Differences in the expression of genes involved in the somatotrophic axis in divergent strains of Holstein-Friesian dairy cows during early and mid lactation

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ABSTRACT

Differences in genetic selection criteria for dairy cows internationally have led to divergence in the Holstein-Friesian breed. The objective of this study was to compare hepatic expression of genes of the somatotrophic axis in the North American Holstein-Friesian and the New Zealand Holstein-Friesian strains of dairy cow at early and mid lactation. Mature cows of both the North American Holstein-Friesian ($n = 10$) and New Zealand Holstein-Friesian ($n = 10$) strains were selected. Liver tissue was collected by percutaneous punch biopsy from all cows at 35 and 140 d postpartum, representing early and mid lactation, respectively. Total RNA was extracted and the hepatic expression of genes involved in the control of the somatotrophic axis was examined. Abundance of insulin-like growth factor (IGF)-1 mRNA was greater in the New Zealand strain, concomitant with a tendency for increased expression of acid-labile subunit mRNA. Across strains, mRNA abundance of IGF-binding protein-1, IGF-binding protein-2, and growth hormone receptor 1A decreased from d 35 to 140 postpartum, whereas expression of IGF-1 and acid-labile subunit tended to increase. Abundance of suppressor of cytokine signaling-3 mRNA was increased at d 140 postpartum. Both the strain of Holstein-Friesian cow and the stage of lactation influenced expression of genes controlling the somatotrophic axis in hepatic tissue.

Key words: Holstein-Friesian, liver, gene expression, somatotrophic axis

INTRODUCTION

Strain comparison studies in New Zealand and Ireland have reported lesser milk volume, greater BCS

throughout lactation, and superior reproductive performance for the New Zealand (NZ) Holstein-Friesian (NZHF) compared with the North American (NA) Holstein-Friesian (NAHF; Horan et al., 2005b). The NAFH strain has been selected for increased milk yield, body size, and angularity in a production system based on year-round calving and greater levels of concentrate supplementation, with little emphasis on traits such as fertility (Hoekstra et al., 1994; Pryce and Veerkamp, 2001; Macdonald et al., 2007). The NZHF strain, on the other hand, has been selected for increased concentrations of milk fat and protein, reduced maintenance energy costs, and improved fertility and survival traits under pasture-based systems of production.

The occurrence of negative energy balance (NEB) during early lactation is a widely reported phenomenon (Bauman and Currie, 1980). During NEB, mobilization of body reserves is necessary to meet the energy requirements of lactogenesis. It is generally accepted that the growth hormone (GH)-IGF (or somatotrophic) axis in the liver becomes uncoupled, whereby elevated plasma GH concentrations fail to stimulate an increase in hepatic IGF-1 synthesis (Thissen et al., 1994; Fenwick et al., 2008; Lucy, 2008). Insulin-like growth factor-1 plays a critical role in stimulating the anabolic and mitogenic activity of GH in various tissues (Laron, 2001). The liver is the major source of circulating IGF (Miller et al., 1981; Schwander et al., 1983; Thissen et al., 1994); therefore, this organ plays a central role in the metabolism of the cow.

Numerous reports have suggested that nutritionally compromised cows have reduced systemic concentrations of insulin and IGF-1 (Patton et al., 2006; Lucy, 2008), and Lucy et al. (2009) reported that NAFH cows experience an uncoupling of the somatotrophic axis in conjunction with reduced BCS. It has been reported that irreversible glucose loss leads to some degree of uncoupling of the somatotrophic axis as plasma concentrations of IGF-1 decline concomitantly with a reduction

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in hepatic mRNA abundance of IGF-1 and GH receptor (**GHR**; Meier et al., 2008). Furthermore, insulin is hypothesized to be a key metabolic signal regulating the coupling of the somatotrophic axis (Butler et al., 2003).

Current knowledge indicates that the IGF system is composed of 2 ligands (IGF-1 and IGF-2); 2 receptors (**IGF-1R** and **IGF-2R**); 6 binding proteins [IGF binding protein (**IGFBP**)-1 to IGFBP-6] and the IGFBP acid-labile subunit (**ALS**). The somatotrophic axis also includes the GHR, which is expressed as multiple mRNA variants in different tissues. In liver, the GHR1A variant is the principal GHR mRNA transcript, and its expression plays a key role in the coupling of the somatotrophic axis. The effects of GH on the IGF system are primarily mediated by the Janus kinase 2 (**JAK2**) protein and 2 members of a family of DNA-binding proteins called signal transducers and activators of transcription (**STAT**), namely, STAT5a and STAT5b (Udy et al., 1997; Teglund et al., 1998).

Previous studies have demonstrated that systemic concentrations of the metabolic hormone IGF-1 in early lactation are positively associated with the subsequent calving-to-service interval and ultimately the pregnancy outcome in dairy cattle (Taylor et al., 2004; Patton et al., 2007; Wathes et al., 2007). Therefore, a greater understanding of the molecular regulation of hepatic expression of IGF-1 and its associated molecules is of critical importance in elucidating how this system may influence cow fertility. Recently, it was reported that although the NAHF and NZHF strains had similar postpartum energy balance (**EB**) profiles, plasma concentrations of IGF-1 were greater in NZHF cows during the posttransition period (30 to 90 d postcalving; Patton et al., 2008). Thus, the objective of this study was to determine the effect of strain of Holstein-Friesian cow on the transcriptional regulation of key hepatic genes controlling the somatotrophic axis during early and mid lactation.

MATERIALS AND METHODS

Animals, Experimental Design, and Tissue Collection

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the Cruelty to Animals Act (Ireland 1876) and European Community Directive 86/609/EEC. The animal model used in this study was described previously (Horan et al., 2005a). Briefly, the NA strain was developed by breeding the top 50% of cows in the Moorepark herd (based on the pedigree index for milk production) with 5 NAHF sires, selected as the highest available in Ireland for pedigree index for

milk production. The animals in the NZ strain were imported from NZ as embryos and implanted into Holstein heifers. These embryos were generated by mating high-genetic-merit NZHF cows with 5 high-genetic-merit NZHF sires (based on breeding worth; the NZ genetic evaluation system). For the purposes of the current study, 10 mature NAHF cows and 10 mature NZHF cows were selected from their respective strains within the Moorepark strain comparison study (Horan et al., 2005a). Milk production, DMI, EB, and blood hormone and metabolite profiles of these animals have been reported previously (Patton et al., 2008). Briefly, NA cows had greater peak milk yields and total lactation milk yields (7,387 vs. 6,208 kg; SE of the difference = 359), lower milk fat, and similar protein concentrations compared with NZ cows. Body condition scores tended to be lower for NA cows, and this strain tended to have greater DMI (17.2 vs. 15.7 kg/d; $P = 0.07$) for wk 1 to 20 of lactation. Similar magnitudes of EB nadir were recorded for the NA and NZ strains [−6.88 vs. −7.31 Unité Fourragère Lait (**UFL**)/d; $P = 0.72$]. One UFL is the NE_L equivalent of 1 kg of air-dry standard barley as described by Jarrige (1989). All cows were managed in a similar manner as a single group throughout the lactation period. The prepartum diet was composed of ad libitum grass silage, with 2 kg/d of the lactating concentrate introduced from 2 wk before the expected calving date. The early postpartum diet consisted of ad libitum grass silage and 8 kg of concentrate. From March 20, all lactating cows were offered ad libitum zero-grazed grass (*Lolium perenne* spp.) supplemented with 4 kg of concentrate. Grass was harvested and fed each morning. The diet offered was designed to simulate normal feeding practices in seasonal-calving systems of milk production in Ireland. All cows were fed ad libitum grass silage and 8 kg of concentrate at the time of the first biopsy, and ad libitum zero-grazed grass supplemented with 4 kg of concentrate at the time of the second biopsy. The concentrate ingredients and the chemical composition of the concentrate and forages were reported previously (Patton et al., 2008).

Liver tissue was collected from all cows at 35 (± 0.5) and 140 (± 2.4) d postpartum by percutaneous punch biopsy as described by Smith et al. (2003). Briefly, a biopsy site between the 11th and 12th ribs was shaved, sanitized with 7.5% povidone-iodine and 70% ethanol, and anesthetized (lidocaine hydrochloride: 2%). An incision of approximately 1 cm was made through the skin, and the biopsy instrument was used to pierce the intercostal muscles and peritoneum. Approximately 1 to 1.5 g of liver tissue was collected, washed with sterile PBS, snap-frozen in liquid nitrogen, and stored at −80°C until further analysis.

RNA Extraction and cDNA Synthesis

Total RNA was isolated from 100 mg of the frozen liver biopsy using TRIzol reagent and chloroform and subsequently precipitated using isopropanol. Ribonucleic acid quantity and purity were determined by absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). All RNA samples had a 260/280 nm absorbance between 1.8 and 2.0. Ribonucleic acid quality and integrity were also assessed by measuring the 18S:28S ratio, and RNA integrity number was assessed by automated capillary gel electrophoresis on a Bioanalyser 2100 instrument with the RNA 6000 Nano LabChip kit according to the instructions of the manufacturer (Agilent Technologies Ireland Ltd., Dublin, Ireland). Ribonucleic acid samples with 18S:28S ratios ranging from 1.8 to 2.0 and RNA integrity numbers of between 8 and 10 were deemed high quality and suitable for gene expression analysis.

Five micrograms of total RNA was treated for potential genomic DNA carryover with RNase-free DNase according to the instructions of the manufacturer (Promega Corporation, Madison, WI). Ribonucleic acid was purified using the RNeasy Mini Kit in accordance with the instructions supplied (Qiagen, Crawley, West Sussex, UK). Complementary DNA was obtained by reverse-transcribing 1 µg of DNase-treated RNA using reverse transcriptase and 500 ng of random hexamer primers in a 20-µL reaction (reverse transcription system, Promega Corporation). A master mix of reagents was prepared for the above reaction to minimize potential variation from pipetting. Selected negative control samples were also prepared by including all reagents as above, minus the reverse transcriptase. A quantity of 0.39 ng of kanamycin resistance gene mRNA (Promega Corporation) was spiked into each sample as an exogenous control.

Primer Design and Optimization of Real-Time Reverse Transcription-PCR

Real-time PCR assays were designed for genes of the somatotrophic axis and their transcriptional regulators. Gene-specific primer sequences were as described previously (Fenwick et al., 2008) or were designed (Table 1) online using the Primer3 Web-based software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), based on coding regions of core bovine nucleotide sequences on the National Center for Biotechnology Information Web site (<http://www.ncbi.nlm.nih.gov/>). Primer alignment specificity and compatibility were determined by using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>).

All oligonucleotides were commercially synthesized as highly purified, salt-free products (Sigma-Aldrich Ireland Ltd., Dublin, Ireland). Primers were first tested by conventional PCR amplification using the Platinum PCR super mix containing *Taq* polymerase (Invitrogen Ltd., Paisley, UK) and the addition of 50 to 75 ng of DNase-treated reverse transcribed RNA and primers (20 µM) to optimize amplification conditions. Once optimized, external standards were amplified from cDNA identical to real-time PCR products and purified using QIA Quick PCR purification columns (Qiagen). Concentrations of purified cDNA product were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.) and the presence of a single product as confirmed by 1.5% (wt/vol) agarose gel electrophoresis. Standards for each gene analyzed were diluted in nuclease-free water (Promega Corporation), and concentrations ranged from 1×10^{-1} to 1×10^{-9} ng/µL of DNA. All amplified PCR products from this study were also sequenced to verify their identity (Eurofins MWG, Operon, Germany). In all cases, the DNA sequence of the gene being verified had the maximum identity with the published sequence.

Real-Time Reverse Transcription-PCR

The stability of the expression of several control or housekeeping genes across all samples in this study was investigated, including GAPDH, β-actin, RPL-19, and 18S rRNA, as part of a preliminary study. Resulting expression data were analyzed using the geNorm software package (version 3.4, Excel add-in, Microsoft, Redmond, WA; Vandesompele et al., 2002). Because statistically significant differences in expression of these genes existed between samples, it was concluded that none of these genes were suitable as housekeeping genes; therefore, relative real-time PCR could not be conducted. Thus, an absolute real-time reverse transcription-PCR (RT-PCR) methodology using an exogenous reference control to normalize real-time PCR results was performed to measure the expression of genes involved in the somatotrophic axis (Table 1), using the ABI 7500 Fast Real-Time PCR System with Power SYBR Master Mix (Applied Biosystems, Warrington, UK). The application of this method was described previously (Smith et al., 2003; Gilsbach et al., 2006). The exogenous control gene used in this study was the kanamycin resistance gene, as reported previously (Fenwick et al., 2008). Real-time RT-PCR was also performed to detect expression of the exogenous kanamycin control in generated cDNA. Reactions were carried out in a 96-well plate format and prepared in a total volume of 20 µL, with 1 µg of cDNA, 10 µL of Power SYBR Master Mix, and 1 µL of 20 µM forward and reverse primer

Table 1. Oligonucleotide primer sequences, PCR product sizes, and annealing temperatures for all gene transcripts analyzed by real-time reverse transcription-PCR

Gene ¹	Primer	Sequence (5' to 3')	Size (bp)	Accession	Annealing temperature (°C)
IGF-2R	L	CTACGTCAACGGGGACAAAGT	102	NM_174352	60.0
	R	TCGTTCTGGAGCTGAAAGGT			
IGFBP-5	L	AAAGAAGCTGACCCAGTCCA	99	NM_001105327	60.0
	R	CCCCTGCTCAGATTTCTGTC			
JAK2	L	TTGGCAATGACAAACAAGGA	100	XM_865133	60.0
	R	ATCTCATCTGGGCATCCATC			
HNF-4 α	L	GCTCCATGGTGTTCAAGGAT	119	NM_001015557	59.9
	R	CAGCACCAACTCATCAAGGA			
SOCS-3	L	CCCCCAGGAGAGCCTATTAC	112	NM_174466	60.1
	R	CGGTCTTCCGACAGAGATGT			
STAT5b	L	TGCATCCGCCATATTCTGTA	137	NM_174617	60.1
	R	AGTCGCAGCTCCTCAAATGT			

¹IGF-2R = IGF-2 receptor; IGFBP-5 = IGF binding protein-5; JAK2 = Janus-activated kinase 2; HNF-4 α = hepatocyte nuclear factor-4 α ; SOCS-3 = suppressor of cytokine signaling-3; STAT5b = signal transducer and activator of transcription 5b.

mix. External standards were run on the same plate in triplicate. Nontemplate controls were included on every plate for each gene product. To minimize variation, all samples included in each analysis were derived from the same cDNA batch and prepared under the same conditions, and samples were run in duplicate. Thermal cycling conditions applied to each assay consisted of an initial *Taq* activation step at 95°C for 15 min, followed by 38 cycles of 95°C for 15 s, then 54.3 to 62.3°C for 30 s, followed by an amplicon dissociation stage (95°C for 15 s, 60°C for 1 min, increasing 0.5°C/cycle until 95°C was reached). All samples for a particular gene were assayed on the same plate, thereby eliminating intraassay variation, and specificity of the reaction products was also confirmed by dissociation curve analysis and gel electrophoresis.

Statistical Analysis

Gene expression data were log-transformed for normalization of variances and were analyzed using the MIXED procedure (SAS Institute, 2003). Cow was treated as a random effect, with terms for day and strain and their interaction included as fixed effects in the model. The expression of several internal control genes was investigated, including GAPDH, β -actin, RPL-19, and 18S rRNA; however, no suitable internal control gene was identified because of consistently high variation between samples. Expression of the kanamycin resistance gene was used as a covariate, and this accounted for the combined effects of pipetting, reverse transcription efficiency, and the presence of any contaminants that could inhibit the PCR reaction. For comparison of expression data, absolute values expressed as picograms per microgram of reverse-transcribed RNA were derived from the mean cycle threshold value of all unknown samples. The cutoff for significant effects

on gene expression was $P < 0.05$, and differences in gene expression with P -values between 0.05 and 0.15 were considered to have tendencies or trends toward significance. Pearson correlation analysis was carried out to determine relationships between plasma variables (glucose, NEFA, BHBA, IGF-1) and mRNA abundance using the CORR procedure (SAS Institute, 2003). This analysis was carried out for each strain independently at d 35 and 140.

The r^2 and amplification efficiency (E) values for real-time RT-PCR were calculated from linear regression analysis of log (input cDNA) versus cycle threshold number plot (Table 2). The slope for each set of standards was used to determine $E = 10^{(-1/\text{slope})} - 1$. Intraassay variation was determined from the average standard deviation across the quantification range and is presented as a percentage using the following formula: $\pm \text{Percentage variation} = [(E + 1)^{\text{SD}} - 1] \times 100$ (Rutledge and Cote, 2003).

RESULTS

Physiological Measurements

The EB, DMI, BCS, and plasma concentrations of metabolic hormones and metabolites of the animals used in the current study are discussed in detail by Patton et al. (2008). In summary, the NAHF and NZHF strains had similar mean daily EB during wk 1 to 20 of lactation. The degree of BCS loss during wk 1 to 20 of lactation was also similar for both strains; thereafter, the NZHF cows began to increase in BCS, whereas NAHF cows continued to partition nutrients toward milk production, resulting in an increased BCS for NZHF by the end of lactation. Plasma insulin was greater for NAHF during the transition period, whereas plasma IGF-1 was similar for the strains during this

Table 2. Linearity (r^2), efficiency (E), and percentage of intraassay variation of real-time reverse transcription-PCR reactions

Gene ¹	r^2	E^2	Variation, ³ %
IGF-1	0.996	1.09	±0.7
IGF-1R	0.999	1.09	±3.8
GHRtot	0.987	0.85	±26.5
GHR1A	0.99	1.17	±3.4
IGFBP-ALS	0.991	1.28	±3.3
IGF-2	0.998	0.76	±12.8
IGF-2R	0.996	1.23	±7.1
IGFBP-1	0.991	1.00	±0.5
IGFBP-2	0.995	0.92	±0.5
IGFBP-3	0.997	0.95	±13.9
IGFBP-4	0.998	0.95	±10.1
IGFBP-5	0.994	1.32	±6.8
IGFBP-6	0.999	0.90	±7.4
ER α	0.995	1.04	±10.0
INSR	0.999	0.89	±3.8
HNF-4 α	0.993	1.15	±21.6
SOCS-3	0.997	0.97	±12.5
JAK2	0.983	1.41	±4.7
STAT5b	0.986	1.38	±6.9

¹IGF-1R = IGF-1 receptor; GHRtot = growth hormone receptor; GHR1A = growth hormone receptor 1A variant; IGFBP-ALS = IGF-binding protein acid-labile subunit; IGF-2R = IGF-2 receptor; IGFBP-1 to IGFBP-6 = IGF binding protein-1 to IGF binding protein-6; ER α = estrogen receptor α ; INSR = insulin receptor type A; HNF-4 α = hepatocyte nuclear factor-4 α ; SOCS-3 = suppressor of cytokine signaling-3; JAK2 = Janus-activated kinase 2; STAT5b = signal transducer and activator of transcription 5b.

²Efficiency (E) = $10^{(-1/\text{slope})} - 1$.

³Variation, % = $[(E + 1)^{\text{SD}} - 1] \times 100$.

time. Mean systemic concentrations of IGF-1 were increased to a greater extent in NZHF than NAHF during the posttransition period (Patton et al., 2008).

mRNA Abundance

Results of the real-time RT-PCR analysis for independent time and strain effects are summarized in Table 3. Strain $\times$ time interaction effects are displayed in Table 4. Insulin-like growth factor-1 gene expression was affected by strain of cow, with mRNA abundance 1.6 times greater in the NZHF strain ($P < 0.05$). The mRNA abundance of suppressor of cytokine signaling-3 (**SOCS-3**) was also 1.6 times greater for the NZ strain ($P < 0.01$), and abundance of ALS mRNA tended to be greater for the NZ strain (1.67-fold increase; $P = 0.06$). There was no effect of strain on mRNA abundance of GHR1A, total GHR (**GHRtot**), IGF-2, IGF-1R, IGF-2R, insulin receptor type A (**INSR**), IGFBP-1 to IGFBP-6, or the remaining transcription factors and signaling molecules examined (Table 3).

Across strains, several genes were upregulated or downregulated at d 140 compared with d 35 postpartum. Upregulated genes (fold increase in parentheses) included IGF-1R (1.5), estrogen receptor α (**ER α** ; 1.5), and SOCS-3 (2.48; all $P < 0.001$), and trends for in-

Table 3. Real-time reverse transcription-PCR analysis for independent strain and time effects¹

Gene ²	Strain ³		P -value	Days postpartum		
	NAHF	NZHF		35	140	P -value
IGF-1	0.32 (0.23–0.44)	0.51 (0.37–0.72)	<0.05	0.32 (0.22–0.47)	0.51 (0.34–0.76)	0.15
IGF-1R	0.004 (0.004–0.005)	0.005 (0.004–0.006)	0.30	0.004 (0.004–0.005)	0.006 (0.005–0.008)	<0.001
GHRtot	0.51 (0.37–0.71)	0.47 (0.33–0.66)	0.71	0.6 (0.42–0.88)	0.4 (0.27–0.58)	0.15
GHR1A	0.03 (0.02–0.04)	0.03 (0.02–0.04)	0.65	0.04 (0.03–0.05)	0.02 (0.02–0.03)	<0.05
IGFBP-ALS	0.06 (0.04–0.09)	0.1 (0.07–0.14)	0.06	0.06 (0.04–0.09)	0.1 (0.07–0.16)	0.11
IGF-2	15.0 (11.9–18.9)	14.6 (11.5–18.5)	0.85	14.9 (11.7–18.9)	14.7 (11.5–18.8)	0.95
IGF-2R	0.14 (0.13–0.16)	0.14 (0.13–0.16)	0.97	0.16 (0.15–0.18)	0.13 (0.12–0.14)	<0.01
IGFBP-1	4.3 (2.8–6.8)	4.3 (2.7–6.8)	0.98	7.7 (4.9–12.3)	2.4 (1.5–3.8)	<0.01
IGFBP-2	3.4 (2.6–4.4)	3.1 (2.3–4.1)	0.62	5.5 (4.0–7.5)	1.9 (1.4–2.6)	<0.001
IGFBP-3	0.48 (0.37–0.62)	0.5 (0.39–0.65)	0.82	0.64 (0.49–0.84)	0.38 (0.29–0.5)	<0.05
IGFBP-4	0.25 (0.21–0.30)	0.24 (0.2–0.29)	0.85	0.25 (0.2–0.3)	0.24 (0.2–0.3)	0.93
IGFBP-5	0.23 (0.2–0.27)	0.23 (0.2–0.27)	0.98	0.2 (0.18–0.25)	0.25 (0.21–0.3)	0.21
IGFBP-6	0.003 (0.002–0.004)	0.003 (0.003–0.004)	0.79	0.003 (0.003–0.005)	0.004 (0.003–0.0005)	0.20
ER α	0.41 (0.37–0.47)	0.46 (0.41–0.53)	0.22	0.36 (0.31–0.4)	0.54 (0.47–0.61)	<0.001
INSR	0.03 (0.03–0.04)	0.02 (0.02–0.03)	0.18	0.031 (0.03–0.04)	0.025 (0.02–0.03)	<0.01
HNF-4 α	0.3 (0.26–0.35)	0.32 (0.27–0.37)	0.56	0.47 (0.4–0.54)	0.2 (0.18–0.24)	<0.001
SOCS-3	0.5 (0.41–0.6)	0.8 (0.66–0.98)	<0.01	0.4 (0.33–0.49)	0.99 (0.8–1.23)	<0.001
JAK2	0.0004 (0.0002–0.0005)	0.0005 (0.0003–0.0007)	0.28	0.0005 (0.0003–0.0007)	0.0004 (0.0002–0.0006)	0.45
STAT5b	0.1 (0.095–0.11)	0.1 (0.09–0.11)	0.51	0.11 (0.1–0.12)	0.1 (0.09–0.11)	0.29

¹Real-time reverse transcription-PCR values are back-transformed least squares means, followed by the 95% confidence limits in parentheses, and are expressed in picograms per microgram of reverse-transcribed RNA.

²IGF-1R = IGF-1 receptor; GHRtot = growth hormone receptor; GHR1A = growth hormone receptor 1A variant; IGFBP-ALS = IGF-binding protein acid-labile subunit; IGF-2R = IGF-2 receptor; IGFBP-1 to IGFBP-6 = IGF binding protein-1 to IGF binding protein-6; ER α = estrogen receptor α ; INSR = insulin receptor type A; HNF-4 α = hepatocyte nuclear factor-4 α ; SOCS-3 = suppressor of cytokine signaling-3; JAK2 = Janus-activated kinase 2; STAT5b = signal transducer and activator of transcription 5b.

³NAHF = North American Holstein-Friesian; NZHF = New Zealand Holstein-Friesian.

Table 4. Strain $\times$ time (S $\times$ T) interaction effects with respective *P*-values, where NA 35 and NA 140 represent North American Holstein-Friesian strain at d 35 and 140, respectively, and NZ 35 and NZ 140 denote New Zealand Holstein-Friesian at d 35 and 140, respectively¹

Gene ²	S $\times$ T NA 35	S $\times$ T NA 140	S $\times$ T NZ 35	S $\times$ T NZ 140	<i>P</i> -value
IGF-1	0.25 (0.14–0.42)	0.41 (0.24–0.71)	0.42 (0.26–0.69)	0.62 (0.38–1.04)	0.79
IGF-1R	0.003 (0.003–0.005)	0.006 (0.004–0.008)	0.004 (0.003–0.005) ^a	0.007 (0.006–0.01) ^b	0.52
GHRtot	0.61 (0.36–1.03)	0.43 (0.25–0.72)	0.59 (0.37–0.95)	0.37 (0.23–0.6)	0.80
GHR1A	0.03 (0.02–0.05)	0.02 (0.02–0.03)	0.04 (0.02–0.05)	0.02 (0.02–0.03)	0.58
IGFBP-ALS	0.05 (0.03–0.09)	0.08 (0.04–0.13)	0.07 (0.04–0.12)	0.14 (0.08–0.24)	0.58
IGF-2	17.2 (11.9–24.7)	13.1 (9.2–18.6)	12.9 (9.3–17.9)	16.5 (11.6–23.3)	0.15
IGF-2R	0.16 (0.14–0.19)	0.12 (0.11–0.14)	0.16 (0.14–0.18)	0.13 (0.11–0.15)	0.50
IGFBP-1	6.4 (3.4–12.2)	2.9 (1.5–5.6)	9.4 (5.3–16.7) ^a	2.0 (1.1–3.6) ^b	0.12
IGFBP-2	6.0 (3.8–9.2) ^a	1.9 (1.2–3.0) ^b	5.1 (3.4–7.4) ^a	1.9 (1.3–2.8) ^b	0.68
IGFBP-3	0.74 (0.5–1.1) ^a	0.32 (0.22–0.46) ^b	0.56 (0.39–0.8)	0.45 (0.31–0.66)	0.11
IGFBP-4	0.3 (0.22–0.4)	0.21 (0.16–0.27)	0.2 (0.16–0.27)	0.29 (0.22–0.38)	<0.05
IGFBP-5	0.2 (0.16–0.27)	0.26 (0.2–0.33)	0.22 (0.17–0.27)	0.25 (0.19–0.32)	0.74
IGFBP-6	0.003 (0.002–0.005)	0.003 (0.002–0.004)	0.002 (0.002–0.003)	0.004 (0.003–0.006)	0.06
ER α	0.34 (0.28–0.41) ^a	0.51 (0.42–0.61) ^b	0.37 (0.31–0.45) ^a	0.57 (0.47–0.68) ^b	0.93
INSR	0.04 (0.03–0.06) ^a	0.02 (0.02–0.03) ^b	0.03 (0.02–0.03)	0.02 (0.02–0.03)	<0.05
HNF-4 α	0.44 (0.36–0.55) ^a	0.2 (0.16–0.25) ^b	0.49 (0.4–0.6) ^a	0.2 (0.17–0.25) ^b	0.62
SOCS-3	0.25 (0.19–0.34) ^{a,A}	0.97 (0.72–1.3) ^b	0.64 (0.48–0.84) ^B	1.0 (0.75–1.4)	<0.01
JAK2	0.0003 (0.0002–0.0006)	0.0004 (0.0002–0.0007)	0.0006 (0.0004–0.001)	0.0004 (0.0002–0.0007)	0.34
STAT5b	0.12 (0.095–0.13)	0.1 (0.09–0.11)	0.1 (0.09–0.12)	0.1 (0.09–0.11)	0.64

^{a,b}Within a strain, means not sharing a common superscript differ between time points ($P < 0.05$).

^{A,B}Within a time point, means not sharing a common superscript differ between strains ($P < 0.05$).

¹Values are back-transformed least squares means, followed by the 95% confidence limits in parentheses, and are expressed in picograms per microgram of reverse-transcribed RNA.

²IGF-1R = IGF-1 receptor; GHRtot = growth hormone receptor; GHR1A = growth hormone receptor 1A variant; IGFBP-ALS = IGF-binding protein acid-labile subunit; IGF-2R = IGF-2 receptor; IGFBP-1 to IGFBP-6 = IGF binding protein-1 to IGF binding protein-6; ER α = estrogen receptor α ; INSR = insulin receptor type A; HNF-4 α = hepatocyte nuclear factor-4 α ; SOCS-3 = suppressor of cytokine signaling-3; JAK2 = Janus-activated kinase 2; STAT5b = signal transducer and activator of transcription 5b.

creased mRNA abundance were observed for ALS (1.66; $P = 0.11$) and IGF-1 (1.6; $P = 0.15$). Downregulated genes (fold decrease in parentheses) included GHR1A (2.0; $P < 0.05$), IGF-2R (1.23; $P < 0.01$), IGFBP-1 (3.2; $P < 0.01$), IGFBP-2 (2.9; $P < 0.001$), IGFBP-3 (1.68; $P < 0.05$), INSR (1.24; $P < 0.01$), hepatocyte nuclear factor-4 α (**HNF-4 α** ; 2.35; $P < 0.001$), and a trend for reduced mRNA abundance of GHRtot (1.5; $P = 0.15$). There was no effect of stage of lactation on mRNA abundance of IGF-2, IGFBP-4 to IGFBP-6, JAK2, or STAT5b (all $P > 0.2$).

Significant ($P < 0.05$) strain $\times$ time interactions were observed for IGFBP-4, INSR, and SOCS-3 genes. Abundance of IGFBP-4 mRNA tended to increase between d 35 and 140 postpartum in NZ cows, but tended to decline during this interval in NA cows. Abundance of INSR mRNA declined ($P < 0.05$) by half in NA cows between d 35 and 140, but declined to a lesser extent in NZ cows. Abundance of SOCS-3 mRNA increased significantly in the NA strain between d 35 and 140, whereas a smaller, nonsignificant increase was observed in the NZ strain (3.9-fold increase vs. 1.6-fold increase, NA vs. NZ, respectively; strain $\times$ time interaction, $P < 0.01$). A trend ($P = 0.06$) for a strain $\times$ time interaction was observed for IGFBP-6, for which mRNA abundance tended ($P = 0.1$) to increase between d 35 and 140 for NZ cows but to remain constant in NA

cows. Conversely, IGFBP-3 decreased ($P < 0.05$) in NA cows between d 35 and 140 (>50% reduction), but remained relatively constant in NZ cows (strain $\times$ time interaction, $P = 0.11$). The IGFBP-1 declined ($P < 0.05$) in the NZ strain between d 35 and 140, but a non-significant decrease ($P = 0.3$) was observed in the NA strain (2.2-fold decrease vs. 4.7-fold decrease, NA vs. NZ, respectively; strain $\times$ time interaction, $P = 0.12$). There was a trend for a strain $\times$ time interaction for mRNA abundance of IGF-2 ($P = 0.15$) because values appeared to decline in NAHF and increase in the NZHF between d 35 and 140. However, within-strain differences between d 35 and 140 for IGF-2 mRNA abundance were nonsignificant ($P > 0.6$ for both strains). There were no trends for a strain $\times$ time interaction for the remaining genes examined (all $P > 0.3$).

Correlation Analysis

Pearson correlation analysis indicated that circulating IGF-1 was positively correlated with IGFBP-5 mRNA abundance in the NZ strain ($R = 0.74$; $P < 0.05$) and was negatively correlated with IGFBP-2 mRNA abundance in the NAHF strain ($R = -0.65$; $P < 0.05$). No relationships ($P > 0.05$) were observed between plasma metabolites and the expression of genes analyzed in the current study.

DISCUSSION

This study examined the expression of key genes of the somatotrophic axis in 2 genetically divergent strains of Holstein-Friesian dairy cows during early and mid lactation. As such, the study provides a suitable model to compare the effects of selection for milk yield on expression of components of the somatotrophic axis and to examine the influence of these genes on physiological changes, such as differences in milk yield and altered nutrient partitioning. The study is a comprehensive molecular investigation into the effects of strain of Holstein-Friesian on the expression of these genes during early and mid lactation. Recently, Lucy et al. (2009) reported that NAHF cows experience a more severe uncoupling of the somatotrophic axis in early lactation compared with NZHF cows. We did not observe the same differences in GHR1A and GHRtot expression in the current study; however, NZ cows had increased mRNA abundance of IGF-1 compared with NA cows.

Nearly all the IGF-1 secreted by the liver (>90%) circulates as a bound ternary complex with IGFBP-3 and ALS (Baxter, 1993; Clemmons, 1997). Patton et al. (2008) reported previously that both strains of cows used in our study had a similar EB status for the first 20 wk of lactation. Under normal conditions, the ternary complex of IGF-1, IGFBP-3 (or IGFBP-5), and ALS (almost all of which is produced by the liver) associate to prevent IGF degradation, prolong half-life in circulation, and increase IGF-1 availability to other tissues (Boisclair et al., 2001). Both hepatic IGF-1 and ALS production are GH dependent (Kim et al., 2006) and occur via the JAK/STAT signaling cascade. The marked increase in IGF-1 mRNA expression observed in the NZHF strain compared with the NAHF strain coincided with increased ALS mRNA abundance in the NZHF strain. Interestingly, signaling molecules associated with activating IGF-1 gene transcription were not increased in the NZHF strain, but the mRNA abundance of the negative feedback molecule SOCS-3 was greater in the NZHF strain compared with the NAHF strain. The observation that mRNA expression of both the IGF-1 and ALS genes was greater in NZHF cows compared with NAHF cows may indicate enhanced coupling of the somatotrophic axis via the JAK/STAT pathway in the NZHF strain. The trend for a strain $\times$ time interaction for IGFBP-3, for which IGFBP-3 mRNA abundance declined more than 2-fold in NAHF cows from d 35 to 140, suggests that the proportion of the circulating IGF-1 in the stable ternary complex may be reduced in NAHF cows compared with NZHF cows. Wu et al. (2008) recently reported that feed-restricted steers had greater degradation of IGF-1 in circulation

because of reduced IGFBP-3 and ALS production in the liver.

Patton et al. (2008) reported that plasma IGF-1 concentrations were higher in NZHF cows compared with NAHF cows, despite both strains having similar EB profiles and despite NAHF cows having greater plasma insulin concentrations. This result was not consistent with other studies that reported an increase in circulating IGF-1 during hyperinsulinemia, coincident with increased hepatic IGF-1 mRNA and GHR1A mRNA (Butler et al., 2003; Rhoads et al., 2004). Two recent reports indicated that NAHF may experience more severe insulin resistance compared with NZHF cows during early (Chagas et al., 2009) or mid lactation (Patton et al., 2009). A similar scenario may have occurred in the current study, whereby NA cows had moderately greater systemic concentrations of insulin, yet the somatotrophic axis remained uncoupled for longer because of greater insulin resistance in this strain.

In both strains, mRNA abundance of IGFBP-1 and IGFBP-2 decreased from d 35 to 140, consistent with improving the EB status (Vicini et al., 1991; Vandehaar et al., 1995; Fenwick et al., 2008). Additionally, a negative correlation between circulating IGF-1 concentrations and IGFBP-2 mRNA abundance at d 35 was observed, in agreement with previous reports (Sharma et al., 1994; Vleurick et al., 2000; Fenwick et al., 2008). The mRNA abundance of GHR1A was also significantly reduced during mid lactation compared with early lactation in the current study. An explanation for this is not readily apparent because cows were in an improved state of EB at d 140 compared with d 35. However, the first biopsy was carried out at 35 d postpartum in the current study, in contrast with biopsy collection on the day of parturition in previous studies, when substantial increases in GHR1A with advancing stage of lactation were reported (Lucy et al., 2001, 2009). Conversely, Rhoads et al. (2008) recently reported no change in GHR1A mRNA abundance in liver tissue between d 40 and 160 postpartum. However, GHR1A mRNA levels do not always reflect abundance of the GHR protein. Rhoads et al. (2007) reported reduced GHR protein without corresponding reductions in GHR1A mRNA in the liver tissue of nutritionally stressed cows. Therefore, our GHR1A expression data may indicate that protein translation of GHR1A increases with improving the nutrient intake and EB status, thus making reduced levels of GHR1A mRNA adequate for efficient hepatic GH action.

Both in vitro and gene knockout studies indicate that the effects of GH are primarily mediated by the JAK2 and the STAT5a and STAT5b proteins (Udy et al., 1997; Teglund et al., 1998). Growth hormone binding

to the GHR results in a conformational change in the GHR that activates JAK2, and JAK2 then phosphorylates STAT5b (Carter-Su and Smit, 1998). We did not observe any strain, time, or strain $\times$ time interaction effects for mRNA abundance of JAK2 or STAT5b. Suppressor of cytokine signaling genes are expressed after early JAK/STAT signaling and act as negative regulators of transcription (Wormald and Hilton, 2004). Previously, it was reported that SOCS-3 mRNA in the liver of mice was stimulated by GH (Adams et al., 1998; Lieskovska et al., 2003). Suppressor of cytokine signaling-3 is a target of the JAK/STAT signaling cascade and causes a negative feedback loop, which inhibits STAT phosphorylation, thereby inhibiting GH signaling (Ram and Waxman, 1999). The mRNA abundance of SOCS-3 was greater in NZHF compared with NAHF in the current study. Additionally, an increase in mRNA expression of SOCS-3 was observed from d 35 to 140 during a time of improving EB status. Because SOCS genes are expressed after early JAK/STAT signaling, the data indicate that this component of the GH signaling cascade is more efficient in NZHF cows compared with NAHF cows and is also more efficient in mid lactation compared with early lactation. Collectively, these observations paradoxically suggest that greater mRNA abundance of SOCS-3 was observed during efficient coupling of the somatotrophic axis. Few published reports have examined hepatic SOCS-3 gene expression in ruminants. Hyatt et al. (2007) reported that SOCS-3 mRNA abundance was reduced in the liver tissue of young adult male sheep that had been nutritionally restricted in utero, and this was coupled with reduced GHR mRNA expression. Further work examining the abundance and phosphorylation status of the protein products of the JAK2, STAT5b, and SOCS-3 genes is necessary to determine if mRNA abundance of these genes corresponds to protein abundance.

Between d 35 and 140, a reduction in the mRNA abundance of the liver-enriched transcription factor HNF-4 α was observed. Hepatocyte nuclear factor-4 α is known to play a critical role in the transcriptional regulation of many liver-specific gene promoters, and is necessary for the development and proper functioning of liver tissue (De Simone and Cortese, 1992; Sladek, 1993; Costa et al., 2003). Odom et al. (2004) reported that HNF-4 α was involved in the regulation of a large proportion of the liver transcriptome by binding directly to almost half of the actively transcribed genes. The transcription factor HNF-4 α is involved in hepatic GH-dependent signaling mechanisms (Rastegar et al., 2000). Growth hormone receptor 1A mRNA is expressed only in liver tissue (Lucy et al., 1998; Jiang and Lucy, 2001b), and the GHR1A promoter contains a common *cis*-regulatory DNA element for the liver-specific transcription factors

HNF-4 α and HNF-4 γ (Jiang and Lucy, 2001a; Lucy et al., 2001; Xu et al., 2004). In the current study, HNF-4 α expression levels declined as lactation progressed. This is consistent with Looor et al. (2005), who reported that liver HNF-4 α mRNA abundance increased from 14 d prepartum to peak at 28 d postpartum, after which time levels began to decrease again. Of note is that the decline in HNF-4 α mRNA abundance between d 35 and 140 was mirrored by reductions in GHR_{tot} and GHR1A mRNA.

Messenger RNA abundance of the INSR was significantly reduced at d 140 compared with d 35, and a significant strain $\times$ time interaction for this gene was observed; the decline in INSR mRNA abundance between d 35 and 140 was greater for NAHF compared with NZHF. Simultaneously, a trend for a strain $\times$ time interaction for IGF-2 was observed, whereby IGF-2 tended to decline in NA cows and increase in NZ cows between d 35 and 140, potentially allowing for strain differences in IGF-2 signaling through INSR. The physiological importance of the observed changes in INSR and IGF-2 gene expression are unclear at present, however, and warrant further study. Hepatic ER α mRNA abundance was greater at d 140 compared with d 35 in the current study. Activation of the ER is known to result in increased IGF-1/IGF-1R activity (Klotz et al., 2002). It was demonstrated previously that the abundance of ER α mRNA in liver tissue is reduced during severe NEB (Fenwick et al., 2008), but the importance of this result in relation to the functionality of the somatotrophic axis is unclear. Administration of estradiol-17 β to steers increases GH binding in liver tissue (Breier et al., 1988); however, in the current study ER α mRNA abundance increased from d 35 to 140, whereas GHR1A mRNA abundance was reduced and GHR_{tot} also tended to decline. Again, further research is necessary to determine the importance of ER α for liver IGF-1 synthesis, and the importance of temporal changes in ER α mRNA abundance in regulating the somatotrophic axis.

CONCLUSIONS

The current study investigated the effects of genetic background and stage of lactation on key genes regulating the somatotrophic axis in 2 strains of Holstein-Friesian cow. During the early postpartum period, orchestrated changes in the endocrine system and whole-body metabolism are necessary to support mammary milk synthesis. Most cows experience at least some degree of NEB and consequent body tissue mobilization during early lactation, and this is associated with uncoupling of the somatotrophic axis. Data from this study suggest that early-lactation adaptations may have more severe

effects in the NA strain compared with the NZ strain of dairy cow. The NZHF strain had greater expression of IGF-1 and also tended to have greater mRNA abundance of ALS. Coupled with the observation that IGFBP-3 mRNA declined in the NA strain between d 35 and 140, the results suggest that a greater proportion of circulating IGF-1 may have been in the stable ternary complex for the NZHF strain. Taken together, the results suggest that strain of Holstein-Friesian dairy cow and stage of lactation influence expression of genes in the somatotrophic axis. These data advance our understanding of the biological mechanisms regulating the somatotrophic axis and nutrient partitioning in lactating dairy cows.

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