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Growth hormone and ghrelin receptor genes are differentially expressed between genetically lean and fat selection lines of sheep

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ABSTRACT: The objective of this study was to determine whether differences in mRNA levels of key pituitary genes that regulate GH production, pituitary development, and growth were present and/or associated with divergent body composition phenotypes observed between sheep from genetically divergent lean and fat selection lines. Real-time PCR transcription profiles for pituitary specific transcription factor 1, prophet of pit1, GH, GH receptor, GH secretagogue receptor, GHRH receptor, leptin receptor, and somatostatin receptors 1 and 2 were determined in pituitary tissue. There was a difference in the amount of both GH ($P < 0.001$) and GH secretagogue receptor ($P < 0.001$) mRNA between the selection lines (5 females and 5 males per line; 20 wk of age); the lean line had greater abundance than the fat line, irrespective of which endogenous control gene was used. The results obtained for GHRH receptor

were equivocal but suggestive; there were greater GHRH receptor mRNA levels ($P < 0.001$) in the lean line using beta-2-microglobulin as the endogenous control but not when hypoxanthine phosphoribosyltransferase and glyceraldehyde-3-phosphate dehydrogenase were used. No difference in pituitary specific transcription factor 1, prophet of pit1, GH receptor, leptin receptor, or somatostatin receptors 1 and 2 mRNA concentration was observed between the lines. The greater abundance of GH mRNA in the pituitary somatotropes from genetically lean animals appears to be associated with increased levels of GH secretagogue receptor mRNA and possibly GHRH receptor mRNA. This suggests that the difference in GH secretion between the lines may be due to differences in the afferent signals, such as ghrelin and/or GHRH, arising from the hypothalamus, or as a result of differential pituitary sensitivity to these hormones.

Key words: growth hormone, growth hormone secretagogue receptor, lean and fat selection lines, pituitary, real-time polymerase chain reaction, sheep

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INTRODUCTION

Divergent selection lines of Coopworth sheep have been selected for high or low weight-adjusted backfat depth since 1979 (Morris et al., 1997). The major differences between these lean and fat selection lines occur in the somatotrophic axis; lean sheep have different plasma GH profiles (Suttie et al., 1993), a greater GH secretory capacity, a greater release of GH in response to GH-releasing factor challenge (Suttie et al., 1991), and greater concentrations of IGF-I (Francis et al., 1997) compared with those of the fat selection lines. The difference in GH status may be due in part to a heavier pituitary gland found in genetically lean animals (Francis et al., 1998). This difference in pituitary weight appears to be the result of a nonspecific increase in size

of the whole gland through increased cell number (Francis et al., 2000).

The concentration of GH in the pituitary gland does not differ between lean and fat sheep, but total pituitary content of GH is greater in lean sheep (Fleming et al., 1997). However, it is still unclear whether the difference in plasma GH between the selection lines is a result of the larger pituitary gland or a greater synthetic capacity of individual somatotropes. Pituitary GH is secreted in a highly episodic pulsatile pattern that is tightly controlled by the hypothalamic peptides GHRH, somatostatin (**SRIF**), and ghrelin (Frohman et al., 2000).

Relationships between GH secretion and carcass composition have also been reported in backfat-based selection lines in other sheep breeds and species (Carter et al., 1989; te Pas et al., 2001). However, the molecular mechanisms regulating these selection-induced changes in hormone status and body composition remain largely unknown. The aim of this study was to determine if differences in mRNA levels of key genes, known to regulate GH production, pituitary develop-

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ment, and growth occurred between the AgResearch genetically divergent lean and fat lines of sheep.

MATERIALS AND METHODS

All experiments were performed with approval granted by the Animal Ethics Committee at Invermay Agricultural Research Center in accordance with the 1999 Animal Welfare Act of New Zealand.

Animals

Twenty lambs born in September 2003 from the Invermay Coopworth lean and fat selection lines (Morris et al., 1997) were used in the experiment. The experimental groups were composed of 5 males and 5 females within each lean or fat line.

Collection of Tissue Samples

The lambs were euthanized at 20 wk of age by intravenous injection of sodium pentobarbitone 500 mg/mL (Pentobarb 500; National Veterinary Supplies Ltd., East Tamaki, Auckland, New Zealand). Whole pituitary and pineal glands were immediately removed, weighed, snap frozen in liquid N, and stored at -70°C .

Carcass Composition Measurements

Following slaughter, carcasses were chilled overnight, and the cold carcass weight and external length of the carcass and tibia were measured as well as depth and width of the LM. Linear fat measurements from the cut face of the LM between the 12th and 13th ribs, shoulders, and hind leg, as described by Kirton and Johnson (1979), were also recorded.

Extraction and Quantification of Total RNA

Total RNA was extracted from the whole pituitary gland using TRIzol Reagent (Invitrogen, Auckland, New Zealand) according to the manufacturer's protocol. The yield of RNA was estimated spectrophotometrically from absorbance at 260 nm (A_{260}) and the purity from the A_{260}/A_{280} ratio. The concentration of the RNA was adjusted to 100 ng/ μL before being used for cDNA synthesis.

Quantitative Real-Time PCR

Gene sequences for primer design were obtained from the National Center for Biotechnology Information's GenBank (Table 1). The bovine GAPDH sequence (GenBank U85042) was aligned to the human refseq NM_002046.2. The intron/exon structure was obtained by alignment to the human genomic sequence. Primers (Invitrogen) were designed using Primer Express (Applied Biosystems, Scoresby, Victoria, Australia). Table 1 lists the forward and reverse primer sequences used in the pituitary expression experiment.

In the first series of experiments the endogenous control (housekeeping) gene beta-2-microglobulin (**B2M**) was used. Where a significant difference between the lines was observed for a candidate gene using B2M [and also for SRIF receptor (**SSTR**) 1 and SSTR2, for which there were no introns], the experiment was repeated using the endogenous control genes hypoxanthine phosphoribosyltransferase (**HPRT**) and glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**).

The total RNA sample (400 ng) was reverse transcribed using Superscript III (Invitrogen) and random primers (Roche, Auckland, New Zealand) after being treated with DNase I (Roche) to minimize potential contamination with genomic DNA. Controls with no reverse transcription were also included, and the results showed that there was no PCR product produced for these samples. Quantitative real-time PCR was carried out using the ABI PRISM 7700 Sequence detection system (Applied Biosystems) according to the manufacturer's instructions; housekeeping and target genes were run in separate wells. A primer matrix was generated for each gene to establish the optimal primer ratio, which was then checked against a serial dilution of cDNA from a random pituitary sample (Table 2). Efficiency curves were generated using the optimized primer ratios. The 25- μL real-time PCR reaction included a cDNA template corresponding to 10 ng of the original total RNA, forward and reverse primers at concentrations based on the PCR efficiency results and differing for each pair of genes, 0.5 μL of the internal passive reference dye ROX, and 12.5 μL of Platinum SYBR Green qPCR SuperMix UDG (Invitrogen). Polymerase chain reaction conditions were as follows: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min, 1 cycle at 95°C for 15 s, and 1 cycle at 60°C for 20 s, ramping to 95°C with a ramping time of 19.59 min.

Data Analysis

After the real-time PCR, data acquisition, and subsequent data analysis were performed using the 5700 Sequence Detection System (SDS Version 1.3; Applied Biosystems). In the 5700 Sequence Detection System, the fluorescence of SYBR Green against ROX (ΔR_n) is measured at the end of each cycle. A sample is considered positive when the ΔR_n exceeds the threshold value. The threshold value is set at the midpoint of ΔR_n vs. cycle number plot. For all the amplifications described in this report, the threshold value of ΔR_n was 0.25.

The threshold cycle (C_T) is defined as the cycle at which a statistically significant increase in the R_n is first detected. Target cDNA copy number and C_T values are inversely related. A sample containing greater copy numbers of the target cDNA will cross the threshold at an earlier cycle compared with samples with fewer copies of the same target. Reactions were performed in duplicate, and the mean of the C_T values was calculated. The normalized expression of each target gene, the

Table 1. Forward and reverse primer sequences used for real-time PCR

| Gene ¹ | Intron/exon | Forward primer sequence (5'-3') | Reverse primer sequence (5'-3') | GenBank accession no. | PCR product size (bp) |
|-------------------|-------------|---------------------------------|---------------------------------|-----------------------|-----------------------|
| GAPDH | Exon 2/3 | GGTCGGAGTGAACGGATTTG | TGGCAACGATGTCCACTTTG | U85042 | 83 |
| HPRT | Exon 3/4 | TTCTATGACTGTGGATTTTATCAGACT | CAATTACTTTTATGTGCGCTGTTGAC | AF176419 | 74 |
| HPRT | Exon 4/5_6 | TTGGTGGAGATGATCTCTCAACTTT | CTTCCCAGTGTCAATTATATCTTCGA | AF176419 | 75 |
| B2M | Exon 1/2 | GGCCTGCTGTCGCTGTCT | TTCTGGCGGGTGTCTTGAGT | AB098890 | 78 |
| Pit1 | Exon 1/2 | GCCTACCGGTCTCCAACCA | TCACGCCATAGGTCGATGACT | U88399 | 108 |
| Prop1 | Exon 1/2 | CCCAGCCGCTGAGACTGT | AAGCTTTGGTCTCCCCACTCTT | NM_174678.2 | 91 |
| GH | Exon 1/2 | CTTGTCCGGCCTGTTTGC | CTCTGTCCCTCCGGGATGTA | A09118 | 108 |
| LepR | Exon 8/9 | GGAGCGCCCTTCTTACCTTTA | CCCAACCGCTGTCAGAATTT | AY278244 | 71 |
| GHR | Exon 5/6 | GTGGCAGGCTCCAGTGATG | CTGCAGACTCTGAGATGCTCTGA | M82912 | 78 |
| GHRHR | Exon 6/7 | CCTCTTTCACCGGGAGAACA | GAAATGAGAGGGCGGTCACAGA | AY008834 | 79 |
| GHSR | Exon 1/2 | GAGACCAGAACCACAAACAAACC | GGAGAATAAATATCGTCTACATGGAA | AY093948 | 101 |
| SSTR1 | Exon 3 | TTACAGCGTGGAGGACTTCCA | AGGTGCCATTGCGGAAGA | AJ314853 | 65 |
| SSTR2 | Exon 2 | GAACGTCCTCTGCTTGGTCAA | GGTCTCATTCAGCCGGGATT | AF335550 | 88 |

¹GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HPRT = hypoxanthine phosphoribosyltransferase; B2M = beta-2-microglobulin; Pit1 = pituitary specific transcription factor 1; Prop1 = prophet of pit1; LepR = leptin receptor; GHR = GH receptor; GHRHR = GHRH receptor; GHSR = GH secretagogue receptor; SSTR1 = somatostatin receptor 1; and SSTR2 = somatostatin receptor 2.

Δ CT, was derived by subtracting the mean C_T of the endogenous control genes from the mean C_T of the target gene for each sample.

Sequencing

The identity of the products generated in real-time PCR was confirmed by DNA sequencing. Polymerase chain reaction products were separated by electrophoresis in 3% Nusieve/agarose gels (Biolab Scientific, Auckland, New Zealand), the amplified fragments were excised, and DNA was purified using QIAquick Gel ex-

traction kit (Biolab Scientific). Deoxyribonucleic acid was sequenced using ABI Big Dye terminator Ver 3.1 cycle sequencing chemistry (Applied Biosystems) on an ABI 3100 (Applied Biosystems).

Statistical Methods

Phenotypic data (pituitary and pineal weights, BW, and carcass fat measurements) were analyzed by ANOVA; the model included sex, line (fat or lean), and their interaction, with a covariate adjustment for carcass weight where appropriate. The Δ CT values were

Table 2. Summary of relative efficiency results for primer optimization

| Housekeeping gene ¹ | Intron/Exon | Primer concentration, nM (forward/reverse) | Test gene ¹ | Primer concentration, nM (forward/reverse) | Relative efficiency |
|--------------------------------|-------------|--|------------------------|--|---------------------|
| B2M | Exon 1/2 | 900/900 | GH | 900/900 | -0.08 |
| B2M | Exon 1/2 | 900/900 | GHR | 900/900 | -0.06 |
| B2M | Exon 1/2 | 900/900 | GHRHR | 50/900 | 0.05 |
| B2M | Exon 1/2 | 900/900 | GHSR | 300/900 | 0.03 |
| B2M | Exon 1/2 | 900/900 | LepR | 300/900 | -0.01 |
| B2M | Exon 1/2 | 300/900 | Pit1 | 900/900 | 0.07 |
| B2M | Exon 1/2 | 900/900 | Prop1 | 300/900 | 0.06 |
| B2M | Exon 1/2 | 900/900 | SSTR1 | 300/900 | 0.09 |
| B2M | Exon 1/2 | 900/900 | SSTR2 | 900/900 | 0.11 |
| GAPDH | Exon 2/3 | 300/300 | GH | 900/900 | 0.05 |
| GAPDH | Exon 2/3 | 300/300 | GHRHR | 300/300 | 0.05 |
| GAPDH | Exon 2/3 | 300/300 | GHSR | 900/300 | -0.03 |
| GAPDH | Exon 2/3 | 300/300 | SSTR1 | 300/50 | -0.02 |
| GAPDH | Exon 2/3 | 300/300 | SSTR2 | 300/900 | -0.07 |
| HPRT | Exon 4/5_6 | 900/900 | GH | 300/300 | -0.02 |
| HPRT | Exon 3/4 | 900/900 | GHRHR | 50/900 | -0.10 |
| HPRT | Exon 4/5_6 | 300/900 | GHSR | 900/300 | -0.03 |
| HPRT | Exon 3/4 | 900/900 | SSTR1 | 300/900 | -0.02 |
| HPRT | Exon 3/4 | 300/300 | SSTR2 | 900/900 | -0.04 |

¹B2M = beta-2-microglobulin; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HPRT = hypoxanthine phosphoribosyltransferase; GHR = GH receptor; GHRHR = GHRH receptor; GHSR = GH secretagogue receptor; Pit1 = pituitary specific transcription factor 1; Prop1 = prophet of pit1; SSTR1 = somatostatin receptor 1; SSTR2 = somatostatin receptor 2.

Table 3. Mean of phenotypic measurements classified by selection line and sex and adjusted for carcass weight where appropriate

| Item | Lean | | Fat | | SED ¹ | P-value | | | |
|--------------------------------|----------------|----------------|----------------|----------------|------------------|---------|--------|------------|------------------|
| | Ewe (n = 5) | Ram (n = 5) | Ewe (n = 5) | Ram (n = 5) | | Sex | Line | Line × Sex | Covariate |
| Birth wt, kg | 4.1 | 4.6 | 3.7 | 4.1 | 0.60 | 0.306 | 0.306 | 0.945 | N/A ² |
| BW, kg at 20 wk | 28.8 | 29.4 | 27.0 | 31.6 | 2.39 | 0.144 | 0.907 | 0.254 | N/A |
| Carcass wt, kg | 11.6 | 11.5 | 12.0 | 13.7 | 1.42 | 0.438 | 0.215 | 0.374 | N/A |
| Pituitary wt, g | 0.48 | 0.45 | 0.37 | 0.41 | 0.037 | 0.697 | 0.011 | 0.167 | 0.046 |
| Pineal wt, g | 0.050 | 0.058 | 0.042 | 0.049 | 0.013 | 0.371 | 0.392 | 0.946 | 0.652 |
| Carcass length, cm | 77.3 | 77.2 | 70.8 | 73.7 | 1.51 | 0.144 | <0.001 | 0.185 | <0.001 |
| Leg length, cm | 25.9 | 27.3 | 24.4 | 23.9 | 0.87 | 0.316 | 0.002 | 0.142 | 0.272 |
| LM width, mm | 50.9 | 49.6 | 47.5 | 48.3 | 2.1 | 0.910 | 0.132 | 0.481 | 0.134 |
| LM depth, mm | 21.7 | 20.2 | 22.2 | 21.6 | 1.54 | 0.319 | 0.419 | 0.678 | 0.008 |
| Shoulder fat (S1) ³ | 1.0 | 0.8 | 3.6 | 2.7 | 0.59 | 0.140 | <0.001 | 0.424 | 0.635 |
| Shoulder fat (S2) ³ | 2.0 | 0.9 | 6.9 | 5.8 | 0.71 | 0.017 | <0.001 | 1.000 | 0.015 |
| Hind leg fat ⁴ | 3.4 | 1.9 | 8.6 | 7.9 | 1.54 | 0.202 | <0.001 | 0.748 | 0.001 |
| Rib fat ⁵ | 1.3 | 1.2 | 6.9 | 4.1 | 1.02 | 0.036 | <0.001 | 0.083 | 0.004 |
| Rib fat ⁶ | 3.2 | 2.8 | 10.9 | 11.1 | 2.5 | 0.750 | <0.001 | 0.884 | 0.031 |

¹SE of the difference among means.

²Not applicable for this analysis.

³Subcutaneous shoulder fat depth at the sixth rib.

⁴Subcutaneous leg chump fat depth at the second last lumbar vertebrae.

⁵Linear subcutaneous fat measurement perpendicular to the deepest part of the LM measured on the cut face between the 12th and 13th ribs.

⁶Tissue depth measured at the 12th rib 11 cm from the midline of the backbone.

analyzed by ANOVA; the model included sex, line, housekeeping gene, and all 2- and 3-way interactions, with housekeeping gene within animals as the block. For the ANOVA of genes for which only the B2M housekeeping gene was used, the fitted terms were sex, selection line, and their interaction. Correlation coefficients between the Δ CT values for the target genes were calculated and analyzed separately for each of the housekeeping genes used for normalization, and were tested using the normal approximation. Genstat V8 was used for all the statistical analyses.

RESULTS

Phenotype Line Association

The mean pituitary weight of animals from the lean selection line was greater than that from the fat line ($P = 0.011$) when adjusted for carcass weight (Table 3), but the difference with sex was not significant ($P = 0.067$). There was no evidence that pineal gland weight varied with selection line ($P = 0.39$) or sex ($P = 0.37$).

A summary of the effect of selection line and sex on all of the animal BW and body composition traits is presented in Table 3. As in previous studies, there were differences between the lines for fat and growth traits.

Quantification of Pituitary Gene mRNA Levels Using Real-Time PCR

A summary of the mean within-group Δ CT for all pituitary candidate genes is presented in Table 4. There was a difference in GH mRNA concentration between

the selection lines ($P < 0.001$); the lean line had greater mRNA levels than the fat line. The amount of GH secretagogue receptor (**GHSR**) mRNA was also greater ($P < 0.001$) in the lean line (using all 3 housekeeping genes).

The between line comparison of GHRH receptor (**GHRHR**) mRNA levels using the 3 different endogenous control genes gave inconclusive results. Although the overall selection line effect for all 3 housekeeping genes showed no difference for GHRHR [($P = 0.052$), unless 2 outliers were removed ($P = 0.034$)], the line \times housekeeping gene interaction was very strong ($P < 0.001$). In fact, when normalized by housekeeping genes, there was a difference in the amount of GHRHR mRNA between the lines for B2M ($P < 0.001$) but not for HPRT and GAPDH, whereas the difference between sexes was significant for HPRT but not significant for other housekeeping genes.

There was no difference in mRNA levels of the 2 pituitary developmental genes, pituitary-specific transcription factor 1 (**Pit1**; $P = 0.46$) and prophet of pit1 (**Prop1**; $P = 0.24$) between the selection lines. However, Prop1 did show a sex \times line interaction that was greater in the lean rams than the fat rams ($P = 0.019$). There were no selection line differences observed for the quantity of GH receptor (**GHR**; $P = 0.33$), leptin receptor (**LepR**; $P = 0.89$), SSTR1 ($P = 0.47$), or SSTR2 ($P = 0.99$) mRNA.

Correlations of mRNA Levels Between Pairs of Genes

The data were also examined for correlations of mRNA levels between pairs of genes to provide evidence

Table 4. Mean Δ CT classified by selection line and sex and normalized to the housekeeping genes used for each target gene

| Gene ¹ | Housekeeping gene ² | Mean Δ CT | | | | SED ³ | P-value | | | |
|-------------------|--------------------------------|------------------|-------------|-------------|-------------|------------------|---------|--------|-------------------|------------------|
| | | Lean | | Fat | | | Sex | Line | Line \times Sex | Line \times HK |
| | | Ewe (n = 5) | Ram (n = 5) | Ewe (n = 5) | Ram (n = 5) | | | | | |
| Pit1 | 1 | 1.70 | 1.67 | 1.99 | 1.60 | 0.218 | 0.191 | 0.457 | 0.262 | N/A ⁴ |
| Prop1 | 1 | 6.53 | 6.21 | 6.37 | 6.64 | 0.160 | 0.814 | 0.244 | 0.019 | N/A |
| LepR | 1 | 7.09 | 6.81 | 6.72 | 7.07 | 0.522 | 0.924 | 0.886 | 0.408 | N/A |
| GHSR | 1,2,3 | 2.95 | 2.75 | 3.55 | 3.07 | 0.161 | 0.009 | <0.001 | 0.241 | 0.026 |
| GH | 1,2,3 | 0.83 | 0.40 | 1.53 | 1.26 | 0.159 | 0.007 | <0.001 | 0.507 | 0.064 |
| GHRHR | 1,2,3 | 0.14 | -0.03 | 0.49 | 0.23 | 0.205 | 0.166 | 0.052 | 0.766 | 0.003 |
| GHRHR | 1,2,3 ⁵ | 0.14 | -0.03 | 0.45 | 0.29 | 0.192 | 0.254 | 0.034 | 0.963 | <0.001 |
| GHR | 1 | 7.37 | 7.43 | 7.41 | 7.66 | 0.194 | 0.281 | 0.325 | 0.488 | N/A |
| SSTR1 | 1,2,3 | 7.63 | 7.56 | 7.62 | 7.87 | 0.293 | 0.672 | 0.470 | 0.444 | 0.372 |
| SSTR2 | 1,2,3 | 7.01 | 7.11 | 7.03 | 7.09 | 0.272 | 0.694 | 0.992 | 0.932 | 0.787 |

¹Pit1 = pituitary specific transcription factor 1; Prop1 = prophet of pit1; LepR = leptin receptor; GHSR = GH secretagogue receptor; GHRHR = GHRH receptor; GHR = GH receptor; SSTR1 = somatostatin receptor 1; and SSTR2 = somatostatin receptor 2.

²1 = B2M; 2 = HPRT; and 3 = GAPDH.

³SED = SE of difference.

⁴Not applicable for this analysis.

⁵GHRHR data was reanalyzed with 2 outliers (samples that had unusually high residuals) removed from the data.

of possible gene coregulation or interaction. There was a high correlation between GHRHR, GH, and GHSR mRNA levels regardless of the housekeeping gene used (Table 5; $P < 0.001$ for each correlation when averaged across housekeeping genes used for normalization). The correlation between the quantity of mRNA for GH and its receptor GHR was 0.34 when B2M was the housekeeping gene used ($P > 0.05$, compared with a critical value of 0.39).

Correlations between Prop1 and each of the following genes: SSTR2 (0.555; $P < 0.01$), GHSR (0.493; $P < 0.05$), GHRHR (0.403; $P < 0.05$), and GHR (0.427; $P < 0.05$), were detected when B2M was used as the housekeeping gene (correlations were not carried out for Prop1 with GAPDH and HPRT because correlations with these housekeeping genes were only performed when a significant difference was shown with B2M). Similarly,

Table 5. Correlations between GH, GH secretagogue receptor (GHSR), and GHRH receptor (GHRHR) mRNA levels for each housekeeping gene used for normalization¹

| Housekeeping gene ² | Gene | GH | GHSR |
|--------------------------------|-------|----------|----------|
| B2M | GHSR | 0.819*** | |
| | GHRHR | 0.887*** | 0.898*** |
| GAPDH | GHSR | 0.730*** | |
| | GHRHR | 0.501* | 0.663** |
| HPRT | GHSR | 0.719*** | |
| | GHRHR | 0.682** | 0.666** |

¹Critical value for $n = 20$: * = 0.388, $P < 0.05$; ** = 0.526, $P < 0.01$; *** = 0.686, $P < 0.001$.

²B2M = beta-2-microglobulin; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; and HPRT = hypoxanthine phosphoribosyl-transferase.

correlations were found between GHR and the GHRHR (0.555; $P < 0.01$), and GHSR (0.410; $P < 0.05$) genes with B2M, but GHR was not repeated with the other 2 housekeeping genes.

There was no correlation ($P > 0.05$) between the amount of mRNA for LepR or Pit1 and the mRNA levels of any of the other genes.

DISCUSSION

As reported previously, the mean pituitary weight of animals from the lean selection line was found to be significantly greater than those from the fat line when adjusted for carcass weight (Francis et al., 1998). Increased pituitary weights have also been described in Yorkshire and Duroc pigs that were selected for at least 14 generations for low backfat (Althen and Gerrits, 1976). The high-backfat pigs also had lower serum GH concentrations at weaning and slaughter.

Pituitary-specific transcription factor 1 and Prop1 are 2 of several homeodomain transcription factors that are required for the development of the anterior pituitary gland and the regulation of gene expression in the adult pituitary (Savage et al., 2003). Polymorphisms in Pit1 have been associated with variations in growth and carcass traits in pigs (Yu et al., 1995, 1996; Stancekova et al., 1999; Brunsch et al., 2002; Sun et al., 2002). However, in this study, neither Pit1 nor Prop1 showed a difference in mRNA levels between the selection lines, although Prop1 did show a sex \times line interaction that was greater in the lean males than in the fat males.

There was a difference in the amount of pituitary GH but not GHR mRNA between the selection lines; the lean line had a greater concentration of GH mRNA than the fat line. Although the differences in GH secretory capacity in animals between the selection lines (Suttie

et al., 1991) is probably due to differences in pituitary size and hence total numbers of somatotropes (Francis et al., 2000; total GH content), our results suggest that differences in GH concentration, or biosynthesis, within pituitary somatotropes may also have a role.

In contrast, a previous study of this population (Fleming et al., 1997) found no significant differences in GH mRNA between the selection lines. However, in their study mRNA levels were determined by northern blotting, which is likely to be less sensitive than real-time PCR. Although this study uses the same selection lines, an additional 8 years of divergent selection has occurred, enhancing the differences between the fat and lean lines. Other differences between the 2 studies that could contribute to these observations are differences in age and hence puberty status, sex of the sheep, and the amount of pituitary tissue sampled [pituitaries halved (Fleming et al., 1997) vs. whole pituitary in the current study].

The difference in GH mRNA levels may be, in part, a consequence of increased numbers of ghrelin receptors, and possibly GHRH receptors, on the surface of the somatotropes. It has been shown in sheep pituitary cells, *in vitro*, that GHSR and GHRHR act synergistically in the stimulation of GH secretion, and that GHRH and ghrelin act to regulate their own receptor mRNA levels (Kineman et al., 1999; Yan et al., 2004). However, GHRH treatment did not significantly influence the mRNA levels of GHSR, nor did GHRP-2 (a synthetic ghrelin) change GHRHR expression with short-term treatment (Kineman et al., 1999; Yan et al., 2004).

We found that the amount of pituitary GHSR mRNA was greater in the lean line regardless of which housekeeping gene was used. Growth hormone-releasing hormone receptor mRNA levels were found to be greater in the lean line, using all 3 housekeeping genes, only when outliers were removed. This might indicate that a difference in GHRHR gene expression exists between the selection lines, but further research is necessary to confirm this. In lean sheep, the increase in GHSR, and potentially GHRHR, expression may increase pituitary sensitivity to GH secretagogues and could contribute to the observed differences in GH synthesis and release in these animals.

Leptin is produced primarily in adipocytes and regulates food intake and energy expenditure (Zhang et al., 1994). In previous work with the AgResearch lean and fat selection lines, the relative levels of leptin mRNA in fat depots was approximately 2-fold greater in the fat line compared with the lean line of ram lambs fed *ad libitum* or fasted for 48 h (Kumar et al., 1998). It was suggested that this elevation in leptin mRNA was most likely a secondary consequence of obesity rather than a cause. Leptin also appears to be involved in ovine somatotrope function since pituitary somatotropes possess leptin receptors (Iqbal et al., 2000). Leptin inhibits the GHRH-stimulated secretion of GH in ovine pituitary cells *in vitro* (Roh et al., 1998). Leptin also modifies the cellular function of ovine pituitary

somatotropes by decreasing the synthesis of GH and GHRHR mRNA and increasing levels of GH-releasing peptides receptor mRNA (Roh et al., 2001).

The LepR gene expression was increased by GH and GHRH in GHRH transgenic mice (Cai and Hyde, 1998, 1999). Increased LepR mRNA levels have also been observed in the hypothalamus of feed-restricted compared with well-fed ewes (Dyer et al., 1997). We were unable to detect a difference in the LepR mRNA levels in the pituitary between our lean and fat selection lines, suggesting that differences in GH secretion between lean and fat lines are not mediated by LepR.

Somatostatin inhibits GH release but not its biosynthesis (Tannenbaum et al., 1989) and can potentially act through at least 5 SSTR subtypes (Moller et al., 2003). Somatostatin receptor 1 and SSTR5 have been cloned in sheep (Debus et al., 2001, 2002), and the expression of SSTR1, SSTR2, and SSTR5 has been described in the sheep pituitary (Debus et al., 2001, 2002; Yan et al., 2004). In this study we could find no differences in SSTR1 and SSTR2 mRNA levels between the selection lines. Since mRNA levels of SSTR3, SSTR4, and SSTR5 were not examined, we cannot completely discount a role for SRIF in modulating this differential GH secretion. However, our data indicate that SRIF is not acting via up- or downregulation of either SSTR1 or SSTR2.

The results suggest that the GH activation (rather than the feedback repression) system is biologically set at a greater level in the lean line compared with the fat line. The difference in GH secretion between the lines may be due to enhanced pituitary sensitivity to the GH secretagogues ghrelin and GHRH through an increase in GHSR and GHRHR expression. Alternatively, greater GH secretion in the lean line may be the result of increased secretion of ghrelin and possibly GHRH into the hypothalamic-pituitary portal vessels. The increased GH pulse frequency in lean compared with fat ram lambs also suggests direct hypothalamic effects or differences in feedback control of GH release (Suttie et al., 1993). Although an increased response to GHRH analogues has been demonstrated in lean sheep compared with fat sheep (Suttie et al., 1991), there are, as yet, no data on endogenous concentrations of hypothalamic GHRH, ghrelin, or SRIF in these sheep, nor expression of their cognate pituitary receptors.

If greater concentrations of GHRH are secreted by animals in the lean selection line, it could account for the heavier pituitaries, especially if this difference was present during pituitary development. Growth hormone-releasing hormone is a hypothalamic hormone essential for expansion of the somatotropic lineage during pituitary development, and excessive GHRH in animal models has been shown to result in enlarged pituitaries primarily due to somatotrope hyperplasia (Frohman et al., 2000). Somatotropes in mouse and human are the dominant pituitary cell type, normally accounting for about one-half of the pituitary volume (Baumann, 2001). Ghrelin may also be involved in the prolifer-

eration of somatotropes and of other pituitary lineages as seen in the lean animals. It has been reported that ghrelin stimulates the proliferation of a somatotrope pituitary tumor cell line, GH3, via activation of the MAPK pathway (Nanzer et al., 2004).

The correlation observed between the abundance of GHRHR, GH, and GHSR mRNA, and also the correlation between the amount of GHR, and of GHRHR and GHSR mRNA, could be expected for genes that are acting in a common regulatory pathway. Interestingly, there was also a correlation between mRNA levels of Prop1 mRNA and SSTR2, GHSR, GHRHR, and GHR mRNA, suggesting perhaps that Prop1 acts as a transcription factor in GH-related gene regulation in the adult sheep pituitary.

The transcription factor Prop1 has been shown to participate in the regulation of porcine FSH- β subunit gene expression (Aikawa et al., 2004). It was, however, surprising that no correlation was seen between the abundance of Pit1 mRNA and the other genes examined, because Pit1 is required for GH expression (Tuggle and Trenkle, 1996), and Pit1 gene expression is regulated by GHRH (Soto et al., 1995), IGF-I, and ghrelin (Garcia et al., 2001). However, this may be a reflection of age-dependent differential regulation of Pit1 (Garcia et al., 2001). For example, it has been shown that the synthetic oligopeptide growth hormone releasing peptide 6 (GHRP-6) does not activate the Pit1 promoter in pituitary cells derived from adult rats (Soto et al., 1995).

In summary, it appears there is a greater concentration of GH mRNA in the pituitary of genetically lean animals, which is associated with increased concentrations of pituitary GHSR and possibly GHRHR mRNA. This result suggests that the difference in GH secretion between the lines may result from differential pituitary sensitivity to ghrelin and/or GHRH signals. An additional mechanism that warrants further investigation is that the observed differential GH expression in these lines may be due in part to differences in extrapituitary signals of ghrelin and GHRH arising in the hypothalamus, other areas of the brain, or from other parts of the body such as fat depots.

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