Effect of growth hormone on the liver transcriptome profile during established lactation in the dairy cow

D. PACHECO, K. NONES, Q. SCIASCIA and S.A. MCCOARD*

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

ABSTRACT

Milk production involves a coordinated metabolic response from multiple tissues. Administration of growth hormone (GH) to lactating cows provides an endocrine signal that redirects nutrients towards milk production. Hepatic responses to GH define the nutrient supply to peripheral organs both directly and indirectly, through processes such as gluconeogenesis and endocrine modulation. However, the mechanisms controlling these processes in the liver itself have not been completely elucidated. Our objective was to evaluate the effect of GH on gene expression in the liver of lactating dairy cows. Using a 22,690 expressed sequence tags (EST) bovine cDNA microarrays, we examined differences in liver mRNA transcript profiles between four cows treated with a slow-release formulation of GH and four control cows treated with saline solution. We identified 38 unique transcripts in liver that met the criteria of having expression changes greater than +/- 1.2 fold and false discovery rates <0.05. Of these genes affected by GH, 29 were down-regulated and nine were up-regulated. The pathways most affected by GH were carbohydrate and lipid metabolism, molecule transport and small molecule biochemistry. This preliminary study provides further insights into the molecular changes involved in the effects of GH on liver gene expression in the lactating dairy cow.

Keywords: bovine; liver; gene expression; milk; growth hormone.

INTRODUCTION

Lactation is an orchestrated process in which the metabolic activity of multiple tissues is modulated to direct nutrients towards the mammary gland. Growth hormone (GH) is a critical part of the endocrine controls modulating the utilisation of nutrients among different organs and tissues. In the lactating dairy cow, this modulation increases milk production by partitioning nutrients towards lactation (Bauman, 1992). The liver is an important organ in this process, as it is the point of confluence of endocrine signals, nutrient-rich blood flow from the gastrointestinal tract and blood flow from peripheral tissues, including the mammary gland. The contribution of liver metabolism to lactation is exemplified by the fact that hepatic gluconeogenesis provides most of the glucose requirement for lactose synthesis in a lactating dairy cow (Etherton & Bauman, 1998). The liver also acts as a mediator of the responses of muscle and adipose tissue through its role in lipid and amino acid metabolism (Burton *et al.*, 1994; Rhoads *et al.*, 2007). However, milk production is not just the result of hepatic nutrient "spill over" (Doepel *et al.*, 20090, but rather the mammary gland, liver and other organs influence each other in a complex nutrient and endocrine coordination.

Although the metabolic responses to GH in lactating ruminants have been extensively studied, the molecular pathways coordinating inter-organ nutrient metabolism and endocrine signals are not fully understood.

The objective of this study was to gain a better understanding of the effects of GH on nutrient partitioning in support of lactation, by evaluating the mRNA transcript profile (transcriptome), in the liver using cDNA microarrays.

MATERIALS AND METHODS

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

Liver tissue was collected from lactating dairy cows treated with either commercially available GH (Treatment) or saline (Control) as previously described (Hayashi *et al.*, 2009). Briefly, eight non-pregnant, second parity spring-calved Jersey cows (300 ± 9 kg; 189 ± 11 d postpartum) were housed indoors, milked twice daily, and offered *ad libitum* a total mixed ration diet consisting of approximately 6.3 kg DM of pasture baleage (11.5 MJ ME/kg DM), plus approximately 2.7 kg of milking cow concentrate (11.5 MJ of ME/kg DM). The diet, which was formulated to exceed by 10% the National Research Council's recommendations for metabolisable energy, protein and essential amino acid requirements (National Research Council, 2001), was offered thrice daily. Following a two-week adaptation period, four cows were randomly assigned to each treatment groups and received either a slow-release formula of commercially available recombinant GH (Lactotropin®, 500 mg GH per cow; Elanco Animal Health, South Africa) or sterile saline solution (0.9% sodium chloride;

Baxter Healthcare, Australia) via a single subcutaneous injection on Day 0. Liver tissue was harvested on Day 6 (Treatment) or Day 7 (Control) within five minutes of euthanasia by barbiturate overdose (Pentobarb, Provet, New Zealand). Tissue was snap frozen in liquid nitrogen and stored at -85°C for later analysis.

Microarrays

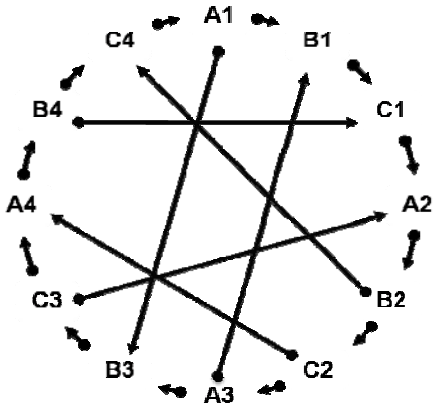
RNA extraction

Total RNA was extracted using Trizol® Reagent (Invitrogen Life Technologies, Carlsbad, California, USA) according to the manufacturer's protocol. Extracted total RNA was quantified and quality checked using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA). Only samples with OD_{260} to OD_{280} ratios greater than 2.0 were used for microarray hibridisations.

cDNA generation, slide hybridisation and scanning

Bovine cDNA microarrays were prepared as described by Diez-Tascón *et al*. (2005). The microarrays contained 22,690 unique amplified cDNA expressed sequence tags (EST), representing 52 tissue libraries, including the liver, and 17,307 unique genes. The liver libraries represented 465 ESTs. Fluorescently-labelled cDNA (SuperScript Indirect cDNA Labeling System, Invitrogen Life Technologies, Carlsbad, California, USA; Cy5 or Cy3 dyes, Amersham Biosciences, Piscataway, New Jersey, USA) was generated from 10 µg total RNA per tissue sample and purified to remove unincorporated dyes using the QIA-Quick PCR purification kit (Qiagen, Hilden, Germany). Microarray slides were pre-hybridised in a preheated (42°C) 0.22 µm filtered solution of 5x SSC (3.0 M sodium chloride; 0.30 sodium citrate), 0.1% SDS and 0.25% bovine serum albumin (Sigma A-788) for 20 minutes. Slides were rinsed twice in deionised water, once in isopropanol and air dried prior to hybridisation. Samples were heat-denatured (95°C x 10 minutes) and 60 µL pre-warmed (68°C) SlideHyb No1 (Ambion, Foster City, California, USA) added. Hybridisations were conducted for 20 hours in sealed humidified chambers (CMT Hybridisation Chambers, Corning Inc., Corning New York, USA). Following hybridisation, slides were washed for 5 minutes each in (1) 2x SSC, 0.1% SDS, (2) 1x SSC and (3) 0.1x SSC and centrifuged at 1000 *g* for 5 minutes. The slides were stored in the dark to minimise photo-bleaching, and scanned for both dye channels at 532 and 635 nm within two days using an Axon Professional 4200A scanner (Molecular Devices, Sunnyvale, California, USA) at 10 µm resolution. Data from the image combination and processing were generated in GPR format using GenePix Pro 6.0 software (Axon Instruments, Sunnyvale, California, USA) with automatic flagging of spurious spots. For this experiment, 18 microarray slides were used in modification of the loop design described by Oleksiak *et al*. (2002) with a balanced, partial dye swap (Figure 1). Only results from the Control and GH treatments are presented herein.

FIGURE 1: Diagramatic representation of the loop design used for the microarray experiment. Three treatments were included (A, B, C), with four cows each (eg. A1, A2). Each arrow symbolises a microarray slide, and letters connected by the arrows indicate the pair of samples hybridised in each slide. Round end and point end of the arrow indicate which sample in the pair was hybridised to Cy3 and Cy5 dyes, respectively. Only contrasts between treatments A (Control) and B (GH Treatment) are presented in this manuscript.



Statistical analysis of microarray data

All data quality checks, normalisation and tests of treatment effects were conducted in R 2.7.0 using the IPLOTS (http://www.iPlots.org) and LIMMA (Smyth, 2005) packages. Data diagnostics included boxplots of foreground and background readings per dye, within and across slides. Data flagged, internal negative controls (bacterial genes) and blank spots were weighed zero prior normalisation of the data using the print-tip-loess method with no background correction. 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 to generate a false discovery rate (FDR). Genes with a FDR <0.05 and a +/-1.2 fold differential expression levels were considered significantly affected by treatment.

Gene annotation and pathway analysis

Ingenuity pathway analysis (IPA) software (http://ingenuity.com) was used to identify the biological functions and metabolic pathways of the genes differentially expressed in response to GH treatment. To annotate the bovine cDNAs, all the microarray EST sequences, including those publicly deposited in the National Center for Biotechnology Information (NCBI), were assembled into contigs

TABLE 1: Complete list of differentially expressed genes in liver in response to growth hormone (GH) treatment versus Control. Genes are ordered in relation to fold difference in expression. Molecule type assigned by ingenuity pathway analysis software is shown.

Reference sequence identification number	Gene symbol	Description	Molecule type	Fold change
NM-004265	FADS2	Fatty acid desaturase 2	Enzyme	1.63
NM_001443	FABP1	Fatty acid binding protein 1, liver	Transporter	1.43
NM_001639	APCS	Amyloid P component, serum	Other	1.36
NM_001891	CSN2	Casein beta	Kinase	1.35
NM_001512	GSTA4	Glutathione S-transferase alpha 4	Enzyme	1.32
NM_003617	RGS5	Regulator of G-protein signalling 5	Other	1.28
NM_000371	TTR	Transthyretin	Transporter	1.24
NM_006759	UGP2	UDP-glucose pyrophosphorylase 2	Enzyme	1.21
NM_000151	G6PC	Glucose-6-phosphatase, catalytic subunit	Phosphatase	1.20
NM_016172	UBAC1	UBA domain containing 1	Other	-1.20
NM_004713	SDCCAG1	Serologically defined colon cancer antigen 1	Other	-1.20
NM_006755	TALDO1	Transaldolase 1	Enzyme	-1.21
NM_024297	PHF23	PHD finger protein 23	Other	-1.21
NM_006547	IGF2BP3	Insulin-like growth factor 2 mRNA binding protein 3	Translation regulator	-1.22
NM_005926	MFAP1	Microfibrillar-associated protein 1	Other	-1.22
NM_004282	BAG2	BCL2-associated athanogene 2	Other	-1.25
NM_006546	IGF2BP1	Insulin-like growth factor 2 mRNA binding protein 1	Translation regulator	-1.25
NM_019049	DENND1B	DENN/MADD domain containing 1B	Other	-1.26
NM_005854	RAMP2	Receptor (G protein-coupled) activity modifying protein 2	Other	-1.26
NM_021964	ZNF148	Zinc finger protein 148	Transcription regulator	-1.26
NM_001145	ANG	Angiogenin, ribonuclease, RNase A family, 5	Enzyme	-1.27
NM_002966	S100A10	S100 calcium binding protein A10	Other	-1.28
NM_001253	CDC5L	CDC5 cell division cycle 5-like (S. pombe)	Other	-1.29
NM_015054	UHRF1BP1L	UHRF1 binding protein 1-like	Other	-1.29
NM_006049	SNAPC5	Small nuclear RNA activating complex, poly	Transcription regulator	-1.31
NM_001357	DHX9	DEAH (Asp-Glu-Ala-His) box polypeptide 9	Enzyme	-1.32
NM_004467	FGL1	Fibrinogen-like 1	Other	-1.33
NM_005502	ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	Transporter	-1.33
NM_005651	TDO2	Tryptophan 2,3-dioxygenase	Enzyme	-1.36
NM_004295	TRAF4	TNF receptor-associated factor 4	Other	-1.39
NM_002084	GPX3	Glutathione peroxidise 3 (plasma)	Enzyme	-1.41
NM_001381	DOK1	Docking protein 1, 62kDa (downstream of tyrosine kinase 1)	Other	-1.42
NM_005946	MT1A	Metallothionein 1A	Other	-1.44
NM_016218	POLK	Polymerase (DNA directed) kappa	Enzyme	-1.44
NM_052870	SNX18	Sorting nexin 18	Transporter	-1.44
NM_133436	ASNS	Asparagine synthetase	Enzyme	-1.48
NM_144611	CYB5D2	Cytochrome b5 domain containing 2	Other	-1.49
NM_002755	MAP2K1	Mitogen-activated protein kinase kinase 1	Kinase	-1.57

using the CAP3 program (Huang & Madan, 1999) after an initial clustering step using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These contigs were used for all future ontology identification. For the protein annotation, the contig to which each EST belonged was blasted against the GenBank SwissProt database and the best match extracted. These annotations allowed for mapping of the genes to those in the IPA database. The significance value (P value) generated by IPA denote the probability that the association of a given gene to a biological function or metabolical canonical pathways is due to chance alone. The software also assigns ratio scores that represent the number of genes from the dataset that map to the pathway divided by the total number of genes in that pathway.

RESULTS

Using the fold-difference and FDR criteria described above, liver transcriptome analysis identified a significant effect of treatment on transcripts of 38 distinct genes, of which 29 were down-regulated in GH treated cows compared to Control cows, and nine were up-regulated (Table 1). These genes encoded proteins of the following

TABLE 2: Top four biological functions assigned to the dataset by ingenuity pathway analysis software (http://ingenuity.com), the range of P values for each classification, and the genes differentially expressed within a functional group. P values generated by ingenuity pathway analysis denote the probability that the association of a given gene to a biological function or metabolical canonical pathways is due to chance alone. The identity, fold changes, and P values for each of these genes are detailed in Table 1.

Biological function	P value	Gene symbols
Carbohydrate metabolism	1.64E-05 - 4.01E-02	UGP2, APCS, TALDO1, FABP1, G6PC, RAMP2, ABCA1
Lipid metabolism	1.64E-05 - 4.97E-02	TTR, FADS2, FABP1, G6PC, DOK1, ANG, ABCA1, S100A10
Molecular transport	1.64E-05 - 4.97E-02	TTR, UGP2, TDO2, FABP1, G6PC, DOK1, ABCA1, S100A10
Small molecule biochemistry	1.64E-05 - 4.97E-02	TTR, TDO2, TALDO1, ABCA1, GPX3, UGP2, APCS, FADS2, FABP1, G6PC, DOK1, ANG, S100A10

types: enzymes (n = 10), transporters (n = 4), kinases (n = 2), phosphatases (n = 1), translational regulators (n = 2), transcriptional regulators (n = 2), with 17 genes classified as "other" (Table 1).

The four biological functions identified by IPA analysis as being most significantly (P <0.0001) affected by GH treatment were carbohydrate metabolism, lipid metabolism, molecular transport and small molecule biochemistry. The genes associated with these biological functions are presented in Table 2.

The canonical metabolic pathways, their involved genes, the associated P values and the ratio scores generated by IPA analysis that were considered significant (threshold >0.05) included: galactose metabolism (*UGP2*; *G6PC*) P = 0.004, 2/116; glutathione metabolism (*GPX3*, *GSTA4*) P = 0.007, 2/98; starch and sucrose metabolism (*UGP2*, *G6PC*) P = 0.02, 2/98; nicotinate and nicotinamide metabolism (*G6PC*, *MAP2K1*) P = 0.02, 2/136; nucleotide sugars metabolism (*UGP2*) P = 0.03, 1/74; inositol phosphate metabolism (*G6PC*, *MAP2K1*) P = 0.04, 2/176.

DISCUSSION

Growth hormone has a well known effect on carbohydrate metabolism to meet the increased glucose requirement for milk production. Our microarray approach confirmed its influence on the genes underlying this biological response. Glucose-6-phosphatase, catalytic subunit (*G6PC*) is a key enzyme in glucose homeostasis, functioning in both gluconeogenesis and glycolysis. Up-regulation of the expression level of this gene is consistent with the known stimulatory effects of GH on gluconeogenesis in the liver. Increased glucose production via hepatic gluconeogenesis and decreased glucose oxidation by body tissues provides the increase in glucose supply required for GH-induced increases in milk production (Knapp *et al.*, 1992; Etherton & Bauman, 1998;;). Asparagine synthetase (*ASNS*) expression level was decreased by GH treatment, suggesting that the increase in GH levels may reduce the biosynthesis of asparagine from aspartate and glutamine. *ASNS* expression has been reported as a "nutrient-sensing" reaction, as it is increased by either amino acid or glucose deprivation (Hutson *et al.*, 1996; Zhong *et al.*, 2003). Thus down-regulation of *ASNS* expression in response to elevated levels of GH could signal amino acid sufficiency to the liver, allowing the channelling of aspartate, and other amino acids, to gluconeogenesis.

GH has a catabolic effect on lipid metabolism in adipocytes, with increased lipolysis observed when cows are in negative energy balance or reduced lipogenesis when the energy balance is positive (Burton *et al.*, 1994; Etherton & Bauman, 1998). Importantly, despite increasing circulating concentrations of non-esterified fatty acid (NEFA), GH administration to lactating dairy cows does not lead to hepatic lipid accumulation and subsequent ketosis (Etherton & Bauman, 1998). Differentially expressed genes in response to GH that are involved in lipid metabolism comprised *TTR*, *FADS2*, *FABP1* and *G6PC* which were up-regulated, and *DOK1*, *ANG*, *ABCA1* and *S100A10* which were down-regulated. *FADS2* and *FABP1* were the most up-regulated genes in response to GH. Down-regulation of *FADS2* is associated with ketosis in cows (Loor *et al.*, 2007), while up-regulation of *FADS2* in response to GH suggests increased synthesis of polyunsaturated fatty acids, which may influence hepatic concentrations of cholesterol esters and/or phospholipids, as observed in mice (Song *et al.*, 2006). *FABP1*, the liver fatty acid binding protein, increases cellular uptake and metabolism of long-chain fatty acids in the liver, and is essential for peroxisomal β-oxidation of fatty acids (Brandes *et al.*, 1990; Hanhoff *et al.*, 2002; Newberry, *et al.*, 2003), producing short-chain fatty acyl-CoA intermediates that are completely oxidised in mitochondria (Drackley, 1999). Up-regulation of *FABP1* in response to GH may function to channel fatty acids towards peroxisomal oxidation. A role for *FABP3* in channelling fatty acids towards β-oxidation has been observed in exercising muscle in

humans (Schmitt *et al.*, 2003). Also, *FABP3* is down-regulated in cows with ketosis, consistent with decreased fatty acid oxidation (Loor *et al.*, 2007).

Collectively, these findings lead us to speculate that GH effects hepatic gene expression during lactation to provide a mechanism by which the liver is able to cope with the increased influx of NEFAs mobilised from adipose tissue, thereby providing a protective function against ketosis and liver lipidosis. In addition to the potentially important effects of GH on genes involved in fatty acid desaturation, gluconeogenesis, lipid accumulation and cholesterol synthesis and transport, GH also up-regulated *GSTA4*, which is involved in oxidative stress responses (Desmots *et al.*, 2002). *GSTA4* expression is down-regulated in cows with ketosis (Loor *et al.*, 2007), therefore up-regulation of *GSTA4* is consistent with increased detoxification of reactive oxygen species in the liver arising from lipid peroxidation and further supports a potential mechanism that enables the liver to protect against ketosis.

Metabolic pathways accounted for six of the 11 canonical pathways identified by IPA analysis as being significantly ($P < 0.05$) affected by GH treatment, of which galactose and glutathione metabolism were the top two pathways. The other metabolic pathways included starch and sucrose metabolism, nicotinate and nicotinamide metabolism, nucleotide sugars metabolism and inositol phosphate metabolism. Conservative interpretation of the relevance of these pathways is cautioned because the total number of differentially expressed genes entered into the program was relatively small. Nevertheless, these findings suggest that GH affects the metabolism of carbohydrates, lipids, amino acids, and cofactors and vitamins in the liver. Differentially expressed genes associated with molecular transport included up-regulation of *TTR*, a carrier protein that transports thyroid hormones and retinol (Vitamin A), and down-regulation of *TDO2*, which has a role in the rate-limiting step in the kynurenine pathway, the major pathway of tryptophan metabolism.

The expression of β-casein transcript (CSN2) in the liver is surprising as *CSN2* is thought to be spatially restricted to the mammary gland (Akers, 2002). The authors acknowledge that these preliminary results require validation by qRT-PCR as transcript expression differences detected by cDNA microarrays can differ from the more sensitive qRT-PCR method (Rajeevan *et al.*, 2001). However, many of the GH-responsive genes identified in the present study using microarrays are known to be expressed in the liver, such as *FADS2, FABP1, G6PC*, and many of the transcripts are involved carbohydrate and lipid metabolism in the liver.

We speculate that in addition to enhancing hepatic gluconeogenesis, GH may influence the expression of key hepatic genes such as *FADS2*, *FABP1* and *GSTA4*, which provide a mechanism by which the liver is able to cope with increased concentration of circulating NEFAs arising from tissue mobilisation or reduced lipogenesis, thereby reducing the risk of ketosis and fatty liver. Further mining of the data and validation of microarray results by real time PCR will contribute to our understanding of the effects of GH on liver gene expression in the lactating dairy cow.

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