Interferon-tau promotes luteal endothelial cell survival and inhibits specific luteolytic genes in bovine corpus luteum

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Abstract

Interferon-tau (IFNT), a maternal recognition of pregnancy (MRP) signals in domestic ruminants, suppresses the release of luteolytic pulses of uterine prostaglandin F2α (PGF2α), thus extending the corpus luteum (CL) life span. We hypothesized that IFNT also exerts anti-luteolytic actions in bovine CL. To examine the direct effects of IFNT on bovine CL, luteal slices and enriched luteal endothelial cells (LECs) were utilized. We found that recombinant ovine IFNT (roIFNT) markedly elevates interferon-associated genes (STAT1, STAT2 and IRF9) and interferon-stimulated genes (ISGs: MX2, ISG15 and OAS1Y) in both models. Furthermore, IFNT time-dependently induced STAT1 phosphorylation in LECs without affecting total STAT1. roIFNT-stimulated viable LECs numbers and the knockdown of protein inhibitor of activated STAT1 (PIAS1) abolished this effect, suggesting that PIAS1 may mediate the proliferative effect of IFNT. IFNT significantly downregulated luteolytic genes such as TGFβ1, thrombospondin-1 (THBS1), endothelin-1 (EDN1) and serpin family E member1 (SERPINE1) in LECs. However, less robust effects were observed in luteal slices. Moreover, PGF2α alone induced THBS1, SERPINE1 and EDN1 mRNA in CL slices whereas in the presence of IFNT, THBS1 and SERPINE1 stimulation was abolished. Collectively, these results indicate that IFNT acts via STAT1-IRF9-dependent and independent pathways and affects diverse luteal functions. Most interestingly, this study suggests the existence of an anti-luteolytic effect of IFNT in bovine CL, namely, inhibiting key PGF2α-induced luteolytic genes. The proliferative effect of IFNT may constitute an additional mechanism that promotes luteal cell survival, thus, extending the luteal life span during early pregnancy in cows.

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Introduction

Maternal recognition of pregnancy (MRP) is a prerequisite for the successful establishment of pregnancy and maintenance of the corpus luteum (CL) function. In ruminants, the mononuclear trophectoderm of the conceptus secretes interferon-tau (IFNT) during the peri-implantation period, which serves as the MRP signal (Spencer et al. 1996, Roberts et al. 1999). Bovine IFNT transcripts were detected in uterine flushes in samples from days 12–28 (Takahashi H. et al. 2007), also prevents secretion of luteolytic pulses of prostaglandin F2α (PGF2α) by uterine epithelia and blocks effects of exogenous estradiol and oxytocin to stimulate uterine release of PGF2α. Expression of estrogen receptor 1 (ESR1) and oxytocin receptor (OXTR) mRNAs is either silenced or the receptors are not responsive to estradiol and oxytocin in endometria of both pregnant cows and cyclic cows treated with intrauterine injections of IFNT (Thatcher et al. 1989, Meyer et al. 1995). In this way, it prevents the release of luteolytic pulses of PGF2α from the endometrium and allows the prolongation of the CL life span and progesterone (P4) production. Recent studies performed by Arosh and coworkers also emphasized the role of the prostaglandin transporter (PGT), in the transport of PGF2α from the endometrium to the ovary (Banu et al. 2010, Lee et al. 2010).

In the absence of a functional/live conceptus in the uterus, the CL undergoes luteolysis (Niswender et al. 2000, Schams & Berisha 2004). Recent evidence highlighted certain genes such as endothelin1 (END1), transforming growth factor beta1 (TGFβ1), thrombospondin1 (THBS1) and serpin family E member1 (SERPINE1) are specifically induced during luteolysis in the PGF2α-responsive CL (Miyamoto et al. 1997, Kliem et al. 2007, Hou et al. 2008, Mondal et al. 2011, Romero et al. 2013, Farberov & Meidan 2016). Their gene products are expected to promote vascular instability, as well as apoptosis and matrix remodeling in the regressing CL. These genes are highly expressed...
in luteal endothelial cells (LECs) (Levy et al. 2001, Zalman et al. 2012). Moreover, prostaglandin F receptor (PTGFR) that are present in LECs (Mamluk et al. 1998, Zannoni et al. 2007, Lee et al. 2010, Shirasuna et al. 2012), can mediate direct PGF2a actions on some of these genes (Girsh et al. 1996b, Shirasuna et al. 2008, Zalman et al. 2012).

IFNT may also act as an endocrine hormone, inducing the expression of several interferon-stimulated genes (ISGs; ISG15, MX1 and 2, OAS1Y and others) in extra-uterine tissues such as the CL, peripheral blood cells and the mammary gland (Oliveira et al. 2008, Yang et al. 2010, Nitta et al. 2011, Matsuyama et al. 2012). Indeed, the uterine infusion or endocrine delivery of recombinant ovine (roIFNT) via the uterine or jugular vein extended the life span of CL in ewes (Oliveira et al. 2008, Bott et al. 2010, Antoniazzi et al. 2013). However, the underlying mechanism that mediates CL rescue has not been established yet. To gain a better understanding about IFNT-induced luteal events that may prevent luteolysis of bovine CL during MRP, we investigated the direct effects of IFNT on whole luteal tissue, using CL slices and on LECs, a specific cell type within the CL.

**Materials and methods**

Unless otherwise stated, all biochemicals were from Sigma-Aldrich and the cell culture materials were from Biological Industries (Kibbutz Beit Haemek, Israel).

**Isolation and culture of LECs**

The procedures for isolation and enrichment of LECs were detailed previously (Levy et al. 2001, Farberov & Meidan 2014, 2016). Briefly, CL was dispersed using sequential incubations with collagenase. Then cells were incubated with BS-1-coated magnetic beads. The adherent cells were washed and concentrated using a magnet until the supernatant was free of cells. BS-1-positive cells (enriched LECs fraction) were plated in DMEM/F-12 containing 10% fetal calf serum (FCS) on collagen type I-coated plates (Surecoat; Advanced BioMatrix). Colonies of LECs were trypsinized with 1% crystalline trypsin (Biological Industries), collected and reseeded; this process was repeated until homogenous cell cultures were visualized. Cell identity was verified using endothelial cell markers (CD31, EDN1) and the lack of contamination by smooth muscle cells (ACTAG2 expression) and fibroblasts (COL1A1 expression) was evaluated. Cells from passages 7–10, derived from at least three different CLs, were used in the present study. The LECs were seeded in 12-well plates (70,000 cells/well) and cultured overnight in DMEM/F-12 containing 10% FCS. The next day, the cells were transferred to starvation medium (0.5% BSA in 0.1% FCS) for 24 h. Then, the LECs were incubated for the time indicated in the legends with or without roIFNT (0.01–10 ng/mL; a generous gift from Prof. Fuller W Bazer, Texas A&M University). At the end of the incubation period, the total RNA was extracted from the cells.

**Experimental animals and synchronization**

Holstein Friesian primiparous cows from commercial dairy farms in central Poland were utilized in this experiment. The experimental protocols and procedures were approved by the Local Ethics Committee. Cows received two intramuscular injections of PGF2a (5 mL dinolytic 5 mg/mL, Zoetis, Belgium) 11 days apart. Estrus was detected 24–72 h after the second PGF2a administration and reproductive tracts including ovaries were monitored by transrectal ultrasonography using a 5–8 MHz linear-array probe (MyLabOne VET, Italy) throughout the experiment. At mid-luteal phase (days 12–14 of the estrous cycle), ovaries with CL were surgically removed under anesthesia. Four CLs derived from individual cows were harvested and used for the experiment described below.

**Culture of CL slices**

CL (above) was cut into slices (300 μm thick, 8 mm diameter, 10–15 mg wet weight) using a Krumdick Tissue Slicer (K&F Research, Birmingham, AL, USA) as previously described (Przygodzka et al. 2014). Then, the slices (2 slices/well) were incubated for 12 h in M-199 supplemented with 0.1% bovine serum albumin (BSA; ICN Bio Medicals, Inc., Costa Mesa, CA, USA), antibiotics (penicillin-streptomycin; Sigma-Aldrich) and an anti-fungal drug (amphotericin B; Sigma-Aldrich) in the presence or absence of roIFNT (1 ng/mL) or PGF2a (Cayman Chemical; 30 nM) alone or in combination of roIFNT and PGF2a. Each treatment (for each cow) was examined in duplicates and averaged. At the end of the incubation, slices were collected, rapidly frozen in liquid nitrogen and stored at −80°C until total RNA isolation.

**Western blot analyses**

Proteins were extracted by adding sample buffer (×2), separated by 7.5%–12.0% SDS-PAGE, and subsequently transferred to nitrocellulose membranes, as previously reported (Zalman et al. 2012). Membranes were blocked for 1 h in TBST (20 mmol/L Tris, 150 mmol/L NaCl and 0.1% Tween 20; pH 7.6) containing 3% BSA or 5% low-fat milk and then incubated overnight at 4°C with the following antibodies: goat polyclonal phosphorylated STAT1 (TYR701; 1:200; sc-7988R; Santa Cruz Biotechnology); goat polyclonal STAT1 (1:200; sc-346-G; Santa Cruz Biotechnology); mouse anti-THBS1 (1:500; ab1823; Abcam) and rabbit anti-p-p44/42 MAPK (1:50,000; Sigma-Aldrich). The membranes were then incubated with peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) or goat anti-mouse immunoglobulin G (heavy + light) (ab6789; Abcam) for 1 h at room temperature. A chemiluminescent signal was generated with SuperSignal (Thermo Fisher Scientific), and the membranes were exposed. The signal was analyzed using the Gel-Pro Analyzer version 3.0 (Media Cybernetics, Inc., MD, USA) and normalized to total p44/42 MAPK in the same sample to correct for the amount of protein loaded.

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Transfection of LECs

Cells were seeded for overnight in 24-well (16,000 cells/well) plates containing 500 µL of DMEM/F-12 medium comprising 10% FCS. The next day, cells were transfected with siRNA (50 nmol/L; GeneCust, Luxembourg; as previously described (Farberov & Meidan 2014)). siRNA targeting protein inhibitor of activated STAT1 (PIAS1) and scrambled siRNA (negative control; 50 nmol/L) using 3.6 µL of lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol at 1% FCS. The siRNA of PIAS1 (siPIAS1) was sense (S) GAGACAAGUCACGACUAUA dCdA, antisense (AS) UUAUAGUCUGAUUGUCUCdCdT, corresponding to bases 1664–1684 (NM_001075396.2). Scrambled siRNA sequence—negative control was (S) UUCUCCGAAACGUGACGUDdTd and (AS) ACGUGACGACUUCCGAGAAdTdT. After 24 h post transfection, LECs were transferred to starvation medium and the next day they were treated with or without IFNT (1 ng/mL) for 24 h.

**Determination of the viable cell numbers**

The viable cell numbers were estimated as previously described (Farberov & Meidan 2014), using the XTT kit (Biological Industries), which measures the reduction of a tetrazolium component by the mitochondria of viable cells. On the day of measurement, XTT was added to the culture media according to the manufacturer’s instructions. Then, plates were incubated at 37°C for 2–3 h. Afterwards, the absorbance was read at 450 nm (reference absorbance, 630 nm).

**RNA extraction and qRT-PCR**

**CL slices**

Collected luteal tissue samples were homogenized in Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer’s instructions. Total RNA was extracted by using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. The integrity and quality of RNA were verified using the Agilent 2100 Bioanalyzer (Agilent Technologies) and NanoDrop (Thermofisher). Genomic DNA contamination was removed according to the manufacturer’s instructions (DNase I Kit, Thermostifher). Gene expression was evaluated by one-step real-time PCR using the Applied Biosystems 7900 HT Sequence Detection System (Applied Biosystems) with TaqMan assays (Thermostifher; Table 1). As detailed previously, each qPCR was performed in triplicates on 384-well plates and was preceded by a reverse transcription reaction (15 min at 40°C (Przygrodzka et al. 2016)).

To select the most stable housekeeping gene among glyceraldehyde 3-phosphate dehydrogenase (GAPDH), beta-actin (ACTB) and hypoxanthine phosphoribosyltransferase 1 (HPRT1), the NormFinder algorithm was applied, based on the report and GAPDH was chosen as a housekeeping gene (Andersen et al. 2004). The expression of selective genes was normalized relative to the abundance of GAPDH mRNA. The threshold cycle number (Ct) was used to quantify the relative abundance of the gene; arbitrary units were calculated as 2−ΔCt=2−ΔCt target gene−ΔCt housekeeping gene (Livak & Schmittgen 2001).

**LECs**

Total RNA was isolated from cells using Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer’s instructions. As previously described, total RNA was reverse transcribed and quantitative RT-PCR (qPCR) was performed using the LightCycler 96 system (Roche Diagnostics) with Platinum SYBR Green (SuperMix, Invitrogen) (Farberov & Meidan 2016). The sequence of primers used for quantitative RT-PCR (qPCR) is listed in Table 2. All primers were designed to have single-product melting curves, as well as consistent amplification efficiencies between 1.8 and 2.2 (Schmittgen & Livak 2008). All amplicons were verified by sequencing. To select the most stable housekeeping gene among glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein S26 (RPS26) in LECs, the NormFinder was used.

**Table 1** Genes, Genbank accessions and the sequences of primers used for real-time PCR performed in LECs.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>TaqMan Assay ID</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Bt03210919_g1</td>
<td>NM_001034034</td>
</tr>
<tr>
<td>STAT1</td>
<td>Bt03252661_m1</td>
<td>NM_001077900.1</td>
</tr>
<tr>
<td>STAT2</td>
<td>Bt04284638_m1</td>
<td>NM_001205689.1</td>
</tr>
<tr>
<td>IRE7F</td>
<td>Bt03220019_m1</td>
<td>NM_001024506.1</td>
</tr>
<tr>
<td>M2</td>
<td>Bt03211948_m1</td>
<td>NM_173941.2</td>
</tr>
<tr>
<td>ISG15</td>
<td>Bt03223508_m1</td>
<td>NM_174366.1</td>
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<tr>
<td>OAS1Y</td>
<td>Bt03211516_m1</td>
<td>NM_001015561</td>
</tr>
<tr>
<td>TGFB1</td>
<td>Bt04259488_m1</td>
<td>NM_001040606.1</td>
</tr>
<tr>
<td>THBS1</td>
<td>Bt03213209_m1</td>
<td>NM_174196</td>
</tr>
<tr>
<td>SERPINE1</td>
<td>Bt03212914_m1</td>
<td>NM_174137</td>
</tr>
<tr>
<td>EDN1</td>
<td>Bt03217448_m1</td>
<td>NM_181010.2</td>
</tr>
</tbody>
</table>

**Table 2** Genes, Genbank accessions and the sequences of primers used for real-time PCR performed in LECs.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence (5′–3′)</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>f-gttccactacccacaggaaggggagtcatcagctggttcaggaagg</td>
<td>NM_001034034</td>
</tr>
<tr>
<td>RPS26</td>
<td>r-tctggctgctgttgcctttg</td>
<td>NM_001015561</td>
</tr>
<tr>
<td>IFNAR</td>
<td>f-tgattttctccagcactatgaggggagtcttctcagagttc</td>
<td>NM_174552.2</td>
</tr>
<tr>
<td>STAT1</td>
<td>r-gagtcgatgaggtcaatgcag</td>
<td>NM_001077900.1</td>
</tr>
<tr>
<td>IRE7F</td>
<td>r-gtgctgctgttccacttcagga</td>
<td>NM_001024506.1</td>
</tr>
<tr>
<td>PIAS1</td>
<td>f-gctgccacctggacatcatttg</td>
<td>NM_001075396.2</td>
</tr>
<tr>
<td>MX2</td>
<td>r-gagccctggacaccaactac</td>
<td>NM_173941.2</td>
</tr>
<tr>
<td>ISG15</td>
<td>f-gtgctgctgttccacttcagag</td>
<td>NM_174366.1</td>
</tr>
<tr>
<td>OAS1Y</td>
<td>r-tctggctgctgttgcctttg</td>
<td>NM_001040606.1</td>
</tr>
<tr>
<td>TGFB1</td>
<td>f-gccagctgctgctgttccacag</td>
<td>NM_001166068</td>
</tr>
<tr>
<td>THBS1</td>
<td>r-tctgcgctgctgttccacag</td>
<td>NM_174196</td>
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<td>SERPINE1</td>
<td>f-ctgccacctgcaatccttg</td>
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</tr>
<tr>
<td>EDN1</td>
<td>r-tctgcgctgctgttccacag</td>
<td>NM_181010.2</td>
</tr>
</tbody>
</table>
algorithm was applied, based on the report, RPS26 was chosen as a housekeeping gene (Andersen et al. 2004). The expression of selective genes was normalized relative to the abundance of RPS26 mRNA.

Statistical analyses

All statistical analyses were conducted using GraphPad Prism version 4.03 Software (GraphPad Software). Data are presented as the means of ± s.e.m.; LECs culture experiments were repeated at least three times; CL slices were collected from four different mid-luteal phase (days 12–14th of the estrous cycle) cows and incubated separately. The expressions of selective mRNA levels were normalized to the relative abundance of GAPDH (for luteal slices) and RPS26 (for LECs). Data were analyzed by either Student's t-test or one-way ANOVA, followed by Bonferroni’s post hoc multiple comparison test, when indicated. Differences were considered as significant at P < 0.05. Asterisks or different letters represent statistical significant differences (P < 0.05). Additional information is provided in legends to figures.

Results

IFNT induces ISGs, IFN-associated genes and STAT1 phosphorylation in LECs

We first examined whether IFNT induces the expression of classical ISGs such as ISG15, MX2 and OAS1Y mRNA in LECs. As shown in Fig. 1 (A, B and C), roIFNT (0.01–10 ng/mL) elevated MX2 (P < 0.001), ISG15 (P < 0.001) and OAS1Y (P < 0.001) levels in LECs in a dose-dependent manner. Among these ISGs, MX2 exhibited the highest induction by roIFNT, followed by ISG15 and OAS1Y (Fig. 1A, B and C).

We then examined STAT1 phosphorylation by IFNT. As shown in Fig. 2, 1 ng/mL of roIFNT significantly induced tyrosine 701 phosphorylation already within 15 min (P < 0.05). STAT1 phosphorylation was evident between 15 and 60 min and its highest induction in response to roIFNT was observed after 60 min of LECs treatment with IFNT (P < 0.001), the response markedly dropped after 120 min (Fig. 2). The content of total STAT1 remained unchanged throughout treatment with IFNT (Fig. 2). The effect of 1 ng/mL roIFNT on mRNA expression of the IFN-associated genes IFNAR1, STAT1, IRF9 and PIAS1 in LECs is shown in Table 3. IFNT markedly elevated as compared to control treatment: STAT1 (5.3-fold; P < 0.01), IRF9 (8.6-fold; P < 0.001) and PIAS1 (1.8-fold; P < 0.01) mRNA levels. However, IFNAR1 levels in LECs remained unaffected after the treatment with IFNT.

IFNT regulates LECs function

To examine whether IFNT is functionally important for the survival of LECs, we assayed the cells viability. roIFNT doubled viable LECs numbers as compared with the control group (cells cultured in basal medium; Fig. 3A). Since PIAS1 is known to be involved in cell proliferation (Munarriz et al. 2004), we examined the effect of PIAS1 silencing on IFN-induced cell proliferation. IFNT maintained its stimulatory effect (1.5 folds) on viable cell numbers with scrambled siRNA transfection (Fig. 3B). However, PIAS1 siRNA significantly reduced PIAS1 mRNA (basal as well as roIFNT-stimulated levels) by 60–70% (Fig. 3B). Concurrently with the decrease in PIAS1 mRNA, cell viability was significantly reduced by PIAS1 silencing (compared with cells transfected with a scrambled siRNA-negative control; Fig. 3C). Furthermore, the positive effect of roIFNT on LECs viability was abolished in PIAS1-silenced cells (Fig. 3C). IFNT had an additional important effect on LECs, i.e., incubation of these cells for 24 h with 1 ng/mL of roIFNT reduced the mRNA levels of luteolytic genes: TGFβ1 (~25%; P < 0.01), THBS1 (~36%; P < 0.001), EDN1 (~40%; P < 0.001) and SERPINE1 (~50%; P < 0.001) (Fig. 4A). THBS1 protein levels in LECs were also strongly reduced (by 60%) in cells incubated with roIFNT (Fig. 4B).

Effects of IFNT, PGF2a and their combination in CL slices

CL slices were treated with 1 ng/mL of roIFNT for 12 h as detailed in the materials and methods section. As shown in Fig. 5A, B, C, D and F, roIFNT-elevated STAT1 (2.2-fold; P < 0.01), STAT2 (6.0-fold; P < 0.01), IRF9 (6.1-fold; P < 0.001), MX2 (230-fold; P < 0.01), ISG15 (30-fold; P < 0.01) and OAS1Y (260-fold; P < 0.001) mRNA levels...
Interferon-tau actions on bovine corpus luteum

5.32 ± 0.87

50%

et al.

50%

et al.

Using LECs, we showed that roIFNT induced

However,

P

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Similar to the responses observed in LECs,

PIAS1 also acts in a STAT-independent manner

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and

et al.

IFNT also induces STAT1 phosphorylation (137%;

Fig. 6). Based on these

et al.

and

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Rosenfeld

et al.

et al.

Serum-starved LECs were

incubated with IFNT (1 ng/mL) for different time points as indicated.

Tyrosine phosphorylated STAT1 (p-STAT1) and total STAT1 proteins

were analyzed using specific antibodies in western blotting.

Phospho-STAT1 and total STAT1 were normalized relative to the

abundance of total MAPK (p44/42). The results represent the

means ± s.e.m. of 4 independent experiments. Asterisks indicate

significant (*P<0.05, **P<0.01, ***P<0.001) statistical differences

from time 0.

as compared to controls. To determine the anti-luteolytic effects of roIFNT in whole bovine CL tissue, slices were incubated with IFNT, PGF2a and their combination (Fig. 6). Similar to the responses observed in LECs, there was a significant downregulation of TGFβ1 (17%; P<0.05), THBS1 (25%; P<0.05) and SERPINE1 (30%; P<0.001) mRNAs in roIFNT-treated CL slices (Fig. 6). PGF2a significantly stimulated THBS1 (137%; P<0.01), SERPINE1 (138%; P<0.01) and EDN1 (134%; P<0.01). Importantly, the stimulation of THBS1 and SERPINE1 induced by PGF2a alone was abolished in the presence of IFNT. TGFβ1 expression was significantly reduced by the combined treatment of IFNT and PGF2a (32%; P<0.001), although there was no induction by PGF2a alone. Contrary to LECs, the expression of EDN1 was stimulated by roIFNT alone or in presence of PGF2a (Fig. 6). Similar, but less pronounced, effects of IFNT were also observed in LECs cultured for 12 h (data not shown).

Table 3. The expression of IFNAR1, STAT1, IRF9 and PIAS1 in LECs cultured for 24 h with roIFNT (1 ng/mL).

Genes | IFN | P value |
------|-----|---------|
IFNAR1 | 0.84 ± 0.08 | 0.203 |
STAT1 | 5.32 ± 0.87 | 0.01 |
IRF9 | 8.66 ± 0.36 | 0.00001 |
PIAS1 | 1.88 ± 0.77 | 0.007 |

Results are the means ± s.e.m. (n = 4); the expression of each gene is relative to its respective controls designated as 1.

Discussion

The present study demonstrates that IFNT affects diverse luteal functions in cows: it stimulated the expression of ISGs, IFN-associated genes, STAT1 phosphorylation, LEC survival and suppressed key luteolytic genes. These actions together may contribute to the maintenance of the bovine CL during early pregnancy.

It was previously shown that several ISGs are stimulated in the CL of ovine and bovine species during early pregnancy (Oliveira et al. 2008, Magata et al. 2012), or after IFNT infusion in ewes (Bott et al. 2010). A direct in vitro effect of IFNT on various luteal cell types was also demonstrated in several studies (Oliveira et al. 2008, Yang et al. 2010, Nitta et al. 2011, Antoniazzi et al. 2013, Romero et al. 2015, Shirasuna et al. 2015). In the present study, we showed that roIFNT dose-dependently stimulated ISGs (MX2, ISG15 and OAS1Y) expression in cultured luteal slices as well as in LECs of bovine CL. Taken together, these results support the contention that IFNT acts directly on bovine CL to stimulate ISGs expression.

IFNT is a multifunctional cytokine that exhibits biological activity similarly to other type-1 IFNs. It acts via binding to the cell surface receptors IFNAR1 and IFNAR2 (Rosenfeld et al. 2002). It is well established that IFNT activates the classical JAK-STAT-IRF signaling pathway in the ruminant endometrium (Staggs et al. 1998, Vitorino Carvalho et al. 2016). Based on these studies, a model has been proposed in which the tyrosine phosphorylation of STAT1 elicited by IFNT leads to the formation of STAT1–STAT2–IRF9 heterotrimers, also known as IFN-stimulated gene factor 3 complex (ISGF3), which is translocated to the nucleus and binds to the interferon-stimulated response element (ISRE) present within the promoter of ISGs and regulates their expression (Vitorino Carvalho et al. 2016). However, IFNT signaling has not yet been validated in tissues other than the endometrium. Findings reported in the present study clearly showed that roIFNT can induce temporal STAT1 phosphorylation and that roIFNT stimulates the expression of interferon-associated genes such as STAT1, STAT2 and IRF9 in LECs and luteal tissue slices. Thus, it seems likely that IFNT-dependent induction of ISGs in the bovine CL occurs via the STAT1–IRF9 pathway.

PIAS proteins are a family of four multifunctional proteins (PIAS1-4), which are known to be important regulators of cytokine actions (Sharrocks 2006). PIAS1 regulates the JAK/STAT pathway by interfering with the DNA binding activity of activated STAT1 (Liu et al. 2004). PIAS1 also acts in a STAT-independent manner to promote mammalian cell proliferation by inhibiting p53 and p73 transcriptional activity (Munarriz et al. 2004). Using LECs, we showed that roIFNT induced PIAS1 expression and increased viable cell numbers. Moreover, PIAS1 silencing abolished roIFNT-elevated cell proliferation without affecting IFNT-induced ISGs.
(data not shown) thus, suggesting STAT-independent action of IFNT in the bovine CL. These findings underscore the importance of PIAS1 in mediating the stimulatory effect of IFNT on LECs survival. Antoniazzi and coworkers (Antoniazzi et al. 2013) demonstrated that endocrine delivery of IFNT upregulated cell survival genes such as BCL2-like 1 (BCL2L1 or Bcl-xL), serine/threonine kinase (AKT), X-linked inhibitor of apoptosis (XIAP) in ewes CL. These genes were inhibited during luteolysis by PGF2α (Antoniazzi et al. 2013). These findings together with the current data support the role of IFNT as survival factor for luteal cells.

It was shown that the luteolytic cascade, initiated by PGF2α, involves the luteal stage and the time-dependent induction of several key genes within the CL: EDN1, THBS1, TGFβ1 and SERPINE1 are among the most extensively studied (Girsh et al. 1996a, Shirasuna et al. 2004, Choudhary et al. 2005, Maroni & Davis 2012, Romero et al. 2013, Meidan et al. 2017). These factors act in concert to inhibit P4 production, angiogenic support, cell survival and extracellular matrix (ECM) remodeling to achieve CL regression as described below. EDN1 and P4 curves showed a close, inverse relationship in response to luteolytic dose of PGF2α (Ohtani et al. 1998) suggested that luteal EDN1 may be the local mediator of PGF2α in reducing P4 output. Indeed, EDN1 was shown to directly inhibit basal and LH-induced P4 in many species, acting via selective EDNRA (Miyamoto et al. 1997, Apa et al. 1998, Meidan et al. 2005). During early pregnancy luteal EDN1 was inhibited (Costine et al. 2007, Przygodzka et al. 2016). PGF2α administration at mid-cycle elevated the expression of several antiangiogenic, pro-apoptotic genes, including THBS1 and TGFβ1 (Hou et al. 2008, Mondal et al. 2011, Zalman et al. 2012, Romero et al. 2013, Berisha et al. 2016, Farberov & Meidan 2016). THBS1 was shown to be a physiological activator of latent TGFβ1 in LECs (Farberov & Meidan 2016). Both THBS1 and TGFβ1 triggered apoptosis, but THBS1 was significantly more effective in causing cell death and activating caspase-3. THBS1 and TGFβ1 also differed in their activation of p38 mitogen-activated protein kinase (Farberov & Meidan 2016). Similarly to THBS1 and TGFβ, SERPINE1 showed marked luteal stage specific expression with extensive responses to PGF2α at mid-cycle (Smith et al. 1997, Kliem et al. 2007, Berisha et al. 2016, Farberov & Meidan 2016). SERPINE1 is known to be a downstream target of TGFβ1; it encodes endothelial PAI-1, a member

![Figure 3](https://www.reproduction-online.org/154/559-568/564.png) Role of PIAS1 in IFNT-induced LEC viability. (A) Non-transfected LEC. (B) PIAS1 mRNA expression and (C) LECs numbers in cells transfected with PIAS1 siRNA (siPIAS1) or scrambled siRNA. (A) Overnight serum-starved LECs were treated with roIFNT (1 ng/mL) for 24 h followed by cell viability determination using XTT. B and C-LECs were transfected with either scrambled siRNA or specific siPIAS1. 24 h post transfection, cells were starved overnight, followed by incubation with or without IFNT (1 ng/mL) for additional 24 h. Control—cells cultured in basal media (without IFNT). The results are presented as the means±s.e.m. from 4 independent experiments. Asterisks indicate significant statistical differences from their respective controls at ***P<0.001 (Student's t-test). The different letters indicate significant statistical differences at P<0.05 analyzed using ANOVA followed by Bonferroni’s post hoc multiple comparison test.

![Figure 4](https://www.reproduction-online.org/154/559-568/564.png) The effect of roIFNT on (A) TGFβ1, THBS1, EDN1 and SERPINE1 mRNA and (B) THBS1 protein in LECs. (A) Serum-starved cells were incubated with either basal medium alone (control) or with roIFNT (1 ng/mL) for 24 h. (B) Cells were incubated without (basal) or with roIFNT (1 ng/mL) for 48 h. THBS1 protein was determined in cell extracts by Western blotting and normalized relative to the abundance of total MAPK (p44/42). Results are presented as the means±s.e.m. from 4 and 3 independent experiments for A and B respectively. Asterisks indicate significant (*P<0.05, **P<0.01, ***P<0.001) differences from their respective controls.
of the serine protease inhibitor family. PAI-1 inhibits tissue plasminogen activator and urokinase plasminogen activator, thus, SERPINE1 is a potent pro-fibrotic factor (Ghosh & Vaughan 2012). In ewes, THBS1 and SERPINE1 were also shown to be concomitantly elevated during luteal regression, and inhibited during MRP (Romero et al. 2013). Furthermore, all of these genes (EDN1, THBS1, TGFB1 and SERPINE1) were reported to be highly expressed in bovine LECs (Levy et al. 2001, Zalman et al. 2012, Farberov & Meidan 2016). In vitro response of luteal cells to IFNT was studied before by Romero and coworkers (Romero et al. 2013) using enriched ovine small, large and mixed luteal cells. The results obtained were not consistent with the expected effects of IFNT or pregnancy. For instance, neither SERPINE1 nor IL6 which were elevated during luteolysis in ewes and inhibited in the early pregnant CL, were not significantly affected by IFNT in vitro in either of these cell preparations. The current study, using specifically enriched bovine LECs, showed that IFNT reduced EDN1, THBS1, TGFB1 and SERPINE1 mimicking the effect of early pregnancy (Costine et al. 2007, Romero et al. 2013, Przygrodzka et al. 2016). Importantly, in CL slices IFNT also abolished the stimulatory effect of PGF2a on THBS1 and SERPINE1. The reason why IFNT elevated EDN1 in slices remains enigmatic, especially as EDN1 is reduced during early pregnancy (Costine et al. 2007, Przygrodzka et al. 2016).

Pregnant ruminants have higher levels of basal PGF2a than cyclic animals do (Wilson et al. 1972, Zarco et al. 1988). However, oxytocin-induced PGF2a, released from endometrium, was significantly inhibited during pregnancy or IFNT infusion (Vallet et al. 1989, Vallet & Lamming 1991, Meyer et al. 1995). This may be because IFNT can inhibit the majority of PGT-mediated release of PGF2a, but not the simple diffusion of PGF2a from endometrial luminal epithelial cells, as suggested by Banu and coworkers (Banu et al. 2010). These studies suggest that PGF2a can reach the CL during early pregnancy, thus requiring an anti-luteolytic mechanism at the level of the CL. In this context, our results demonstrating that IFNT can suppress luteolytic genes are physiologically important.

Precision-cut luteal slices and LECs were utilized to study the direct and functional roles of IFNT on bovine CL. Precision-cut CL slices represent an in vitro model that closely resembles the multi-cellular complexity as well as the structural and functional features of whole tissue. Use of precision-cut luteal slices to study luteal function has been validated in pigs (Przygrodzka et al. 2014). However, luteal slices have limitations such as restricted incubation times. In addition, slices are also an unsuitable model to study functional assays such as cell proliferation and cell transfection. CL is a highly

![Figure 5](image_url) IFNT-induced STAT1 (A), STAT2 (B), IRF9 (C), MX2 (D), ISG15 (E) and OAS1Y (F) mRNA in CL slices. Slices were incubated with either basal medium alone (control) or roIFNT (1 ng/mL) for 12 h. Results are presented as the means ± s.e.m. from 4 individual cows. Asterisks indicate significant (*P < 0.05, **P < 0.01, ***P < 0.001) differences from their respective controls.

![Figure 6](image_url) Luteolytic gene expression in CL slices in response to roIFNT (1 ng/mL), PGF2a (30 nM) or their combination. Levels of TGFB1, THBS1, EDN1 and SERPINE1 were determined in CL slices incubated in basal media (control) or with treatments for 12 h. Results are presented as the means ± s.e.m. from 4 CLs each derived from individual cow. The different letters indicate significant statistical differences at P < 0.05 analyzed using ANOVA followed by Bonferroni’s post hoc multiple comparison test.
vascular gland, where LECs comprise more than 50% of all cell types within it (O’Shea et al. 1989). But in addition to their abundance, these cells are known to be active participants in the endocrine functions of the CL (Levy et al. 2001, Meidan et al. 2005), as highlighted again in this study. The stronger downregulation of luteolytic genes by IFNT in LECs may suggest that these cells are the primary target for IFNT to mediate its anti-luteolytic effects in bovine CL. However, the anti-luteolytic effects of IFNT on other bovine luteal cell types have not been studied yet and cannot be ruled out. Collectively, the findings reported in the present study suggest that IFNT acts in the bovine CL via STAT1-IRF9-dependent and independent pathways to elicit ISGs, type 1-associated genes and anti-luteolytic mechanisms. The increased viability of LECs induced by IFNT may constitute an additional mechanism to promote luteal cell survival, thus extending the luteal life span of cows during early pregnancy.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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