Effects of BST on Oviductal and Uterine Genes Encoding Components of the IGF System in Lactating Dairy Cows¹

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ABSTRACT

Lactating Holstein cows, averaging 80 d in milk, were used to examine effects of exogenous bovine somatotropin (bST) on oviductal and uterine genes encoding components of the insulin-like growth factor (IGF) system. About 12 h before expected ovulation in an Ovsynch protocol, cows were assigned randomly 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 sacrificed on d 3 or 7 postovulation. Samples of oviductal and intercaruncular endometrial tissue from oviducts and uterine horns ipsilateral to the corpus luteum (CL) were collected and immediately frozen at -80°C for subsequent mRNA analyses. Northern blots revealed mRNAs for IGF-II, IGF-binding protein-2 (IGFBP-2), and IGFBP-3 in all oviductal and endometrial tissues. Significant amounts of IGF-I and growth hormone receptor-1A (GHR-1A) mRNAs were detected in uteri but not in oviducts. The bST treatment had no effect on amount of IGF-I mRNA transcript in uterine endometrium. The mRNA encoding IGF-II was induced by bST in oviducts collected on both d 3 and 7 but was down-regulated in endometrium on d 7. Transcript of IGFBP-2 mRNA was greater in endometrial than oviductal tissues and did not differ between treatments. Both oviductal and endometrial IGFBP-3 mRNA concentrations increased between d 3 and 7 postovulation, with a tendency for greater endometrial IGFBP-3 mRNA in bST-treated cows on d 7. On d 7, concentrations of endometrial GHR-1A mRNA were 30% lower in bST-treated cows. Results indicate complex and tissue-specific regulation of the uterine IGF system components by exogenous bST. Some of

those biological responses to bST may be important in early development of bovine embryos.

(Key words: bovine somatotropin, uterus, insulin-like growth factor, cattle)

Abbreviation key: GHR = growth hormone receptor, IGFBP = insulin-like growth factor binding protein.

INTRODUCTION

During the estrous cycle and pregnancy, the mammalian uterus undergoes substantial morphological and functional differentiation that is paralleled by highly coordinated changes in uterine expression of insulin-like growth factors (IGF-I and IGF-II), IGF receptors, and IGF binding proteins (**IGFBP**; Simmen et al., 1995; Wathes et al., 1998). Because uterine endometrium and myometrium contain relatively high concentrations of IGF receptors (Talavera et al., 1990; Hofig et al., 1991), it has been suggested that these ligands may constitute important autocrine and paracrine effectors of uterine differentiation and embryonic development.

Under physiological conditions, essentially all circulating IGF ligands are complexed to soluble IGFBP. These proteins share the ability to bind IGF-I and IGF-II with high affinity, and often are associated with cell membranes and extracellular matrix where they can influence interactions of IGF with IGF receptors and possibly exert IGF-independent functions (Rechler, 1993; Jones and Clemmons, 1995). In the pig uterus, IGFBP-2 gene expression was induced during the periimplantation period and reached maximal concentrations at mid-pregnancy (d 60; Song et al., 1996). The concentrations of IGFBP-3 and IGFBP-4 transcripts were elevated in endometrium after implantation, whereas IGFBP-5 and IGFBP-6 mRNAs were in greater abundance in periimplantation than postimplantation endometrium (Song et al., 1996). In the cow, the abundance of endometrial IGFBP-2 mRNA increased between 10 and 18 d postestrus (Geisert et al., 1991), and pregnancy was associated with reduced amounts of IGFBP-3 in uterine luminal fluids (Keller et al., 1998). The unique temporal and spatial pattern of

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mRNA expression exhibited by each member of the IGFBP family suggests that these binding proteins may have distinct functions in the development of specific tissues.

Recent studies indicated that exogenous bST increases pregnancy rates in lactating dairy cows when used 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; Moreira et al., 2001). This raised the possibility that supplemental bST may stimulate local production of the IGF family of growth factors that are essential for early blastocyst development and embryonic growth. The objective of this study was to characterize bST-induced changes in oviductal and uterine genes that encode components of the IGF system in lactating dairy cows.

MATERIALS AND METHODS

Animals and Experimental Design

The experiment was conducted at the University of Florida Dairy Research Unit (Hague, FL) during the months of March through May, 2000. Lactating dairy cows were kept in free-stall facilities and milked three times daily at approximately 8-h intervals. Cows were fed a TMR that contained 1.65 Mcal of NE_I/kg and 18.1 CP (percentage of DM) during the entire experiment. At approximately 44 d in milk, 21 cyclic, lactating Holstein cows were presynchronized with an injection of GnRH (Cystorelin, Merial, Ltd., Iselin, NJ; 100 µg, i.m.) followed 7 d later with PGF_{2α} (Lutalyse, Pharmacia Animal Health, Kalamazoo, MI; 25 mg, i.m.; Badinga et al., 1994). Twelve d after the PGF_{2α} injection, the Ovsynch program was initiated with an injection of GnRH (Cystorelin, 100 µg, i.m.) followed 7 d later with PGF_{2α} (Lutalyse, 25 mg, i.m.). At 48 h after PGF_{2α}, cows received a second injection of GnRH to induce ovulation. At approximately 16 h after the second GnRH administration, cows were assigned randomly to receive bST (Posilac, Monsanto Co., St. Louis, MO; 500 mg, i.m.; n = 11) or serve as untreated controls (n = 10). Within each treatment group, cows were sacrificed either on d 3 or 7 following initiation of bST treatment. Ovulation was verified by ultrasonography within 48 h of the second GnRH injection and later confirmed at slaughter. All animals were sacrificed in the abattoir of the Meats Laboratory of the University of Florida Animal Sciences Department.

Sample Collection and Analysis

Reproductive tracts were collected, placed on ice, and brought to the laboratory within 15 min of slaughter.

Oviducts and uterine horns ipsilateral to the corpus luteum (CL) were trimmed free of the broad ligament, and samples (4 to 5 g) of oviductal and intercaruncular endometrial tissue were collected and immediately frozen at -80°C for subsequent RNA analyses.

Total cellular RNA was isolated from oviductal and endometrial tissues with TriZol reagent (Life Technologies, Grand Island, NY) following the manufacturer's instructions. For mRNA analyses, 30 µg of total cellular RNA were fractionated in a 1.0% agarose-formaldehyde gel and blotted to a Biotrans nylon membrane (ICN, Irvine, CA) via capillary action. The RNA was cross-linked to the membrane by UV irradiation and baked at 80°C for 1 h. RNA blots were prehybridized with Rapid-Hyb buffer (Amersham-Pharmacia Biotech., Piscataway, NJ) at 60°C for 30 min. The filters were then hybridized consecutively with random primer-labeled bovine IGF-I, IGF-II, IGFBP-2, IGFBP-3, and GHR-1A cDNA probes (Feinberg and Vogelstein, 1983). After hybridization, RNA filters were washed for 20 min in 50 ml 2X SSC, 0.1% SDS at room temperature, followed by two 15-min washes in 0.1X SSC, 0.1% SDS at 42°C. The filters were blotted dry and exposed to X-ray film (X-OMAT, Eastman Kodak, Rochester, NY) for 24 to 48 h at -80°C. Hybridization signals for each target gene were quantified by densitometric analysis.

Statistical Analysis

During the experiment, four cows failed to ovulate (3 control cows and 1 bST cow), resulting in their elimination from the experiment. Thus, all statistical analyses were performed using seven cows for d 3 (3 control and 4 bST cows) and 10 cows for d 7 (5 control and 5 bST cows). Messenger RNA responses to bST were evaluated by Least squares ANOVA using the General Linear Models procedure of SAS (1988). The mathematical models included the main effects of treatment, day of estrous cycle, tissue, and all appropriate two- and three-way interactions. The variance for cow, nested within treatment × day interaction, was used as random error term to test the effects of treatment, day of the estrous cycle, and treatment × day interaction. Densitometric values for target genes were adjusted using the values for 18S ribosomal RNA staining as covariates.

RESULTS

Northern blots revealed mRNAs for IGF-II, IGFBP-2, and IGFBP-3 in all oviductal and endometrial tissues. Significant amounts of IGF-I and GHR mRNA transcripts were detected in the uterine endometrium but not in the oviduct. Short-term bST treatment had no detectable effect on the abundance of IGF-I mRNA tran-

Figure 1. Effects of bST on endometrial IGF-I mRNA expression 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.

script in the endometrium (Figure 1). A treatment $\times$ day $\times$ tissue interaction was detected ($P < 0.002$) for IGF-II gene expression. In the oviduct, steady-state levels of IGF-II mRNA transcript were greater ($P < 0.0001$) in bST-treated than control cows on both d 3 and 7 of the synchronized estrous cycle (Figure 2). In contrast, IGF-II mRNA in the uterine endometrium was lower ($P < 0.002$) in bST-treated than in control cows at d 7 of the estrous cycle, but not on d 3 (Figure 3).

Messenger RNA levels for IGFBP-2 were greater ($P < 0.0001$) in the endometrium than the oviduct but did not differ between stages of the estrous cycle or treatments (Figures 4 and 5). On average, oviductal and uterine endometrial IGFBP-3 mRNA levels increased ($P < 0.0003$) between d 3 and 7 of the estrous cycle (Figures 6 and 7), and there was a tendency ($P < 0.09$) for greater endometrial IGFBP-3 mRNA abundance on d 7 for bST-treated cows (Figure 7).

A treatment $\times$ day interaction was detected ($P < 0.04$) for uterine endometrial expression of GHR-1A mRNA. Compared to control cows, bST decreased the amount

Figure 2. Effects of bST on oviductal IGF-II mRNA expression 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.

of GHR mRNA transcript at d 7 of the estrous cycle (Figure 8).

DISCUSSION

Bovine Somatotropin has been shown to have negative (Burton et al., 1990; Cole et al., 1992; Luna-Dominiguez et al., 2000), positive (Moreira et al., 2000; Morales-Roura et al., 2001; Moreira et al., 2001), or no (Erdman et al., 1990; Pell et al., 1992; Oldenbroek et al., 1993) effects on fertility responses in dairy cattle. Because cows treated with bST do not express estrus as intensely as untreated controls (Lefebvre and Block, 1992; Waterman et al., 1993; Kirby et al., 1997), it has been suggested that bST may increase the percentage of undetectable ovulations through alteration of behavioral centers within the brain that control expression of estrus. When a reproductive management system that eliminates the need for estrous expression was used in lactating dairy cows (Moreira et al., 2000; Moreira et al., 2001), it was observed that administration of bST increased pregnancy rates to the synchronized service. This raised the possibility that supplemental bST may stimulate local production of IGF family of

Figure 3. Effects of bST on endometrial IGF-II mRNA expression 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 (treatment $\times$ day interaction, $P < 0.002$).

growth factors that are essential for oviductal and uterine differentiation and early embryonic development.

This study extends previous observations on unique temporal and tissue-specific expression of IGFs and IGFBPs in the bovine reproductive tract during early stages of the estrous cycle (Geisert et al., 1991; Kirby et al., 1996; Robinson et al., 2000). The mRNAs encoding IGF-II, IGFBP-2, and IGFBP-3 were detected in all oviductal and endometrial tissues examined, whereas significant amounts of IGF-I and GHR mRNA transcripts were detected in the uterine endometrium and not in the oviduct. The abundance of endometrial IGF-I mRNA transcript did not vary between stages of the estrous cycle or treatments. These results agreed with previous studies that showed no significant variations in endometrial IGF-I mRNA transcript between early and mid-luteal phases of the estrous cycle in beef (Geisert et al., 1991) and Holstein (Robinson et al., 2000; Meikle et al., 2001) cows. Other studies have demonstrated that endometrial IGF-I mRNA levels are highest around estrus and lowest during the early and mid-luteal phases of the estrous cycle, suggesting that uterine IGF-I gene expression is up-regulated by estradiol. Thus, the trend for higher IGF-I mRNA levels detected

Figure 4. Effects of bST on oviductal IGFBP-2 mRNA expression 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.

at d 7 of the synchronized estrous cycle in this study may reflect a transient up-regulation of uterine IGF-I gene expression by peripheral estradiol secreted by the first-wave dominant follicle (Badinga et al., 1992). The lack of bST stimulation of IGF-I mRNA in the uterus is in agreement with a previous study (Kirby et al., 1996), but does not rule out that the bST signaling in the uterus may involve pathways that were not measured in the present study. Alternatively, Northern blot hybridization may not have been sensitive enough to detect minor changes in uterine endometrial IGF-I transcript induced by exogenous bST.

Results of this study provide the first direct evidence that bovine oviductal IGF-II mRNA is induced by supplemental bST as early as d 3 of a synchronized estrous cycle. Although the exact mechanism for bST stimulation of oviductal IGF-II gene expression awaits further investigation, our data would support a role for IGF-II in the control of oviductal differentiation and early embryonic cleavage. The observation that bST down-regulated IGF-II transcript in the endometrium at d 7 of the estrous cycle, when IGF-I mRNA transcript tended to increase, would suggest that these two mitogens may have either overlapping or distinct roles in

Figure 5. Effects of bST on endometrial IGFBP-2 mRNA expression 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.

Figure 6. Effects of bST on oviductal IGFBP-3 mRNA expression 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 (day effect, $P < 0.0003$).

the control of oocyte cleavage and subsequent blastocyst development in cattle.

The messenger RNA encoding IGFBP-2 is expressed readily in the porcine (Song et al., 1996), ovine (Cann et al., 1997; Reynolds et al., 1997), and bovine (Geisert et al., 1991; Kirby et al., 1996; Robinson et al., 2000) uterus. In the present study, the abundance of IGFBP-2 transcript was greater in endometrial than oviductal tissues but was unaffected by stage of the estrous cycle or bST treatment. Similar to our data, Keller et al. (1998) found no differences in bovine endometrial IGFBP-2 activity due to stage of the estrous cycle or pregnancy when evaluated on d 13 and 15 postestrus. In uterine tissue of ewes, IGFBP-2 mRNA was not detected until d 29 of gestation, when intense hybridization was localized to the dense stroma adjacent to the luminal epithelium (Reynolds et al., 1997). In the pregnant pig uterus, IGFBP-2 gene expression was induced during the periimplantation period and reached maximal levels by d 60 of pregnancy (Song et al., 1996). The extent to which IGFBP-2 may have a modulatory role in uterine function during embryonic development remains to be substantiated. The fact that IGFBP-2 can interact with the extracellular matrix through an argi-

nine-glycine-aspartic acid sequence has led to the speculation that binding of IGFBP-2 to the extracellular matrix in the reproductive tract may act to increase local concentration of IGFs in the tissue or enhance transport of locally produced IGFs across the tissue into the uterine lumen (Keller et al., 1998). Additionally, IGFBP-2 may regulate oviductal and uterine cell mitogenesis and differentiation through IGF-independent mechanisms, as demonstrated in cultures of porcine endometrial glandular epithelial cells (Badinga et al., 1999). The apparent lack of uterine and oviductal IGFBP-2 response to exogenous bST suggests that this binding protein may not be a major component of bST signaling mechanism in the uterus.

On average, oviductal and endometrial IGFBP-3 mRNA levels increased between d 3 and 7 of the estrous cycle, and there was a tendency for greater endometrial IGFBP-3 mRNA abundance in d-7 bST-treated cows. Robinson et al. (2000) detected no significant changes in the expression of endometrial IGFBP-3 mRNA throughout the estrous cycle, and postulated that, in cows, IGFBP-3 mRNA may not be regulated by ovarian steroid hormones. In contrast, other studies have reported that IGFBP-3 mRNA, like the IGF-I transcript,

Figure 7. Effects of bST on endometrial IGFBP-3 mRNA expression 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 (treatment effect, $P < 0.09$).

is under direct estradiol control (Johnson et al., 1996; Simpson et al., 1997). This led us to the speculation that the greater abundance of IGFBP-3 transcript detected on d 7 of the estrous cycle in this study may be indicative of a transient up-regulation of IGFBP-3 gene expression by circulating estradiol secreted by the newly selected first-wave dominant follicle (Badinga et al., 1992). The tendency for higher IGFBP-3 message in bST-treated dairy cows is consistent with previous studies that showed higher serum IGFBP-3 concentrations in bST-treated beef (Rausch et al., 2002) and lactating dairy (Cohick et al., 1992) cows. Bovine somatotropin appeared to up-regulate IGFBP-3 transcript at a stage of the estrous cycle when luminal IGFBPs undergo substantial proteolytic cleavage in dairy cattle (L. Badinga, R. A. Pershing, and W. W. Thatcher, data not shown). Thus, whether and how bST-induced endometrial IGFBP-3 gene expression may be relevant to uterine differentiation and early embryogenesis warrants further investigation.

Expression of GHR mRNA has been reported in several reproductive tissues including the corpus luteum, ovary, oviduct, endometrium, and myometrium (Kirby et al., 1996). In the present study, Northern blot analy-

Figure 8. Effects of bST on endometrial GHR-1A mRNA expression 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 (treatment x day interaction, $P < 0.005$).

sis revealed significant amounts of GHR-1A transcript in the uterine endometrium but not in the oviduct. Our inability to detect GHR-1A mRNA in bovine oviduct on d 3 and 7 of the estrous cycle indicates that this gene may be developmentally regulated in cattle. Alternatively, the absence of GHR-1A transcript in oviductal tissues may have been related to the less-sensitive Northern blot procedure utilized in this study instead of nuclease protection assay utilized in the aforementioned study (Kirby et al., 1996). Short-term bST treatment reduced the abundance of GHR-1A transcript at d 7 of the estrous cycle. The down-regulation of GHR mRNA by exogenous bST agrees with a previous report (Kirby et al., 1996) and suggests that, at relatively high peripheral concentrations, bST may decrease its own receptor transcription. However, the physiological relevance of this transient receptor down-regulation by the ligand is unclear, because exogenous bST clearly down-regulated endometrial IGF-II mRNA and stimulated uterine IGFBP-3 gene expression at d 7 of the estrous cycle.

CONCLUSIONS

This study provides the first direct evidence that exogenous bST induces oviductal IGF-II mRNA as early

as d 3 of a synchronized estrous cycle, and suggests that IGF-II may play a modulatory role in oviductal differentiation and early embryonic development in cattle. The observation that bST down-regulated IGF-II transcript in the endometrium at d 7 of the estrous cycle, when IGF-I transcript tended to increase, implies that these mitogens may have either overlapping or distinct roles in the control of oocyte cleavage and the ensuing blastocyst development. The physiological significance of bST-induced IGFBP-3 transcript in the uterine endometrium at d 7 of the estrous cycle warrants further investigation, because bST appears to up-regulate IGFBP-3 gene expression at a stage of the estrous cycle when uterine luminal IGFBPs undergo substantial proteolytic cleavage. Collectively, our data indicate complex and tissue-specific regulation of the uterine IGF system components by exogenous bST. Those biological responses to bST may be important in early development of bovine embryos.

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