Effects of Bovine Somatotropin on Uterine Genes Related to the Prostaglandin Cascade in Lactating Dairy Cows*

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ABSTRACT

Multiparous Holstein cows, averaging 80 d in milk, were used to examine the effect of exogenous bovine somatotropin (bST) on uterine expression of estrogen receptor \( \alpha \) (ER\( \alpha \)), prostaglandin endoperoxide synthase-2 (PGHS-2), and peroxisome proliferator-activated receptor \( \delta \) (PPAR\( \delta \)). About 12 h before expected ovulation in a synchronization protocol, cows were assigned to receive bST (500 mg, \( n = 11 \)) or serve as untreated controls (\( n = 10 \)). Cows that ovulated (\( n = 9 \) bST, 8 control) were divided within treatment to be killed on d 3 or 7 postovulation. Samples of intercaruncular endometrial tissue from uterine horns ipsilateral to the corpus luteum were collected and stored at \(-80^\circ\)C for subsequent mRNA analyses. Endometrial concentrations of ER\( \alpha \) and PGHS-2 mRNA transcripts were greater on d 7 than on d 3 of the estrous cycle, but did not differ between treatments. Compared with untreated cows, short-term bST treatment decreased PGHS-2 protein expression at d 7 of the estrous cycle. Concentration of PPAR\( \delta \) mRNA transcript in the uterus decreased between d 3 and 7 of the estrous cycle and was negatively correlated with ER\( \alpha \) and PGHS-2 mRNA concentrations. Short-term administration of bST to lactating dairy cows had minimal effects on uterine genes encoding ER\( \alpha \), PGHS-2, and PPAR\( \delta \) at d 3 and 7 of the estrous cycle but there may be an inverse relationship between PPAR\( \delta \) and uterine expression of ER\( \alpha \) and PGHS-2 genes.

(Key words: uterus, gene, bovine somatotropin, receptor)

Abbreviation key: ER\( \alpha \) = estrogen receptor alpha, PGHS = prostaglandin endoperoxide synthase, PPAR = peroxisome proliferator-activated receptor, TBS = Tris-buffered saline.

INTRODUCTION

Episodic release of PGF\(_{2\alpha}\) from the uterus is responsible for luteolytic signal during the estrous cycle in domestic ruminants (McCracken et al., 1972; Thatcher et al., 1984). It has been proposed that estrogens from ovarian follicles interact with estrogen receptor alpha (ER\( \alpha \)) to increase oxytocin receptor gene expression, which ultimately leads to pulsatile release of PGF\(_{2\alpha}\) (Beard and Lamming, 1994; Kim et al., 2003). The rate-limiting step in eicosanoid synthesis is the cleavage of sn-2 fatty acyl ester bond of membrane phospholipids by cytosolic phospholipase A\(_2\) (Van den Bosch, 1980; Irvine, 1982). The arachidonic acid that is released by phospholipid hydrolysis is acted on by prostaglandin endoperoxide synthase (PGHS) to form PGH-2, which then is converted to PGF by a reductase. There are 2 forms of PGHS that have been characterized, a constitutively expressed PGHS-1, and an induced PGHS-2 (Smith et al., 1996). In cattle, the synthesis and activity of PGHS-2 must be attenuated for pregnancy to be maintained (Thatcher et al., 1997). Whether and how exogenous bST interacts with the PGF cascade in cattle has not been fully elucidated.

Peroxisome proliferator-activated receptors (PPAR) have been studied traditionally for their roles in lipid metabolism and metabolic diseases (Chinetti et al., 2000). There are 3 subtypes of PPAR (PPAR\( \alpha \), \( \delta/\beta \), and \( \gamma \)), all of which have distinct patterns of expression and functional roles (Braissant and Wahli, 1998). Subtype PPAR\( \alpha \) is mainly expressed in tissues in which fatty acid catabolism is significant, such as the liver, heart, and skeletal muscle (Jalouli et al., 2003; Lee et al., 2003; Schiffrin et al., 2003). Subtype PPAR\( \gamma \) is highly expressed in adipose tissue, where it regulates adipocyte differentiation, lipid storage, and insulin sensitivity (Chawla et al., 2003; Schiffrin et al., 2003). Much less is known about the function of PPAR\( \delta \), although it is highly expressed in the brain, colon, and skin (Braissant and Wahli, 1998; Matsuura et al., 1999; Mano et al., 2000). In mice, PPAR\( \delta \) deficiency leads to placental defects and results in frequent midgestational lethality (Barak et al., 2002), suggesting that this nuclear recep-
tor may play an important role in the control of reproductive processes in mammalian species.

Administration of recombinant bST to dairy cows has become a common management practice in the United States for enhancing milk production (Peel and Boman, 1987). However, this practice has raised numerous concerns, as exogenous bST initially was shown to decrease fertility in dairy cattle (Cole et al., 1992; Zhao et al., 1992). It was suggested that the negative effect of supplemental bST on fertility might be caused, in part, by lower estrous detection rates (Kirby et al., 1997; Lefevre and Block, 1992). Recent studies indicated that exogenous bST increased pregnancy rates in lactating dairy cows when administered at estrus in repeat breeding cows (Morales-Roura et al., 2001) or when combined with a regimen for synchronization of ovulation and timed AI (Moreira et al., 2000, 2001). This raises the possibility that bST administration to dairy cows during the peri-ovulatory period may have a positive effect on the endocrine and biochemical signals between the conceptus and maternal uterus at the time of pregnancy establishment.

Given the lack of information relating uterine components of the PGF cascade and supplemental bST, the objective of this study was to examine the effect of exogenous bST on uterine endometrial expression of ERα, PGHS-2, and PPARδ in lactating Holstein cows, and to determine whether those responses vary with stage of the estrous cycle.

MATERIALS AND METHODS

Experimental Design

Twenty-one multiparous Holstein cows (average DIM = 80) were used in a completely randomized design to examine the effect of exogenous bST on uterine endometrial expression of ERα, PGHS-2, and PPARδ during the estrous cycle. Detailed cow management and experimental manipulations were described previously (Per- shing et al., 2002). Briefly, lactating Holstein cows were milked 3 times daily and fed a TMR that contained 1.65 Mcal/kg and 18.1% CP (DM basis) during the entire experiment (March to May 2000). At approximately 44 DIM, cows were presynchronized with an injection of GnRH (Cystorelin, Merial Ltd., Iselin, NJ; 100 mg, i.m.) followed 7 d later with PGF2α (Lutalyse, Pharmacia Animal Health, Kalamazoo, MI; 25 mg, i.m.). Twelve days after the PGF2α injection, the Ovsynch program was initiated with an i.m. injection of GnRH (100 mg) followed 7 d later with PGF2α (25 mg). Forty-eight hours after the administration of PGF2α, cows received a second dose of GnRH to induce ovulation. On the day of expected ovulation (approximately 16 h after the second GnRH administration), cows were assigned randomly to serve as untreated controls (n = 10) or to receive an i.m. injection of bST (Posilac, Monsanto Co., St. Louis, MO; 500 mg). Within each treatment, cows that ovulated (n = 9 bST, 8 control) were killed on either d 3 or 7 following initiation of bST treatment. Ovulation was verified by ultrasonography within 48 h of the second GnRH dose and later confirmed at slaughter.

RNA Isolation and Analysis

Reproductive tracts were collected and brought to the laboratory within 15 min of slaughter. Uterine horns ipsilateral to the corpus luteum were trimmed free of the broad ligament, and samples (~5 g) of intercaruncular endometrial tissue were collected and immediately frozen at ~80°C for subsequent RNA and Western blot analyses. Total cellular RNA was isolated from endometrial tissues using TriZol reagent (Life Technologies, Grand Island, NY) following the manufacturer’s instructions. Samples of RNA (20 µg/lane) were fractionated in a 1% (wt/vol) agarose-formaldehyde gel, blotted to Biotrans nylon membranes (ICN, Irvine, CA), and prehybridized in Rapid-Hyb buffer (Life Technologies, Piscataway, NJ) at 60°C for 1 h. The filters then were hybridized for 2 h at 60°C in Rapid-Hyb buffer with 32P-labeled ERα (Ing et al., 1996), bovine PGHS-2 (a gift from Jean Sirois, Universite de Montreal, St-Hyacinthe, Canada), and bovine PPARδ (amplified by reverse transcription-PCR from bovine endometrial RNA) cDNA probes. The cDNA probe for PPARδ was generated using a set of primers (forward: 5′-CACCTCACTGCTGGACAA-3′; reverse: 5′-TGCGGTTCTTCTCTGAGATT-3′) designed from the bovine PPARδ sequence (GenBank accession number: AF229357). The size (216 bp) and identity of the PCR product were further verified by DNA sequencing before its use in Northern blot analyses. The membranes were washed sequentially in 2× saline sodium citrate/0.1% (wt/vol) SDS at room temperature for 20 min and 0.1× saline sodium citrate/0.1% (wt/vol) SDS at 50°C for 15 min. Hybridization signals were detected by exposing membranes to x-ray film (X-Omat Blue XB 1, Eastman Kodak Co., Rochester, NY) at ~80°C for 24 to 48 h. Hybridization signals for each target gene were quantified by densitometric scanning (Kodak Electrophoresis Documentation and Analysis System 290, Eastman Kodak Co.). Following autoradiography, the membranes were stripped with 1% (wt/vol) SDS and rehybridized with 18S ribosomal RNA probe to verify the consistency of RNA loading and specificity of treatment effect.

Western Blot Analysis

The abundance of ERα, PGHS-2, and PPARδ in uterine endometrium was examined by Western blot analy-
Figure 1. Effects of bST on uterine endometrial expression of estrogen receptor α (ERα) mRNA in control (d 3, lanes 1 to 3; d 7, lanes 1 to 5) and bST-treated (d 3, lanes 4 to 7; d 7, lanes 6 to 10) cows. Each lane represents a different cow. Day of estrous cycle differed, $P = 0.02$.

Total protein was extracted from intercaruncular endometrial tissues (300 to 400 mg) by tissue homogenization in ice-cold lysis buffer [50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2 mM EDTA, 2 mM ethylene glycol-bis (2-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid (EGTA), 50 mM NaF, 1% (wt/vol) Nonidet P-40, 20 mM b-glycerophosphate, 2 mM Na$_3$VO$_4$, 10% (wt/vol) glycerol, 1 mM phenylmethylsulfonyl fluoride, and 10 g/mL each of aprotinin, leupeptin, and pepstatin; Binelli et al., 2000]. Protein concentrations in cell lysates were determined by the Bradford method (Bradford, 1976), with BSA used as the standard. For each endometrial sample, 100 μg of protein was subjected to 7.5% (wt/vol) SDS-PAGE in the presence of β-mercaptoethanol followed by electrophoresis onto a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech) at 4°C overnight. Following electrophoresis, the membranes were transiently stained with Ponceau S solution (Sigma Chemical Co., St. Louis MO) to determine the efficiency of protein transfer. The membranes were blocked in Tris-buffered saline (TBS) containing 5% (wt/vol) Carnation instant nonfat dry milk for 2 h at
Figure 2. Effects of bST on uterine endometrial expression of prostaglandin endoperoxide synthase-2 (PGHS-2) mRNA in control (d 3, lanes 1 to 3; d 7, lanes 1 to 5) and bST-treated (d 3, lanes 4 to 7; d 7, lanes 6 to 10) cows. Each lane represents a different cow. Day of estrous cycle differed, \( P < 0.01 \).

Statistical Analyses

Messenger RNA and protein responses were evaluated by least squares ANOVA using the GLM procedure of the SAS software package (SAS Inst., Inc., Cary, NC). The mathematical model for uterine mRNA and protein responses included treatment, day of estrous cycle, and treatment \( \times \) day interaction. For all target genes, the densitometric values were expressed as ratios of target mRNA or protein.
**Figure 3.** Effects of bST on uterine endometrial expression of peroxisome proliferator-activated receptor δ (PPARδ) mRNA in control (d 3, lanes 1 to 3; d 7, lanes 1 to 4) and bST-treated (d 3, lanes 4 to 7; d 7, lanes 5 to 9) cows. Each lane represents a different cow. Day of estrous cycle differed, $P < 0.01$.

**Table 1.** Pearson (upper right) and partial1 (lower left) correlation coefficients for uterine endometrial estrogen receptor α (ERα), prostaglandin endoperoxide synthase-2 (PGHS-2), and peroxisome proliferator-activated receptor δ (PPARδ) mRNA transcripts.

<table>
<thead>
<tr>
<th></th>
<th>ERα</th>
<th>PGHS-2</th>
<th>PPARδ</th>
</tr>
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<tbody>
<tr>
<td>ERα</td>
<td>—</td>
<td>0.84**</td>
<td>−0.52**</td>
</tr>
<tr>
<td>PGHS-2</td>
<td>0.87**</td>
<td>—</td>
<td>−0.81**</td>
</tr>
<tr>
<td>PPARδ</td>
<td>0.07</td>
<td>0.19</td>
<td>—</td>
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</tbody>
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1Partial correlations were adjusted for treatment, day of estrous cycle, and treatment × day interaction. **Significant correlation at $P < 0.01$.

**RESULTS**

**Uterine ERα, PGHS-2, and PPARδ Genes**

Uterine endometrial mRNA encoding ERα, PGHS-2, and PPARδ were readily detectable on d 3 and 7 of the
Figure 4. Effects of bST on endometrial concentrations of estrogen receptor α (ERα) protein in control (d 3, lanes 1 to 3; d 7, lanes 1 to 5) and bST-treated (d 3, lanes 4 to 7; d 7, lanes 6 to 10) cows. Each lane represents a different cow.

estrous cycle in lactating Holstein cows (Figures 1, 2, and 3). Northern blot analyses identified single mRNA transcripts for ERα (6.8 kb; Figure 1A), PGHS-2 (4.4 kb; Figure 2A), and PPARδ (3.7 kb; Figure 3A) genes. When averaged within treatments and stages of the estrous cycle, concentrations of ERα and PGHS-2 mRNA transcripts in the endometrium were greater (P < 0.05) on d 7 than on d 3 of the estrous cycle (Figures 1B and 2B). Conversely, the abundance of endometrial PPARδ mRNA transcript decreased (P < 0.01) between d 3 and 7 of the estrous cycle (Figure 3B). There were no detectable differences between control and bST-treated cows on either d 3 or d 7 of the estrous cycle for all 3 genes (Figures 1B, 2B, and 3B). Pearson and partial correlation analyses revealed a positive relationship (P < 0.01) between endometrial ERα and PGHS-2 mRNA concentrations (Table 1). When unadjusted for treatment, stage of the estrous cycle, and among-cow variations, both ERα and PGHS-2 mRNA concentrations were negatively correlated (P < 0.01) with that of PPARδ mRNA (Table 1). However, the apparent negative relationships between PPARδ and either ERα or PGHS-2 mRNA were no longer detectable when mRNA responses were adjusted for treatment and stage of the estrous cycle (Table 1).

**Uterine ERα, PGHS-2, and PPARδ Proteins**

Western immunoblotting revealed 3 isoforms for ERα protein (MW = 53 to 67 kDa; Figure 4A), a doublet for PGHS-2 protein (MW = 70 to 72 kDa; Figure 5A), and 4 isoforms for PPARδ protein (MW = 47 to 67 kDa; Figure 6A). Results for the ERα protein are consistent with a previous report, which identified 3 to 4 isoforms
of rat pituitary ERα protein when probed with an anti-ERα polyclonal antibody (Pasqualini et al., 1999). The doublet detected for immunoreactive PGHS-2 protein is also consistent with the literature data on cyclooxygenase-2, which tends to resolve as a doublet on Western immunoblots (Habib et al., 1993; Crew et al., 2000). Western blot analysis revealed multiple bands for the PPARδ protein (Figure 6A). The anti-PPARδ polyclonal antibody (H-74) used in this study reacts with PPARδ of mouse, rat, and human origin but does not cross-react with PPARα or PPARγ. Thus, the multiple bands detected for the PPARδ protein in the current study may represent distinct isoforms of PPARδ in bovine uterine endometrial tissue.

When averaged within treatments and stages of the estrous cycle, the concentration of the full length ERα protein (67 kDa) was unaffected by bST or day of the estrous cycle (Figure 4B). A treatment × day interaction was detected (P < 0.05) for endometrial PGHS-2 protein abundance. Compared with control cows, bST decreased endometrial PGHS-2 concentration at d 7 of the estrous cycle (Figure 5B). When pooled together, the abundance of PPARδ isoforms did not differ between control and bST-treated cows (Figure 6B). There were no apparent relationships among ERα, PGHS-2, and PPARδ protein concentrations in the endometrium of cyclic Holstein cows (Table 2).

**DISCUSSION**

The observation that bST receptors are expressed in several reproductive tissues (Kirby et al., 1996) raises the possibility that bST may play a direct role in the control of mammalian reproductive function. In the
present study, uterine endometrial ER\textsubscript{\alpha} and PGHS-2 mRNA concentrations were greater on d 7 than on d 3 of the estrous cycle, but did not differ between control and bST-treated cows. These findings are consistent with a previous report that detected no growth hormone effects on uterine endometrial ER\textsubscript{\alpha} gene expression in ovariectomized ewes receiving ovarian steroid replacement therapy (Spencer et al., 1999). The lack of growth hormone effect on PGHS-2 agrees with a previous in vitro study which detected no growth hormone effects on steady-state PGHS-2 mRNA concentration in cultured bovine endometrial cells (Badinga et al., 2002).

In contrast with our findings, Guzeloglu et al. (2004) detected a stimulatory effect of bST on endometrial ER\textsubscript{\alpha} and PGHS-2 synthesis at d 17 of the estrous cycle in nonlactating Holstein cows. These observations collectively suggest that the net effect of supplemental growth hormone on endometrial ER\textsubscript{\alpha} and PGHS-2 syn-

Table 2. Pearson (upper right) and partial\textsuperscript{1} (lower left) correlation coefficients for uterine endometrial estrogen receptor \textsubscript{\alpha} (ER\textsubscript{\alpha}), prostaglandin endoperoxide synthase-2 (PGHS-2), and peroxisome proliferator-activated receptor \textsubscript{\delta} (PPAR\textsubscript{\delta}) proteins.

\begin{tabular}{|c|c|c|}
\hline
 & ER\textsubscript{\alpha} & PGHS-2 \\
\hline
ER\textsubscript{\alpha} & — & 0.01 \\
PGHS-2 & -0.46 & — \\
PPAR\textsubscript{\delta} & 0.26 & -0.34 \\
\hline
\end{tabular}

\textsuperscript{1}Partial correlations were adjusted for treatment, day of estrous cycle, and treatment \times day interaction. None of the correlations were significant.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6.png}
\caption{Effects of bST on endometrial concentrations of peroxisome proliferator-activated receptor \textsubscript{\delta} (PPAR\textsubscript{\delta}) protein in control (d 3, lanes 1 to 3; d 7, lanes 1 to 5) and bST-treated (d 3, lanes 4 to 7; d 7, lanes 6 to 10) cows. Each lane represents a different cow.}
\end{figure}
thesis may vary depending on the physiological state of the experimental animal (i.e., lactation, stage of estrous cycle, and pregnancy).

Uterine ERα is known to be up regulated by estradiol (Wu et al., 1996; Ing and Tornesi, 1997; Ing and Ott, 1999) and is generally maximally expressed at estrus in the bovine uterus (Meikle et al., 2001). In the cow, peripheral estradiol exhibits 1 or 2 secondary peaks coinciding with the emergence of 1 or 2 nonovulatory dominant follicles during the interestrous interval (Savio et al., 1988; Sirois and Fortune, 1988; Badinga et al., 1992). It is conceivable that the greater ERα and PGHS-2 mRNA concentrations detected in the bovine endometrium at d 7 of the estrous cycle may reflect the subtle rise in plasma E2 that originates from the endometrium at d 7 of the estrous cycle and PGHS-2 increased between d 3 and 7 of the estrous cycle. Negative correlations between the PPARδ gene and both ERα and PGHS-2 genes were apparently dependent on stage of the estrous cycle because the inverse relationships no longer existed when the mathematical models were adjusted for stage of the estrous cycle. Positive Pearson and partial correlations were detected between endometrial ERα and PGHS-2 mRNA concentrations, further supporting the concept that ERα may be the necessary physiological mediator of endometrial PGF2α biosynthesis in cattle. The apparent lack of bST effect on expression of PGHS-2 was in contrast to a recent report in nonlactating Holstein cows (Guzeloglu et al., 2004), and would suggest that the net effect of supplemental bST on uterine ERα and PGHS-2 synthesis varies depending on the physiological state of the experimental animal.

CONCLUSION

Results provide a direct evidence for PPARδ gene expression in the bovine uterus. Endometrial PPARδ mRNA concentration decreased, whereas that of ERα and PGHS-2 increased between d 3 and 7 of the estrous cycle. Negative correlations between the PPARδ gene and both ERα and PGHS-2 genes were apparently dependent on stage of the estrous cycle because the inverse relationships no longer existed when the mathematical models were adjusted for stage of the estrous cycle. Positive Pearson and partial correlations were detected between endometrial ERα and PGHS-2 mRNA concentrations, further supporting the concept that ERα may be the necessary physiological mediator of endometrial PGF2α biosynthesis in cattle. The apparent lack of bST effect on expression of PGHS-2 was in contrast to a recent report in nonlactating Holstein cows (Guzeloglu et al., 2004), and would suggest that the net effect of supplemental bST on uterine ERα and PGHS-2 synthesis varies depending on the physiological state of the experimental animal.

REFERENCES


