BRIEF COMMUNICATION: Effect of growth hormone on the mammary transcriptome profile during established lactation in the dairy cow

S.A. McCOARD*, A.A. HAYASHI, D. PACHECO, Q. SCIASCIA, K. NONES, J.R. ROUNCE, B.R. SINCLAIR, W.C. MCNABB and N.C. ROY

AgResearch Grasslands, Private Bag 11-008, Palmerston North 4442, New Zealand
*Corresponding author: sue.mccoard@agresearch.co.nz

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INTRODUCTION

Gene expression profiling studies have provided insights into the potential mechanisms involved in a range of physiological processes in the ruminant mammary gland. However, despite the well established galactopoietic effect of growth hormone (GH) in lactating ruminants (Bauman, 1999; Akers, 2006), the molecular mechanism(s) that mediate the effects of GH on milk production are not fully understood.

GH has been implicated in the induction of α-casein gene and protein expression in cultured bovine mammary epithelial cells (Sakamoto *et al.,* 2005) and β-casein mRNA levels are elevated following a 63 day infusion of GH (Yang *et al.,* 2005), while κ-casein gene expression is unaffected by GH treatment in lactating dairy goats (Boutinaud & Jammes, 2004). In addition to transcription regulation, post-transcriptional regulation of casein mRNA has also been proposed (Zeigler & Wicha, 1992; Yang *et al.,* 2005). Hence, milk protein gene expression levels may be regulated through post-transcriptional mechanisms. The wider effects of GH treatment on the mRNA levels of other genes in the mammary gland of the lactating dairy cow have also not been reported.

We have previously reported that GH increases milk and protein yield in dairy cows potentially via both transcriptional and translational mechanisms using a microarray approach (McCoard *et al.*, 2004). The objective of this study was to validate the findings of that study and to gain a better understanding of the effects of GH on milk production by evaluating the wider gene expression profile in the mammary gland.

MATERIALS AND METHODS

All procedures involving these animals were carried out in compliance with the guidelines of the AgResearch Grasslands Animal Ethics Committee.

The animal experiment from which the tissues were derived has been described in detail elsewhere (Hayashi *et al.,* 2009). Briefly, eight non-pregnant second parity mid-lactation spring-calved Jersey cows with a live weight of 300 ± 9 kg were housed indoors, milked twice daily, and offered an *ad libitum* total mixed ration diet (70% as fed pasture silage and 30% concentrate) formulated to exceed metabolisable energy, protein and essential amino acid requirements, thrice daily. Following a two week acclimatisation period, cows were randomly assigned to treatment groups (n = 4/group) and administered with either a 500 mg dose of zinc sometribove - a slow-release formula of commercially available GH (Lactatropin®, Elanco Animal Health, Bryanston, South Africa) or saline (Control) via a single subcutaneous injection

TABLE 1: Gene name, gene bank ID, forward and reverse primer sequences (5'→ 3') and quantitative real time-polymerase chain reaction (PCR) product length (amplicon size). GPAM = Glycerol-3-phosphate acyltransferase 1; BCLAF1 = Bcl-2-associated transcription factor 1

Gene name	GenBank ID	Forward primer	Reverse primer	Amplicon size (bp)
Milk genes				
α_{s1}-casein	NM_181029	TGGGAGTGAATCAACTGAGGA	CAGAGCCAATGGGATTAGGGA	434
α_{s2}-casein	NM_174528	GGACGATAAGCACTACCAGA	TGGCTTCATAGCTTTCTGATGC	358
β-casein	NM_181008	TCCCTCCTCTTACTCAAACC	ACTGAGGAGGAAACATGACA	260
κ-casein	NM_174294	ACCAACAGAAACCAGTTGCAC	CTACAGTGCTCTCTACTGCTT	303
α-lactalbumin	M18780	ACCAGTGGTTATGACACACAAGC	AGTGCTTTATGGGCCAACCAGT	233
β-lactoglobulin	BC108213.1	ATCCCTGCGGTGTTCAAGAT	CCATGCAGACGAGGTACT	365
Reference genes				
GPAM	AY515690	CTGTGCTATCTGCTCTCCAAT	CTCGGCAAGACCAGGAC-3'	156
BCLAF1	NM_001098117	CAAAGACAATCACACCACAG	GCCTCTTTATCCCTGGTATT	175

TABLE 2: Effect of growth hormone (GH) on the mRNA levels and yield of the major milk protein genes in the lactating bovine mammary gland. Data is expressed as fold change in expression (mRNA levels) ± standard error or percentage increase in protein yield in GH treated animals relative to Controls.

Parameter	Expression	Significance	Protein yield[1]
κ-casein	2.1 ± 0.7	P < 0.05	↑ 37%
α_{s1}-casein	2.2 ± 0.6	P <0.001	↑ 42%
α_{s2}-casein	2.1 ± 0.9	P >0.10	↑ 42%
β-casein	2.1 ± 0.6	P <0.01	↑ 42%
α-lactalbumin	2.0 ± 0.6	P <0.05	↑ 79%
β-lactoglobulin	1.4 ± 0.3	P <0.10	↑ 38%

[1] Data derived from McCoard *et al.,* 2004.

(Day 0). Reported changes in milk yield have been described previously (McCoard *et al.,* 2004).

Cows were euthanised on Day 6 using an overdose of barbiturate, lobulo-alveolar mammary tissue was snap frozen in liquid nitrogen and stored at -85°C for later analysis. Total RNA was extracted using Trizol® Reagent (Invitrogen Life Technologies, Carlsbad, California, USA) according to the manufacturer's protocol, and total RNA quality and quantity estimated using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA). Fluorescently-labelled cDNA (SuperScript Indirect cDNA Labeling System, Invitrogen Life Technologies; Cy5 or Cy3 dyes, Amersham Biosciences, Piscataway, new Jersey, USA) was generated and hybridised to AgResearch proprietary bovine cDNA microarrays containing 22,690 unique amplified cDNAs (representing 17,307 different genes) using a loop design balanced for dye bias (Pacheco *et al.,* 2010). Slides were scanned using an Axon Professional 4200A scanner (Axon Instruments, GE Healthcare, San Francisco, California, USA) at 10 μm resolution, and image combination and processing performed using GenePix Pro 6.0 software (Axon Instruments, GE Healthcare, San Francisco, California, USA). Normalised EST expression data were analysed using the LIMMA package in R 2.7.0. A modified *t*-test was performed for each EST and the treatment effect P value adjusted for multiple testing using the Benjamini and Hochberg correction (Benjamini & Hochberg, 1995) to generate a false discovery rate (FDR). Genes with ≥2 ESTs with a FDR <0.05 and a 1.2 fold differential expression levels were considered to be of interest. Ingenuity pathway analysis (IPA) software (http://ingenuity.com) was used to cluster genes into specific pathways/functions.

The expression of milk protein genes were evaluated by a quantitative real time polymerase chain reaction (qRT-PCR) using the Light Cycler (Roche Diagnostics, Mannheim, Germany) and the Light Cycler Fast Start DNA Master Plus SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) and normalised against two reference genes (*GPAM*, *BCLAF1*) selected from the microarrays (Table 1). The qRT-PCR conditions for all primers were denaturation: 95°C, 10 minutes; annealing: 62°C, 10 seconds; elongation: 72°C, amplicon length/25 seconds and melting: 95°C, 10 seconds; with 40 cycles. PCR efficiencies were calculated using the LinReg PCR program (Ramakers *et al.,* 2003). Differences in gene expression levels were assessed by a pair-wise fixed reallocation randomisation test, using the relative expression software tool (REST) (Pfaffl *et al.,* 2002). Primers (Table 1) were designed using the Roche Probe Design Software v4 (Roche Diagnostics, Mannheim, Germany), with specificity to each target gene assessed using gel electrophoresis and amplicon sequencing. Data is presented as fold differences in expression ± standard error in GH-treated relative to Control cows.

RESULTS AND DISCUSSION

The mammary transcriptome profiles of lactating dairy cows was evaluated following six days of treatment with bovine GH to better understand the mechanisms underlying the effects of GH on milk production, notably milk protein synthesis.

Real time PCR analysis indicated that GH increased α_{s1}-casein, β-casein, α-lactalbumin, and κ-casein mRNA levels and tended to increase β-lactoglobulin mRNA levels but α_{s2}-casein mRNA levels were unchanged (Table 2). Using the described selection criteria, with the exception of κ-casein (1.45 fold increase; 1 EST), microarray analysis was unable to detect difference in mRNA levels of the milk protein genes. It is likely that microarray profiling was not sensitive enough to detect small differences in mRNA levels, whilst qRT-PCR has greater levels of sensitivity as classically observed. The mRNA levels reported here differ to our previous report (McCoard *et al.,* 2004) where microarray analysis indicated that GH increased κ-casein, α_{s2}-casein, α-lactalbumin and β-lactoglobulin mRNA levels, but decreased β-casein and α_{s1}-casein mRNA levels. The difference between the current study and our previous report is an increase in the stringency for selection of differentially expressed genes in the microarray and qRT-PCR analysis in this report. The increase in β-casein mRNA levels in response to GH is consistent

with the observations of Yang *et al.* (2005), however the increase in κ-casein mRNA levels contrast observations in goats where GH fails to affect κ-casein mRNA levels (Boutinaud & Jammes, 2004), suggesting potential species-specific effects.

There was no clear relationship between the mRNA levels and the yield of the individual milk proteins previously reported (McCoard *et al.,* 2004) suggesting that mRNA levels are only one of the drivers of milk protein yield. For example, although GH increased the yield of α-casein 42%, α_{s1}-casein mRNA levels were increased 2.2 fold while α_{s2}-casein mRNA levels were unchanged (Table 2). In addition, despite a 2-fold increase in β-casein and α-lactalbumin mRNA levels, GH administration elicited a 42% and 79% increase in the yield of these two proteins respectively (Table 2). These observations suggest that the effects of GH on milk protein yield are achieved through both transcriptional (mRNA) and post-transcriptional mechanisms.

Mammary transcriptome analysis identified 947 genes whose expression levels were altered by GH of which 366 were down-regulated and 579 were up-regulated. Application of more stringent selection criteria of ≥2 ESTs differentially expressed per gene to minimise potential false positives, the analysis identified 99 unique genes of which 27 were down- and 72 were up-regulated in response to GH. These genes consisted of the following ingenuity pathway analysis categories: enzymes (n = 22), transporters (n = 10), transcriptional regulators (n = 5), translational regulators (n = 3), transmembrane receptors (n = 5), kinases (n = 3), peptidases (n = 4) and others (n = 46).

Ingenuity pathway analysis indicated that GH administration influences the mRNA levels of genes from a wide range of pathways, with considerably more genes up- than down-regulated in each of the functional categories evaluated as expected for a stimulatory effect on milk production. Two of the top GH-responsive biological functions were cell growth and proliferation for example, *BCL2*, *COL1A1, FABP3* and cell death for example, *IGFBP5*, *FOXO3A, CD14*, suggesting GH may influence cell number and/or maintenance of secretory cell number consistent with previous reports (Capuco *et al.,* 2001; Boutinaud *et al.,* 2003) as well as apoptosis. Other biological functions of particular interest in this study were cell-to-cell signaling and interaction and molecular transport such as, *ABCG2*, small molecule biochemistry such as, *SAT1*, protein synthesis such as, *EEF1A1*, *EEF1A2*, carbohydrate metabolism such as, *UGP2*, lipid metabolism such as, *LPL*, *FABP2* and amino acid metabolism such as *GLS*, consistent with increased milk and milk protein yield. The mRNA levels of genes involved in the regulation of cell signaling, post-translation modification and gene expression, and transcriptional and translational control, were also modified by GH consistent with both transcriptional and translational regulation of milk production.

These findings provide further insights into the molecular changes involved in the increase in milk production in response to GH in the dairy cow. These observations support the concept that milk protein gene expression is regulated by both transcriptional and post-transcriptional events, and that GH-simulated increases in milk production may be associated with increased cell number and/or the secretory function of the mammary gland.

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