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Complex Induction of Bovine Uterine Proteins by Interferon-Tau[1]

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ABSTRACT

Interferon-tau (IFN-τ) is released by the conceptus and induces two uterine proteins during early pregnancy: ubiquitin cross-reactive protein (UCRP) and granulocyte chemotactic protein-2 (GCP-2). The present experiments were designed to determine whether detection (Western blot) of cytosolic UCRP and release of GCP-2 could be used to examine IFN-τ signal transduction in cultured endometrial explants and primary epithelial cells. Recombinant (r) type 1 IFNs (rboIFN-τ and rboIFN-α; 5, 25, 100 nM) induced UCRP, but only rboIFN-τ induced GCP-2 in explant culture. Recombinant boIFN-τ and conceptus secretory proteins containing native IFN-τ induced UCRP and GCP-2 in cultured primary epithelial cells. All concentrations of rboIFN-α (25, 50, 100 nM) induced UCRP, but only the highest concentration induced GCP-2 in cultured primary epithelial cells. Interestingly, phorbol ester (100, 500, 1000 ng/ml) induced GCP-2, but it had no effect on UCRP. Because type 1 IFNs induce UCRP, IFN-τ probably interacts with the janus kinase (Jak)-associated IFN-α receptor to phosphorylate signal transducers and activators of transcription (STAT) and/or interferon regulatory factor-1 (IRF-1). However, IFN-τ-specific induction of GCP-2 may involve a variant type 1 receptor subunit or activators of transcription that are associated with protein kinase C and the Jak/STAT/IRF-1 pathway.

INTRODUCTION

Pregnancy is established in cows through the release of interferon-tau (IFN-τ) by the conceptus and the inhibitory actions of this cytokine on uterine endometrial prostaglandin $F_{2\alpha}$ (PGF) [1–3]. Because IFN-τ inhibits the release of PGF during early pregnancy, the corpus luteum is maintained. Several reports have described binding of IFN-τ to endometrial receptors [4, 5]. However, little is known about signal transduction that occurs after IFN-τ interacts with its bovine endometrial receptor.

An 8-kDa protein (P8) is released by the bovine endometrium in response to recombinant bovine (rbo) IFN-τ [6, 7]. Recombinant boIFN-α shares 55% amino acid sequence identity with rboIFN-τ [8], but it had no effect on release of endometrial P8, provoking the hypothesis that P8 might be used as a novel marker for IFN-τ-specific action [6]. This uterine protein was purified and digested to yield internal peptides. Peptides had 92–100% amino acid sequence identity with bovine granulocyte chemotactic protein-2 (GCP-2), a member of the alpha chemokine family of chemotactic cytokines [7]. Western blot experiments using antiserum against GCP-2 revealed that rboIFN-τ induced the release of GCP-2 in cultured endometrial explants [7]. To our knowledge, the only other report describing regulation of boGCP-2 was in Madin-Darby bovine kidney (MDBK) cells, but such regulation occurred only when these cells were treated with phorbol ester [9].

Another interferon-stimulated gene product (ISG) has been identified in the bovine uterus as ubiquitin cross-reactive protein (UCRP) [10, 11]. The gene encoding UCRP is transcribed temporally in a manner that is similar to synthesis of the protein on Days 15–26 of pregnancy [10, 12]. Both rboIFN-τ and rboIFN-α induce the synthesis and release of UCRP by cultured endometrial explants [10]. The UCRP gene is transcribed in response to rboIFN-τ and rboIFN-α, and it has interferon stimulatory response elements (ISREs) [13] that probably interact with *trans*-acting proteins of the janus kinase (Jak)/signal transducing activators of transcription (STAT) and the interferon regulatory factor (IRF) pathway [14].

Currently, the only known regulators of GCP-2 are phorbol ester in MDBK cells [9] and rboIFN-τ in bovine endometrium [7]. Thus, the first objective of the present experiments was to determine whether type 1 IFNs (IFN-τ and IFN-α), conceptus secretory proteins known to contain native IFN-τ, and phorbol ester induced secretion of UCRP and GCP-2 in bovine endometrium. The second objective was to determine whether detection of UCRP and GCP-2 could be used to delineate IFN-τ-specific signal transduction from that induced by IFN-α.

MATERIALS AND METHODS

Animals and Collection of Tissues

Estrous cycles were synchronized in Angus crossbred cows using PGF (Lutalyse; Upjohn Co., Kalamazoo, MI). The day of estrus (standing estrous behavior) was defined as Day 0/21 (~21-day estrous cycle). Estrous behavior was monitored three times daily after treatment with PGF. Nonpregnant cows (Day 14; n = 5 cows) were not artificially inseminated. Reproductive tracts were removed from cows on Day 14 of the estrous cycle after slaughter. Endometrial explants representing Day 14 of the estrous cycle produce little if any UCRP and GCP-2 when cultured in the absence of rboIFN-τ [7, 10, 12, 15]. However, when cultured with rboIFN-τ, endometrial explants produce both uterine proteins in significant amounts [7, 10, 12, 15].

Conceptus secretory proteins (CSP) containing native IFN-τ were generated by culturing Day 18 conceptuses for 24 h as described previously for endometrial culture [6, 10, 16]. Cows (n = 3) were artificially inseminated ~12 h after detection of estrus. This day of pregnancy was selected to rep-

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resent a high rate of secretion of IFN-τ [1–3]. Presence of IFN-τ in CSP was confirmed and quantitated using antiserum against rboIFN-τ in Western blots.

Endometrial Explant Culture

Endometrial explants from Day 14 nonpregnant cows (n = 4) were cultured (0.5 g/5 ml) as described previously [6, 10, 16]. Explants were cultured with 0, 0.5, 5, 25, or 100 nM rboIFN-τ (5×10^7 IU/mg) or with rboIFN-α (9×10^7 IU/mg). These concentrations of IFN were based on effective concentrations used in previous experiments [6, 10], saturation of IFN receptors [5], and units of antiviral activity used by others [17]. After culture, medium was treated with the protease inhibitor phenylmethylsulfonyl fluoride (0.1 mM), centrifuged at $2000 \times g$ (20 min) to remove debris, dialyzed (3500 M_r cutoff) against 10 mM Tris-HCl (pH 7.5), and analyzed for the presence of GCP-2 using Western blot. Cytosolic proteins were prepared from explants by homogenizing 100 mg explant tissue in 1 ml Laemmli buffer [18] and analyzed for the presence of UCRP using Western blot.

Primary Epithelial Cell Culture

Enriched epithelial cells were isolated from endometrium collected from a cow on Day 14 of the estrous cycle as described previously [11]. Culture medium consisted of 40% minimal essential medium (MEM; D-valine modified; Sigma Chemical Co., St. Louis, MO), 40% Ham's (D-valine added at 0.00085%), 10% fetal calf serum (heat inactivated), 10% horse serum (heat inactivated), 1% antibiotic antimycotic (Sigma), and insulin (0.2 U/ml). Cells (1×10^6) were plated onto Matrigel-coated (10%; Corning Costar Corp., Cambridge, MA) T75 culture flasks and incubated at 37°C under 5% CO_2.

Growth of stromal cells was inhibited initially through culture with D-valine [19]. Stromal cells also were removed by rinsing flasks in serum-free MEM, adding Dispase II (3 ml; Boehringer-Mannheim, Indianapolis, IN), incubating (2 min), dislodging fibroblasts via agitation, and removing these cells by rinsing twice with serum-free MEM; epithelial cells remained adhered to plates.

After the first passage, epithelial cells were propagated on Matrigel in the presence of serum and MEM/Ham's (with D-valine). After four passages (~1 mo), cells (2×10^6 cells/2 ml) were seeded in six-well plates in the presence of serum but without Matrigel. After reaching ~90% confluence, cells were cultured with CSP (0, 10, 100, or 200 μg/ml), IFNs (0, 25, 50, 100 nM), and phorbol myristate acetate (100, 500, 1000 ng/ml; Sigma) for 24 h in serum-free medium. Medium was dialyzed as described for explant culture and analyzed for the presence of GCP-2 using Western blot. Cells were lysed in 400 μl Laemmli buffer and analyzed for presence of UCRP using Western blot.

Affinity Purification of Antibodies

Synthesis of the UCRP peptide, generation of anti-UCRP antiserum, and affinity purification have been described previously [15]. Synthesis of the GCP-2 peptide and production of anti-GCP-2 peptide antiserum in sheep also have been previously described [7]. Antibodies against GCP-2 were purified from whole serum using affinity chromatography as described for the anti-UCRP antiserum [15]. Titers of both antibodies were 1:25 000 as determined by ELISA using peptide as the antigen [15].

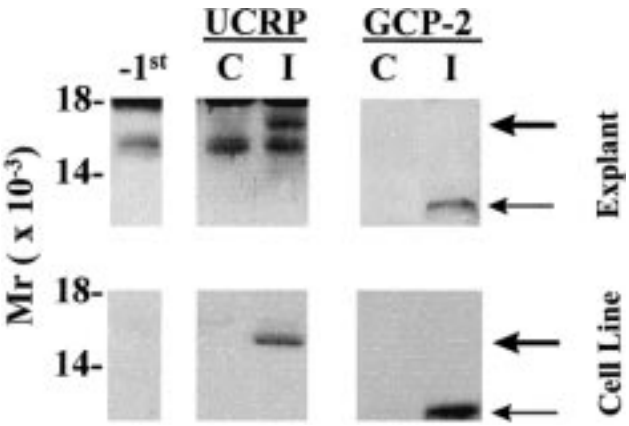

FIG. 1. Representative Western blots using antiserum against UCRP or GCP-2. The control in which primary antiserum was deleted from the Western blot procedure is shown in the first lane (–1st) for endometrial explant (upper blots; Day 14 of the estrous cycle) and primary epithelial cell (lower blots) cultures. Endometrial explants or epithelial cells were incubated in the absence (control: C) or presence (I) of 25 nM rboIFN-τ. UCRP is identified with the larger arrow; GCP-2 is identified with the smaller arrow. Nonspecific interaction by detecting second antiserum did not interfere with detection of UCRP or GCP-2.

Western Blotting

Proteins released into explant and primary epithelial cell culture medium were loaded on one-dimensional (1D) PAGE gels based on amount of protein (100 μg/lane). Volume was not used as an index of equal loading because of variation caused by dialysis. Cytosolic extracts from explants (10 μl) and primary epithelial cells (30 μl) were loaded on 1D-PAGE gels based on equal volume of extract. After 1D-PAGE, proteins were transferred to 0.2-μm nitrocellulose membranes and Western blotted using antiserum against UCRP (1:10 000) or GCP-2 (1:2000) as described previously [7, 15]. Membranes incubated with primary antibodies were washed, incubated at 20°C for 45 min in mouse anti-goat IgG (cross-reacts with sheep IgG; 1:7500; Sigma), washed, and then incubated in anti-mouse IgG coupled to horseradish peroxidase (1:30 000; Sigma). Immunoreactivity with antibodies was detected with Renaissance Western Blot Chemiluminescence Reagent (New England Nuclear, Boston, MA) [15]. All Western blots within an experiment were processed together and exposed to film for the same period of time (5 min). Autoradiographs were scanned from the bottom to the top of the 8-kDa (GCP-2) and 17-kDa (UCRP) bands using densitometry. Nonspecific binding due to second detecting antibodies did not interfere with detection of UCRP and GCP-2 (Fig. 1).

Optical density area was examined using balanced ANOVA procedures. The model for endometrial explant culture was nested for animal (n = 4) because explants from each animal received each concentration of IFN in vitro. Concentration was tested using IFN by animal as the error term. If concentration was shown to be significant ($p < 0.05$), means were separated using Fisher's least significant difference test. The one-way ANOVA model for primary epithelial cell culture (n = 3 replicates) included concentration of CSP, IFN, or phorbol ester. If concentration was shown to be significant, means were separated using Fisher's least-significant-difference test.

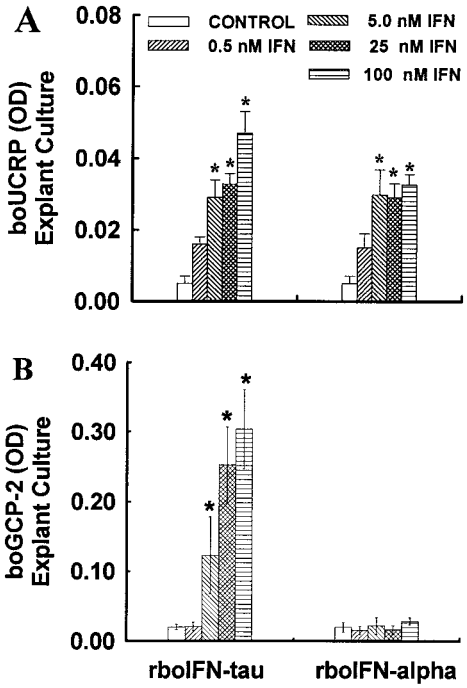

FIG. 2. Induction of cytosolic UCRP (**A**) and GCP-2 (**B**) by rboIFN-τ and rboIFN-α in endometrial explants. Values represent optical density area means ± SE. Means differ from controls when designated with an asterisk ($p < 0.05$).

RESULTS

Induction of UCRP and GCP-2 in Cultured Endometrial Explants

Endometrial explants from nonpregnant cows (Day 14 of estrous cycle) were used to examine induction of UCRP and GCP-2 in vitro. Both rboIFN-τ and rboIFN-α caused concentration-dependent increases in cytosolic UCRP in cultured explants (Fig. 2A). Release of GCP-2 was not affected by rboIFN-α, but it increased in a concentration-dependent response to rboIFN-τ (Fig. 2B). The lowest effective concentration of rboIFN-τ in stimulating release of GCP-2 by cultured endometrial explants was 5 nM.

Induction of UCRP and GCP-2 in Cultured Primary Endometrial Epithelial Cells

Western blot using anti-boIFN-τ antiserum confirmed that IFN-τ was present in CSP (~25 nM IFN-τ/200 μg CSP). All concentrations of CSP caused an increase in cytosolic UCRP in epithelial cells (Fig. 3A). Only the highest concentration of CSP (200 μg/ml) induced release of GCP-2. All concentrations of IFN-τ induced increases in cytosolic UCRP and release of GCP-2 in comparison with control values (Fig. 3B). IFN-α induced cytosolic UCRP, but only the highest concentration (100 nM) caused an increase in GCP-2 (Fig. 3C). The 100 nM concentration of IFN-α was 20 times higher than the lowest effective concentration of IFN-τ used in explant culture. Phorbol ester had no effect on cytosolic UCRP, but all concentrations caused an increase in release of GCP-2 (Fig. 3D).

DISCUSSION

Two uterine proteins, originally called P8 (8 kDa) and P16 (16 kDa), were first identified as IFN-regulated proteins using 1D-PAGE and fluorography [6]. P16 was named

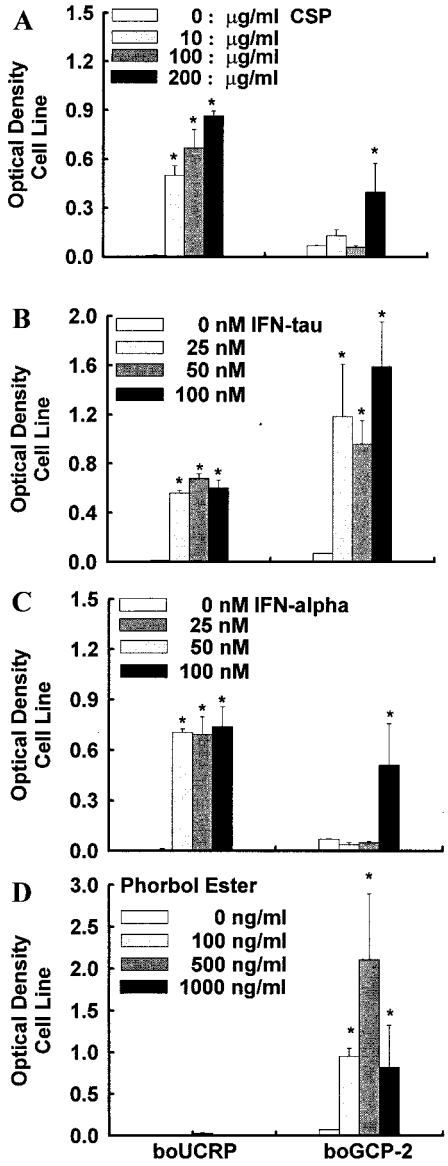

FIG. 3. Induction of UCRP and GCP-2 by CSP known to contain native IFN-τ (**A**), rboIFN-τ (**B**), rboIFN-α (**C**), and phorbol ester (**D**) in cultured primary endometrial epithelial cells. Values represent optical density area means ± SE. Means are different from controls when designated with an asterisk ($p < 0.05$).

UCRP because it cross-reacted with antiserum against ubiquitin [10]. Western blots using antiserum against ubiquitin revealed that endometrial UCRP was present on Days 15–26 of pregnancy, with highest amounts detected on Day 18 of pregnancy. Western blots also revealed that UCRP was released by the endometrium in response to both rboIFN-τ and rboIFN-α. Antiserum against ubiquitin was used to isolate the UCRP cDNA from a bovine endometrial cDNA library. The UCRP cDNA had 30% nucleotide sequence identity with a tandem ubiquitin repeat and 70% identity with the human ISG15 product [11].

Genes stimulated by IFN contain ISREs that bind to phosphorylated STATs [14] and IRFs [20, 21]. The STATs are phosphorylated by receptor-associated tyrosine kinases that are activated after the receptor interacts with type 1 IFNs [22, 23]. Because UCRP shares nucleotide sequence identity with ISG15 and is released in response to IFN-τ and IFN-α, it was suspected that the gene encoding UCRP

had ISREs that were regulated by the Jak/STAT signal transduction pathway. Indeed, recent nucleotide sequence of the gene encoding UCRP revealed that it has five putative upstream consensus ISREs [13].

A second uterine protein that is regulated by IFN-τ has been identified. An 8-kDa endometrial protein called P8 was released by cultured endometrial explants in a concentration-dependent response to rboIFN-τ [6]. Because rboIFN-α had no effect on release of P8, it was hypothesized that P8 might be a marker for IFN-τ-specific action. After purification, analysis of two P8 peptide amino acid sequences revealed 92–100% identity with GCP-2. A GCP-2 peptide was synthesized based on antigenic index and unique amino acid sequence. This peptide was used to generate polyclonal antiserum in sheep. Preliminary Western blot using this antiserum revealed that GCP-2 was secreted by the endometrium in response to rboIFN-τ [7].

In the present experiments, release of UCRP by cultured explants occurred in concentration-dependent responses to rboIFN-τ and rboIFN-α. This response was expected on the basis of previous experiments [10]. However, the present experiments are the first to examine the response of GCP-2 to these rIFNs in vitro. Recombinant boIFN-τ, but not rboIFN-α, caused a concentration-dependent increase in the release of GCP-2 by cultured explants.

It could be argued that rboIFN-τ contains a bacterial contaminant or a toxin that stimulated release of GCP-2. However, CSP known to contain IFN-τ stimulated release of GCP-2 by cultured endometrial epithelial cells. Also, endometrial explants from Day 18–21 pregnant cows released GCP-2 in high amounts in the absence of any exogenous stimulator, whereas endometrium from nonpregnant cows did not release GCP-2 in vitro (our unpublished results).

CSP used in the present experiments (200 μg/ml) contained ~25 nM IFN-τ. Cytosolic UCRP was induced in response to the lowest concentration of CSP (~1.25 nM IFN-τ), whereas the highest concentration of CSP (~25 nM IFN-τ) was required to induce release of GCP-2. These results are interpreted to mean that IFN-τ regulates the uterine endometrium in concentration-dependent mechanisms.

The response to rboIFN-τ and rboIFN-α was similar in endometrial explants and primary epithelial cells with the exception that in the cell line, the highest concentration of IFN-α caused an increase in release of GCP-2. The 100 nM concentration of IFN-α was 20 times higher than the 5 nM concentration of IFN-τ that was effective in eliciting release of GCP-2 in explant culture. Also, the cell line represented purified epithelial cells (and potentially IFN receptors), whereas explant culture represented a mixed cell population. Thus, the cell line may have been more sensitive to the highest concentration of IFN-α. Finally, IFN-α, because it shares 50% sequence identity with IFN-τ, may be able to activate IFN-τ-specific signal transduction at high levels. A precedence for this exists in many endocrine systems: glucocorticoids will activate mineralocorticoid receptors at high concentration [24].

IFNs induce subtle and complex biological effects on target cells. Within the type 1 IFNs, several studies have shown that different molecular forms of IFNs have very different binding kinetics and affinities for their receptors and that receptor binding can vary with source of tissue. For example, IFN-τ binding to endometrial receptors is complex, with both high- and low-affinity components as inferred by curvilinear Scatchard plot and cross-linking experiments [4, 5], whereas binding of rboIFN-α to endometrial membranes is simple as inferred by linear Scatchard plot [5]. Thus, it has been proposed that IFNs exert different biological effects and tissue specificity according to receptor specificity, binding affinity, and transcription factors.

Protein kinases other than the janus kinase family may play a role in IFN signal transduction. The antiviral action of IFN-α can be blocked by the protein kinase C inhibitor, staurosporine [25, 26]. The protein kinase C pathway also has recently been implicated with the action of IFN-τ on the ovine endometrium [27]. IFN-τ suppresses transcription of estrogen and oxytocin receptor genes in ovine endometrium [28]. Using compounds that inhibit or down-regulate protein kinase C, such as staurosporine, phorbol ester, and calphostin C, Flint and coworkers [27] showed that the protein kinase C pathway might be involved in inhibition of the oxytocin receptor by IFN-τ.

How do Jak/STAT/IRF pathways converge or interact with the protein kinase C pathway? Serine in addition to tyrosine phosphorylation is involved with IFN-α signal transduction [29]. STAT-3 binds to the alpha receptor 1 (AR1) subunit of the IFN receptor and becomes tyrosine phosphorylated. The p85 regulatory subunit of phosphatidylinositol 3-kinase then interacts with STAT 3 and becomes tyrosine phosphorylated. Thus, STAT transcription factors may couple other signaling pathways, such as those activated by protein kinase C to IFN receptors.

Bovine GCP-2 is secreted by MDBK cells, but only when treated with phorbol ester [9]. Because GCP-2 was found to be regulated by phorbol ester in MDBK cells, and the protein kinase C pathway had been implicated in IFN-τ action on the uterine endometrium [27], we suspected that endometrial GCP-2 also was regulated by phorbol ester. Both IFN-τ and phorbol ester induced release of GCP-2 by cultured bovine endometrial cells. The intriguing finding in the present experiments was the inability of phorbol ester to induce UCRP. Even though IFN-α induced UCRP, it failed to stimulate release of GCP-2 in endometrial explant culture, and only the highest concentration of IFN-α was effective in inducing release of GCP-2 in the endometrial epithelial cell line.

In summary, GCP-2 is induced by native boIFN-τ (CSP) and by rboIFN-τ in vitro. Because GCP-2 is not induced by rboIFN-α in explant culture and requires high concentrations of rboIFN-α in cultured epithelial cells, we conclude that it can be used as an index of IFN-τ-specific action. The UCRP gene contains five putative ISREs [13]. Both IFN-τ and IFN-α activate the Jak/STAT pathway in the bovine endometrium as suggested by induction of UCRP in endometrial explant and epithelial cell cultures. Finally, phorbol ester had no effect on UCRP but mimicked the effects of IFN-τ by inducing release of GCP-2. It is concluded that IFN-τ signal transduction is complex in the bovine endometrium and that it may involve transcription factors other than or ancillary to those described for the Jak/STAT pathway. Type 1 IFNs compete for binding to a common cell surface receptor. The AR1 subunit of the IFN receptor undergoes ligand-dependent tyrosine phosphorylation. Thus, AR1 may undergo a specific conformational change in response to IFN-τ that allows IFN-τ-specific signal transduction.

Future experiments will delineate cross talk between phorbol ester/diacylglycerol and IFN-τ signal transduction pathways. Perhaps it is as simple as phosphorylation of a transcription factor (STAT, IRF, Jun, or Fos). Or it may be complex and involve a variant IFN-τ receptor subunit, other receptor-associated protein kinases, turnover of phospholipids, or action of novel transcription factors.

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