for IFNT action because it represents the first tissue exposed to IFNT upon its exit from the uterus. Levels of uterine vein *ISG15* mRNA were not affected by pregnancy status (data not shown). For this reason, uterine vein tissue is not a likely target for IFNT signaling during early pregnancy. However, the CL contains *IFNAR1* and *IFNAR2* mRNA, and several genes downstream of these receptors (STATs, IRFs, and ISGs) were induced in response to pregnancy status [38].

Upon phosphorylation, STAT1:STAT2 heterodimers are released from IFN receptors and associate with IRF9, which, along with STAT1, has DNA-binding domains. This complex, known as ISG factor-3 (ISGF3), is able to translocate to the nucleus and initiate transcription of genes by binding to IFN-stimulated response elements (ISREs) [44, 45]. Both STAT1 and STAT2 mRNA concentrations increased in CL by Day 14 of pregnancy. One of the genes induced by ISGF3 binding to the ISRE is IRF7. Following de novo synthesis, IRF7 is phosphorylated and activates transcription of the IFNα/β gene [46], thus possibly preparing the mother's defense system to respond to any viral infections that could cause a loss of pregnancy. IRF3 mRNA concentrations increase between Days 14 and 15 in both NP and P ewes, although the increase was more pronounced in P ewes. This increase in IRF3 mRNA concentrations in the CL after estrus might be augmented by progesterone, as is the case for endometrial ISGs [47]. But by Day 15, concentration of serum progesterone was significantly lower in NP compared with P ewes (Fig. 1). In contrast, STAT1, STAT2, IRF7, IRF9, DDX58, IFIH1, and ISG15 mRNA concentrations did not change very much during the estrous cycle, which we interpret to reflect very little impact of serum progesterone on these mediators of IFN signal transduction in the CL. As pregnancy progressed to Day 15, there was an increase in STAT1, STAT2, IRF7, and IRF9 mRNA concentrations, and all of these early responses to IFN action were associated with increased ISGs, such as DDX58, IFIH1, and ISG15. The upregulation of these classic responses to IFN may contribute to luteal resistance during early pregnancy in the ewe.

The binding of ISGF3 to ISRE results in transcription of ISGs, such as *ISG15*. Concentrations of *ISG15* mRNA increased in the liver by Day 14, and *IFIH1*, *DDX58*, and *ISG15* mRNA were in greater concentrations in the CL on Day 14 and/or Day 15 of pregnancy compared with the estrous cycle. The present studies confirm and expand the scope of the temporal induction of genes encoding IFN signaling in extrauterine tissues, with a specific emphasis on the CL and liver during early pregnancy in sheep.

Glass and coworkers [48] reported that ovine luteal ESR1 was low on Day 4, increased significantly by Day 6, reached

maximum level by Day 8, decreased by Day 12, and then increased by Day 16 of the estrous cycle. OXT from LLCs working through OXTR on small luteal cells inhibited luteinizing hormone-stimulated secretion of progesterone from small luteal cells (M. Mayan and G.D. Niswender, unpublished data). Because IFNT prevents the upregulation of transcription of endometrial *ESR1* and *OXTR* genes during maternal recognition of pregnancy in ruminants [5], we tested whether pregnancy would have similar effects on concentrations of *ESR1* and *OXTR* mRNA in the CL. We did not find differences for *ESR1* or *OXTR* mRNA concentrations in the CL during the estrous cycle or early pregnancy, and we conclude that these genes are not regulated by pregnancy in the CL. However, this does not preclude the possibility of an inhibitory action of pregnancy on OXT synthesis and release from LLCs, which would essentially protect small luteal cells regardless of the actual numbers of OXTR. Also, there might be changes in the membrane of these cells to essentially mask or block binding of OXT to its receptor, as described in the context of the action of progesterone on the CL in vitro (see Davis et al. [49]).

ISG15 mRNA concentrations were first detected in endometrium on Day 13 of pregnancy. One day later (i.e., Day 14), *ESR1* and *OXTR* mRNAs were upregulated in endometria from NP ewes, but this upregulation was blocked in P ewes. STAT1, STAT2, IRF9, DDX58, and IFIH1 mRNA concentrations did not increase until Day 14 of pregnancy, whereas IRF3, IRF7, and ISG15 mRNA concentrations lagged behind by 1 day and did not actually increase until Day 15 of pregnancy in peripheral tissues, such as CL, liver, and PBMCs. The limit of detection of IFNT in uterine vein blood was 148 pg/ml; however, induction of ISGs by lower concentrations of IFNT can be achieved in vitro. Not being able to detect IFNT in uterine vein blood on Day 15 may not actually reflect the action of low concentrations of IFNT when inducing transcription of ISGs in CL, liver, and PBMCs. One reason for a possible 24-h delay in detection of IFNT in serum by Day 16, compared with action when inducing transcription of genes in peripheral tissues, might be the time required for translation of IFNT protein and release of IFNT in the uterine lumen to concentrations that are high enough to pass through the basement membrane of the endometrium and enter endometrial venous drainage. Also, there may be variation in the expression of junctional complex proteins that allow the endometrial cells to become more or less leaky to movement of IFNT into the uterine venous blood [50].

In conclusion, IFNT responses, such as induction of *ISG15* mRNA in the endometrium (paracrine action), can be detected as early as Day 13 of pregnancy. This endometrial response to IFNT is followed 1–2 days later in extrauterine tissues (endocrine action) on Days 14–15. The direct action of IFNT on the CL may confer resistance of the CL to the luteolytic pulses of PGF (see Antoniazzi et al. [34]). In addition, IFNT may control antiapoptotic mechanisms and cell survival genes to ensure luteal cell differentiation that will prolong luteal life span. Ultimately, luteal survival may be driven by ISG15 action, by conjugating to intracellular proteins and altering luteolysis/antiluteolytic/cell survival signaling pathways. These hypotheses are subjects of ongoing experiments.

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