Effects of negative energy balance on liver gene and protein expression during the early postpartum period and its impacts on dairy cow fertility

End of Project Report

Project RMIS 5234

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Summary

Negative energy balance (NEB) is a severe metabolic affecting high yielding dairy cows early post partum with both concurrent and latent negative effects on cow fertility as well as on milk production and cow health. The seasonal nature of Irish dairy production necessitates high cow fertility and a compact spring calving pattern in order to maximise grass utilisation. Poor dairy cow reproductive performance currently costs the Irish cattle industry in excess of €400 million annually. High milk yields have been associated with lower reproductive efficiency, and it has been suggested that this effect is probably mediated through its effects on the energy balance of the cow during lactation. The modern high genetic merit dairy cow prioritises nutrient supply towards milk production in early lactation and this demand takes precedence over the provision of optimal conditions for reproduction. In this study we used the bovine Affymetrix 23,000 gene microarray, which contains the most comprehensive set of bovine genes to be assembled and provides a means of investigating the modifying influences of energy balance on liver gene expression.

Cows in severe negative energy balance (SNEB) in early lactation showed altered hepatic gene expression in metabolic processes as well as a down regulation of the insulin-like growth factor (IGF) system, where insulin like growth factor-1 (IGF-1), growth hormone receptor variant 1A (GHR1A) and insulin-like growth factor binding protein-acid labile subunit (IGFBP-ALS) were down regulated compared to the cows in the moderate negative energy balance MNEB group, consistent with a five-fold reduction in systemic concentrations of IGF1 in the SNEB group.

Cows in SNEB showed elevated expression of key genes involved in the inflammatory response such as interleukin-8 (IL-8). There was a down regulation of genes involved in cellular growth in

SNEB cows and moreover a negative regulator of cellular proliferation (HGFIN) was up regulated in SNEB cows, which is likely to compromise adaptation and recovery from NEB.

The *puma* method of analysis revealed that 417 genes were differentially regulated by EB ($P<0.05$), of these genes 190 were up-regulated while 227 were down-regulated, with 405 genes having known biological functions. From Ingenuity Pathway Analysis (IPA), lipid catabolism was found to be the process most affected by differences in EB status.

Introduction

Successive animal breeding programmes combined with improved nutrition has led to significant improvements in milk production in the dairy cow over the last twenty years. The rate of genetic improvement in Ireland has been aided by the importation of the North American Holstein genotype. However increased milk production has been associated with impaired cow fertility and health and there has been a consistent decline in conception rate to first service in Irish dairy herds over the past 20 years. Similar trends have also been observed in the US and the UK. Decreasing conception rates and delayed resumption of ovarian cyclicity postpartum will extend calving to conception intervals, disrupt seasonal calving and ultimately increase the proportion of cows culled for infertility.

Negative Energy Balance

While such problems are likely to be multi-factorial growing evidence implicates negative energy balance (NEB) as a contributing factor. During the early postpartum period the energy demands of lactation exceed energy intake and cows enter a period of NEB. For example, a cow producing 35kg of milk has a daily net energy requirement of approximately 21 UFL, equivalent to the net energy content of 21kg of standard barley. This amounts to over three times more energy than the cow requires for maintenance alone. Lactogenesis becomes the predominant physiological process in terms of nutrient utilization and these energy deficits are offset by partitioning of body reserves to the liver for recycling and use by the mammary gland, or directly to the mammary gland. This results in increased mobilization of fat and protein reserves and these metabolic adaptations are accompanied by increased accumulation of NEFAs and BHB and reduced systemic concentrations of the insulin like growth factor-1 (IGF-1) hormone. Negative energy balance has being associated with aberrations in several reproductive and immune function variables, including resumption of ovarian cyclicity, uterine function, embryo survival and immune competence. However the relationship between energy balance status, animal physiology and the underlying mechanisms involved are as yet unclear.

Liver

The liver plays an essential role in the body through the expression of genes encoding plasma proteins, clotting factors, and enzymes involved in detoxification, gluconeogenesis, glycogen synthesis, and metabolism of glucose, lipid and cholesterol. Liver function is therefore crucial for maintaining metabolic homeostasis and is central to the adaptation and recovery from NEB. During the early postpartum period liver blood flow and metabolic activity is dramatically increased and the liver undergoes extensive biochemical and morphological modifications. These are necessary to maintain glucose homeostasis but in extreme cases can compromise liver function resulting in metabolic diseases such as ketosis and fatty liver. NEB therefore can have a profound affect on liver function and this is likely to be mediated by alterations in gene expression.

Objective

The object of this study is to investigate the affects of NEB on global patterns of gene expression in liver and reproductive tissues collected during the period of greatest NEB between cows managed to achieve a high or low energy balance status. The biological material for this experiment was collected as part of a larger Moorepark energy balance study (RMIS 4997).

To gain new insights into these events, candidate and global gene expression was analysed in liver obtained from cows managed to achieve a mild (MNEB) or severe negative energy balance (SNEB) status.

Materials & Methods

Animal Model

Twenty-four multiparous dairy cows were blocked according to parity and BCS three weeks prior to expected calving date and were randomly allocated within block to one of two postpartum dietary treatments differing in energy density and designed to create two divergent groups of animals in terms of energy balance (mild versus severely negative) postpartum. From the day of calving onwards animals were individually fed and energy balance was calculated on a daily basis. Follicular dynamics were recorded on a daily basis from seven days postpartum. From the original 24 cows, 12 animals (6 per treatment) were selected for slaughter on the basis of energy balance and possession of a healthy oestrogen active, dominant follicle. MNEB cows were fed *ad lib* grass silage supplemented with 8 kg day^{-1} of a 21% crude protein dairy concentrate and milked once daily. SNEB cows were restricted to 25 kg day^{-1} silage with 4 kg day^{-1} concentrate and milked thrice daily. The chemical composition of silage and concentrate offered was identical across treatments. Daily measurements of milk yield, milk composition, body weight, dry matter intake (DMI) and dietary energy intake were used to calculate EB, based on the French NE_L system (Jarrige, 1989). Net EB was calculated as Unité Fourragère Lait (UFL) day^{-1} in which one UFL is the Net Energy (NE) content of 1 kg of air-dry standard barley for milk production (Jarrige, 1989). Six animals from each treatment group, showing extremes in energy balance status based on measurements of body condition score were slaughtered on days 6-7 of the first follicular wave after calving based on transrectal ultrasonography. Tissues were collected at this point in relation to ovarian function, rather than at a fixed day postpartum in order to minimize confounding effects of hormonal variation and also to examine the effects of NEB in a number of different reproductive tissues for use in other studies. Mean energy balance on the day of slaughter for MNEB and SNEB groups was –1.8 ± 0.96 UFL/day and –6.6 ± 1.01 UFL/day respectively ($P<0.05$). Systemic concentrations of glucose (4.08 vs 2.66 mmol/l) and IGF-1 (51.38 vs 10.56 ng/ml) were higher ($P<0.001$) in the MNEB cows while β-

hydroxybutyrate (3.70 vs 0.53 mmol/l) and non-esterified fatty acids (1.40 vs 0.34 mmol/l) were higher ($P<0.05$) in the SNEB cows, confirming the divergence in energy balance. All procedures were conducted under licence and in accordance with the European Community Directive, 86-609EC.

Blood sampling, hormone and metabolite assays

Blood samples were taken approx 14 days and 7 days before ECD (i.e. 2 pre-calving bleeds). After calving bloods were taken 3 times weekly by jugular venipuncture, collected after morning milking (8:00 h) up to day of slaughter (approx 14 days after calving). Also bloods were taken twice daily between day 7 after calving and day of slaughter for reproductive hormone assays (E2, FSH and IGF etc). Samples were collected into lithium-heparin or K3 EDTA vials and were placed on ice before centrifugation at 2000g for 10 min. Plasma was decanted and stored at -20 ºC for subsequent analysis. For the IGF-1 assay human OCTEIA IGF-1 kits (DIS, Tyne and Wear, UK) were used. Blood samples were also analyzed for glucose, non-esterified fatty acids (NEFA), β-hydroxybutyrates, urea, cholesterol and tryglycerides using appropriate kits and an ABX Mira Autoanalyzer (ABX Mira, Cedex, France). Plasma insulin concentrations were determined using a solid-phase radioimmunoassay (coat-a-count, Diagnostics Products Corp., CA). Plasma oestradiol concentrations were measured using the Estradiol MAIA assay kit (BioStat Diagnostic systems, UK). Blood samples collected on day of slaughter by jugular venipuncture were also underwent haematological analysis. Red blood cell count (RBC), white blood cell count (WBC), granulocyte and monocyte counts, as well as packed cell volume (PCV), haemoglobin (Hb) concentration, mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and platelet numbers were determined with an automated cell counter (Celltac MEK-6108K; Nihon-Kohdon, Tokyo, Japan) within 6 h of blood sampling.

Tissue collection

Metabolic and reproductive tissues were collected under RNase free conditions, rinsed in RNase free phosphate buffer, snap-frozen in liquid nitrogen and stored at -80 ºC. Hepatic glycogen was

determined as previously described (Chun Y et al. 1998), with modifications: Briefly, 50mg liver was homogenised in 1.0ml KOH (30%, w/v) and centrifuged (9500 x g for 5 min). A portion of the clear supernatant (250 µl) was heated for 20 min at 100 ºC and total glycogen was precipitated from solution in 1.0 ml ethanol (-80 ºC). Pellets were reconstituted in distilled water H2O and added to 1.0 ml 0.2% anthrone reagent (Sigma; Sigma-Aldrich Company Ltd., Dorset, UK; dissolved in 98% H_2SO_4) and heated for 10 min at 100 ºC. Optical density measurements were obtained at 620 nm and average values from three separate assays were used to determine concentrations based on standard dilutions of bovine glycogen type IX (sigma). For triacylglyceride (TAG) analysis, total lipids were extracted from 50 mg samples of liver based on the Folch method (Folch et el) and total TAG was determined from lipid fractions using the infinityTM Triglycerides Reagent (Thermo Electron, Noble Park, Australia). Glycerol standards (Sigma) were used to derive equivalent concentrations of TAG by correction for unesterified glycerol (Stinshoff et al). Results are based on average optical density measurements from three separate assays.

RNA isolation and reverse transcription

Total RNA was prepared from 100 to 200mg of fragmented frozen liver tissue using the TRIzol reagent (Sigma). Tissue samples were homogenised in 5 ml of TRIzol® reagent in a RNase free glass scintillation vial (LIP, Galway) using a homogeniser (Ultra-Turras, Ika-Werke GmbH, Staufen, Germany). To avoid cross contamination, the rotor was consecutively washed with 70 % ethanol, RNase Away and RNase free water between samples. Homogenised samples were incubated at room temperature for 5 min and equally divided into four 1.5 ml microcentrifuge tubes. A one fifth volume of chloroform was added and the tubes were shaken vigorously and incubated at room temperature for 2 min. The samples were centrifuged at 12,000 x g for 15 min at 4^{o}C in a bench top centrifuge. The RNA-containing aqueous phase was transferred to an RNase-free microcentrifuge tube containing 0.6 volume of isopropanol (Sigma Chemical Co.)

and incubated at room temperature for 30 min. The isolated RNA was then precipitated by centrifugation at 12,000 x g for 10 min. The supernatant was removed and the RNA pellet was washed with 70% ethanol, air-dried and resuspended in nuclease free water (Promega Corporation, Madison,WI, USA). RNA samples were stored at –80^{o}C. Twenty micrograms of total RNA from each sample was treated for genomic DNA contamination with the RNase-free DNase set (QIAGEN, Crawley, West Sussex, UK) and purified using the RNeasy mini kit in accordance with guidelines supplied (QIAGEN, Crawley, West Sussex, UK). RNA quality and quantity was assessed using automated capillary gel electrophoresis on a Bioanalyzer 2100 with RNA 6000 Nano Labchips according to manufacturers instructions (Agilent, Waldbronn, Germany). All samples displayed intact 28S and 18S rRNA transcripts. Complimentary DNA (cDNA) was obtained by reverse transcribing 1 μg of DNase-treated RNA using AMV reverse transcriptase and 500ng random hexamer primers in a 20 μl reaction (Reverse transcription system kit; Promega WI, USA). A quantitiy of 0.39 ng of Kanamycin mRNA (Promega) was spiked into each sample as an exogenous control (Promega). A mastermix of reagents was prepared for the above reaction to minimize potential variation from pipetting. Negative control samples were also prepared by including all reagents as above for the cDNA synthesis, minus the reverse transcriptase enzyme.

Primer Design

Several house keeping genes were analysed across samples including ribosomal protein L19 (RPPL190) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Gene specific primers were designed (Table 1) online using the Primer3 web based software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) based on the bovine target sequences used on the Affymetrix array. Primer specificity was determined using the BLAST search tool from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). All oligonucleotides were commercially synthesized as highly purified, salt free products (Sigma genosys). For each gene, PCR conditions were optimized by conventional PCR amplification using Go Taq Flexi DNA

Polymerase (Promega, WI, USA), and addition of reverse-transcribed RNA and primers (20 μM). From these PCR products external standards were prepared from cDNAs identical to real-time PCR products and purified using QIquick PCR purification columns (QIAGEN, Crawley, West Sussex, UK). Exact concentrations of purified PCR product was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.,DE) and the presence of a single product confirmed by electrophoresis on a 1.5% (w/v) etidium bromide agarose gel. The identity of the cDNA products was confirmed by DNA sequence analysis (MWG Biotech AG, London, UK)

Real Time RT-PCR

Real-Time RT-PCR analysis was performed on selected genes using the Corbett Rotor-Gene 3000 cycler with the SYBR Green Master Mix (ABgene Surrey, UK). One μl of first strand cDNA synthesis reaction was amplified in a 20 μl volume using 10 μl ABsolute QPCR SYBR Green Mix and 1 μl of 20 μM forward and reverse primer mix. The reaction was made up to 20 μl using 8 μl Nuclease free water (Promega). The Real time PCR temperature profile consisted of 15 mins incubation @ 95ºC for SYBR Green activation. The cycling conditions consisted of 35 cycles of 30 secs @ 95ºC , the chosen annealing temperature for 30 secs, 30 secs @ 72ºC for primer extension followed by an amplicon specific acquisition reading temperature. A melting curve analysis was performed for each amplicon between 50-95ºC to eliminate any non-specific products such as primer dimers prior to acquisition. Non-reverse transcribed total RNA was included as a control for the presence of genomic DNA contaminants. The identity of the RT-PCR products was confirmed by DNA sequencing. All samples were assayed on the same run thereby eliminating inter-assay variation and specificity of the reaction products was confirmed by melt curve analysis and gel electrophoresis. RT-PCR gene expression data between groups was analyzed by ANOVA using PROC GLM (SAS v 9.01).

Micoarray analysis

Microarrays allow for the screening of several thousand genes simultaneously in a single sample. Here, gene expression profiling was conducted using a 24,027 probe set bovine oligonucleotide array (Affymetrix) representing approximately 23,000 bovine transcripts. Hybridisation of samples to arrays and scanning was carried out by the German Resource Centre for Genomics Research, Germany (RZPD). All microarray analysis including, pre-processing, normalisation and statistical analysis was carried out using R version 2.6.0 (www.cran.org), R Development Core Team, 2007) and Bioconductor version 2.1. Differential gene expression analysis was carried using the local the 'propagating uncertainty in microarray analysis' or puma method. Puma is a recent probe level method developed to utilise the probe level uncertainty in gene expression in downstream analysis. Using mutli-mgMOS and a Bayesian hierarchical model the puma method calculates the probability of positive likelihood ratio (PPLR) of differential gene expression ie. the probability that a gene is differentially expressed. A platform consisting of a total of 5787 annotated genes was used to compare the effects of SNEB versus MNEB. To examine the molecular functions and pathways, the microarray data was explored using Ingenuity Pathway Analysis 6.0 (IPA) software. This software programme allows the identification of biological mechanisms, pathways, and functions. Genes were assigned their respective human gene counterparts approved by the HUGO Gene Nomenclature Committee in preparation for IPA. The gene list containing gene names, gene expression fold change values and p-like values was uploaded into the IPA research tool.

Results

Energy balance and Metabolites

Mean energy balance on the day of slaughter for the MNEB and SNEB groups was –1.8 UFL/day and –6.6 UFL/day respectively ($P<0.05$) (Figure 1).

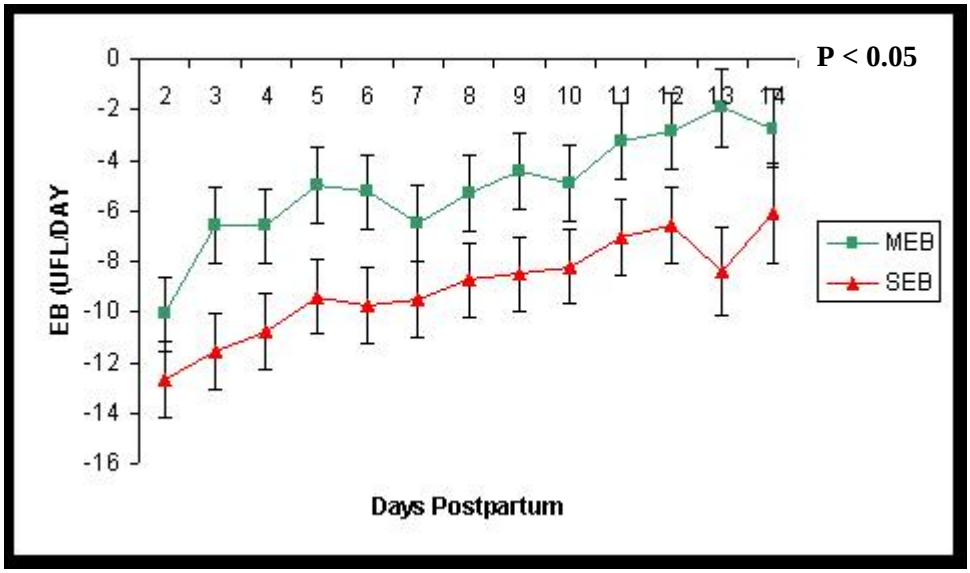

Figure 1. Effect of treatment on daily Energy balance (UFl day^{-1})

This difference in energy balance was further confirmed by divergence in systemic concentrations of metabolites and IGF-1. Cows in SNEB had increased systemic concentrations of non-esterified fatty acids (NEFA) (1.40 vs 0.34 mmol/l), β-hydroxybutyrate (BHB) (3.7 vs 0.53 mmol/l) (P<0.01) and hepatic triacylglyceride (5.8 vs 1.5 mM) (P<0.05). Mean plasma IGF-1 concentrations were not different between groups for the first week of lactation, however after this time concentrations were divergent, with peripheral IGF-1 levels in SNEB cows, much lower than those of the MNEB group (Figure 2).

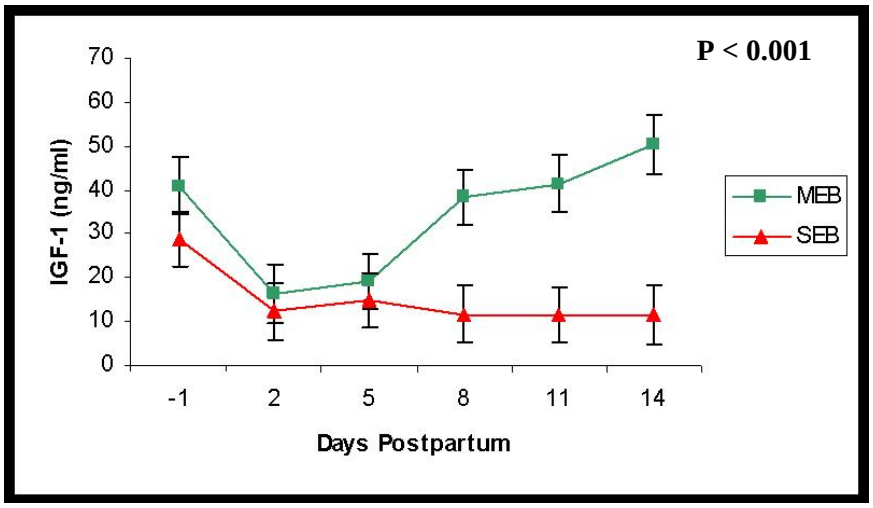

Figure 2. Effect of treatment on plasma IGF-1 concentrations

Mircoarray data

A cut off 'p-like value' of $P<0.05$ resulted in a total of 417 differentially expressed genes using the *puma* method. Of these 190 were up-regulated while 227 were down-regulated. Data were further analysed using Ingenuity Pathway Analysis (Ingenuity Systems®, www.ingenuity.com). IPA is a bioinformatic tool which allows you to maps gene expression data into relevant pathways based on their functional annotation and known molecular interactions. A total of 4995 genes on the array could be mapped to the IPA database. Of the 417 differentially expressed genes a total of 405 genes could be mapped to a known molecular function or pathway and 314 were eligible for generating networks using IPA. Among the network eligible genes, 140 were up-regulated while 174 were down-regulated. A total of 136 up-regulated and 168 down-regulated genes were associated with one or more biological functions, disease and/or developmental process.

The molecular and cellular functions which were most significantly affected by EB status include lipid metabolism, molecular transport, small molecule biochemistry, amino acid metabolism and cell morphology. Canonical signalling pathway analysis revealed that some of the nuclear hormone receptor superfamily including liver X receptors (LXRs) and the retinoid X receptor as well as amino acid metabolism were the pathways most affected by EB status.

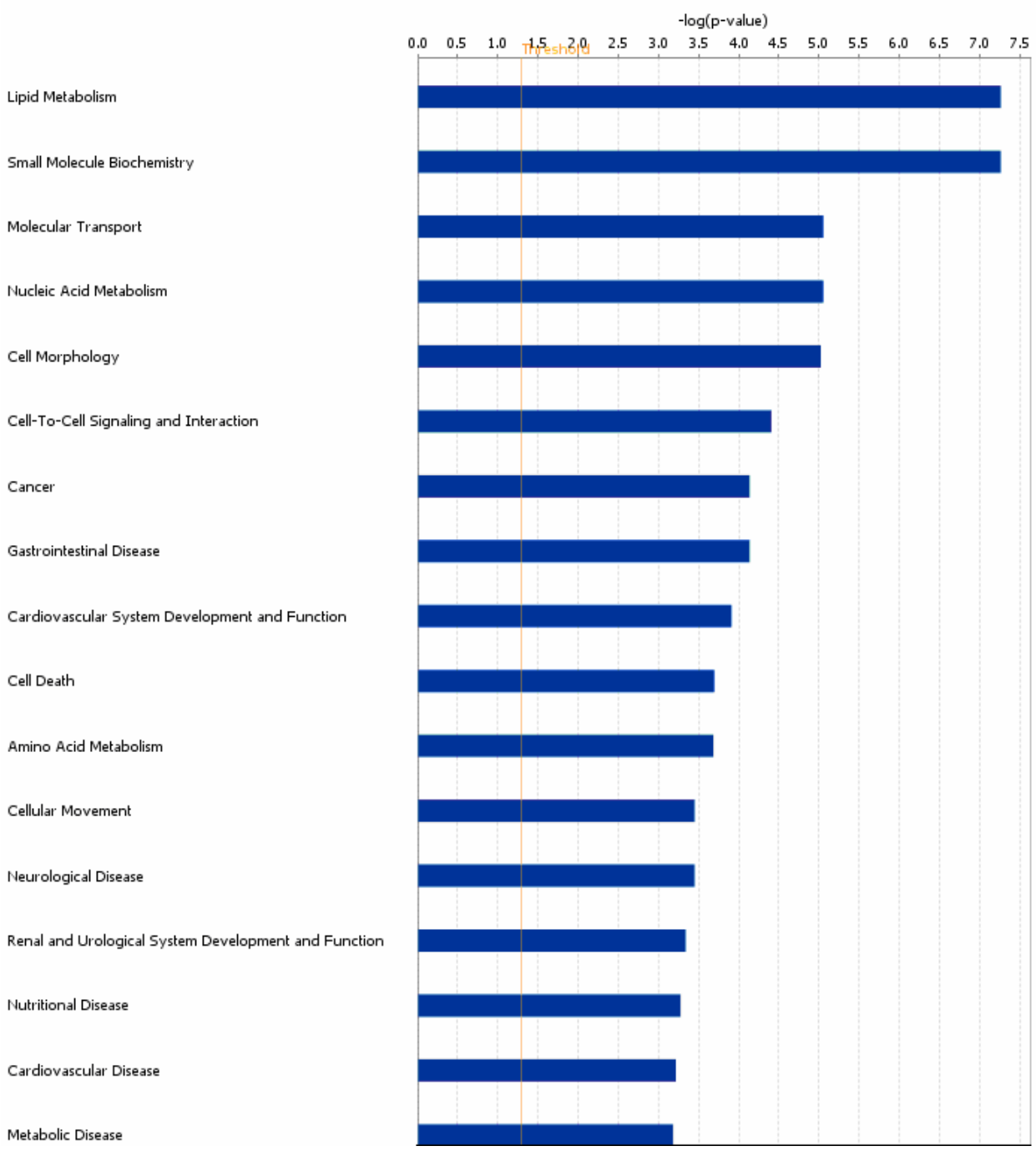

Figure 3. Most highly significant functional groups of differentially expressed genes using IPA. Blue bars indicate the likelihood [-log (P-value)] that the specific functional category was affected by NEB compared with others represented by the list of differentially expressed genes. The threshold line (—) is shown at $P<0.05$ (1.301 log scale).

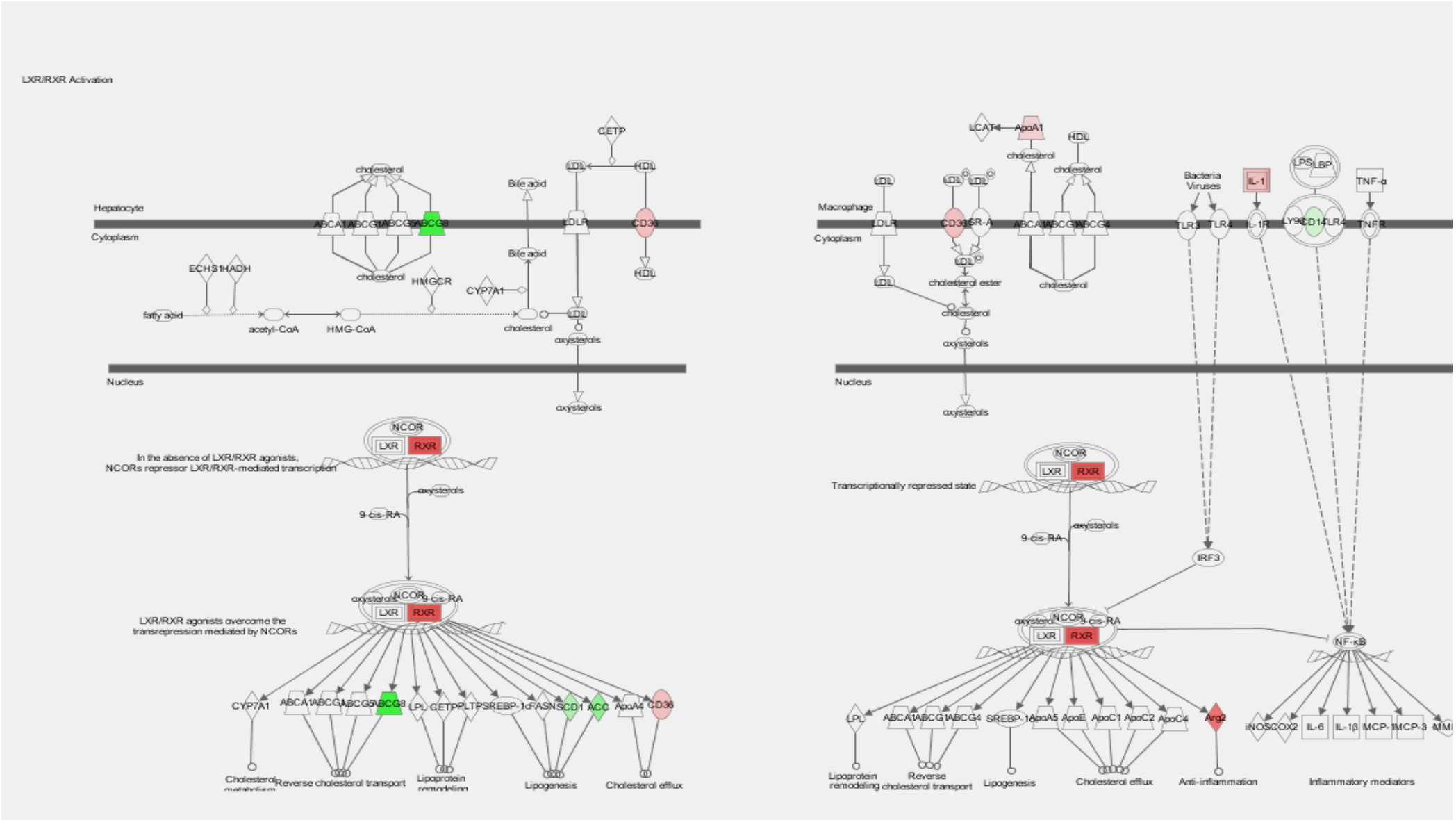

Figure 4: LXR/RXR activation pathways taken from IPA output.

A total of 35 networks were identified by IPA, 30 of which had a score [-log(p-value)] of 2 or greater. 18 networks had between 10 and 27 "focus" genes among the differentially expressed genes. The top IPA networks, displayed functions associated with lipid metabolism (Figure 5), cell-cell signalling, molecular transport, gene expression, and reproductive system disease.

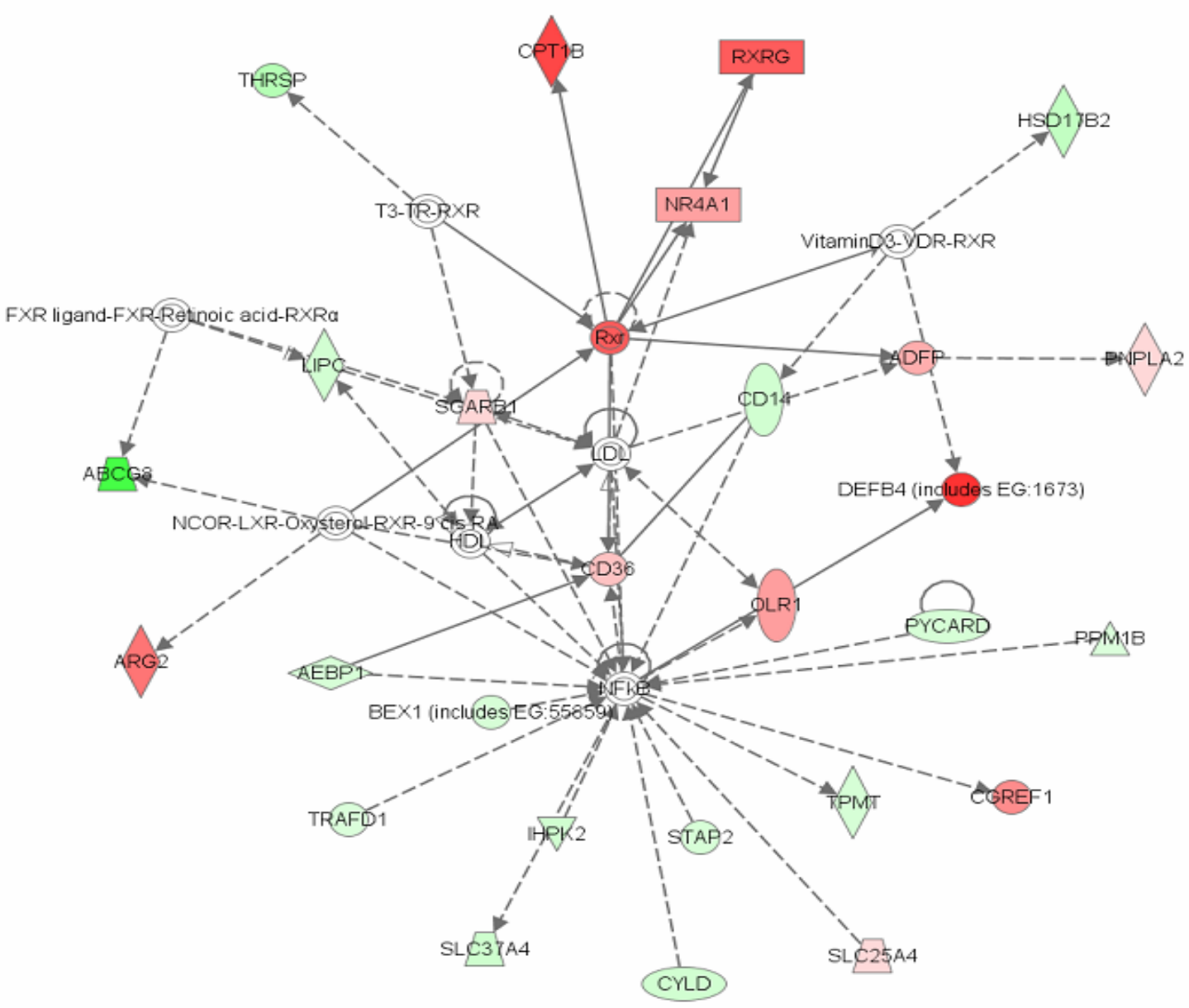

Figure 5: Lipid metabolism network from IPA. The network is displayed graphically as nodes (genes) and edges (the biological distance between nodes). Gene expression is indicated by node colour and intensity; where red indicates up-regulation and green down-regulation.

There were also a number of differentially expressed genes which were involved in immune response, cell cycle, carbohydrate metabolism, as well as nucleic acid metabolism. The highest level of up-regulation was observed for the immune molecule defensin, beta 4 (DEFB4) which had a fold change of 21.36, carnitine palmitoyltransferase 1B (CPT1B) also appeared in the top five with a fold change of 5.52. There was also increased expression of other genes involved in lipid transport; apolipoprotein A1 (ApoA1) and lipid oxidation; acyl-Coenzyme A dehydrogenase, very long chain (ACADVL) and angiopoietin-like 4 (ANGPTL4). Those genes which displayed the most dramatic down regulation included fatty acid desaturase 2 (FAD2) and the hepatocyte nuclear factor forkhead box A3 (FOXA3).

Real time RT-PCR

To validate and support the microarray data a selection of approximately 30 genes, some of which are represented in Table 1., underwent real time RT-PCR analysis. The majority of the genes analysed appeared on the array output, while some of the genes were selected as a candidate approach. In general expression patterns were found to be similar for genes undergoing both types of analysis.

Cows in SNEB had increased expression of genes involved in lipid transport and catabolism. Expression of genes involved in the synthesis and stability of insulin like growth factor-1 (IGF-1) including IGF-1, growth hormone receptor variant 1A (GHR1A) and insulin-like growth factor binding protein-acid labile subunit (IGFBP-ALS) were down regulated in the SNEB compared to the MNEB group, consistent with a five-fold reduction in systemic concentrations of IGF1 in the SNEB group. In addition, higher levels of IGFBP-2 mRNA were observed in the SNEB cows. There was increased expression of genes involved in acute phase response; serum amyloid A3 (SAA3) and antigen presentation; interleukin-8 (IL-8), S100A12, SERPINA3 in the SNEB group. The SNEB cows also showed elevated expression of the oxidative stress response protein; glutathione peroxidase (GPx). In terms of lipid metabolism, cows in SNEB had higher

expression of genes involved in lipid transport; (FABP5, CPT1B) and oxidation; (ACADVL, ANGPTL4) as compared to MNEB animals.

Table 1. Some real time RT-PCR validation of microarray gene expression. Values are back-transformed least square means followed by the 95% confidence intervals and are expressed as fg per µg of reversed transcribed RNA.

Gene	Mild NEB	Severe NEB	Fold change	P value
IGF-1	0.7 (0.3-1.3)	0.1 (0.1- 0.2)	7.0 ↓	<0.01
IGFBPALS	1.4 (0.7-2.7)	0.3 (0.2-0.5)	4.7 ↓	<0.01
IGFBP2	0.6 (0.3-1.0)	2.9 (1.7-5.0)	4.8 ↑	<0.01
GHR1A	0.2 (0.2-0.4)	0.02 (0.01-0.02)	10.0 ↓	<0.001
FABP5	0.5 (0.2-1.3)	0.8 (0.3-2.2)	1.6 ↑	0.36
ACADVL	2.2 (1.8-2.8)	3.3 (2.7-4.1)	1.5 ↑	<0.05
PDK	0.1 (0.04-0.5)	0.4 (0.1-1.2)	4.0 ↑	0.25
SAA3	253 (70-908)	787 (245-2527	3.1 ↑	0.17
GPx	41 (16-102)	437 (191-1000)	10.7 ↑	<0.01
Hsp90	13.1 (9.8-17.5)	10.8 (8.3-14.1)	1.2 ↓	0.32
FOXA3	4.5 (2.1-9.9)	1.9 (0.9-3.9)	2.4 ↓	0.10
FAD	0.24 (0.14-0.4)	0.03 (0.02-0.04)	8.0 ↓	<0.001

Discussion

The problems of low fertility in the modern high yielding dairy cow are of considerable economic cost to the dairy industry with nutritional and metabolic stress playing a possible role in the poor reproductive performance. The early postpartum period is a time of considerable metabolic stress in the dairy cow. Low dietary intake, concurrent with the increasing nutrient demands of lactation, results in a state of NEB. Body fat reserves must be mobilised to offset the deficit in dietary energy intake and results in a reduction in body condition score. The severity and duration of NEB are quite variable among individual cows. Severe NEB in early lactation has been associated with reduced reproductive efficiency both through delayed resumption of cyclicity and reduced subsequent embryo survival /conception rates.

Considering that the aim of this study was to investigate effects of NEB it was critical that the animal model provided two groups of animals divergent in EB status. From physiological measurements and UFL data it was evident that the high intake versus low intake diet and the once daily versus thrice daily milking system produced one group of cows which could be described as having a MNEB and a second group which experienced a SNEB during the early postpartum period. The nature of the differentially expressed genes indicate that SNEB affects expression of genes encoding proteins and enzymes involved in a broad range of biological functions including glucose and lipid metabolism, transcription/translation, growth factors, cell signalling, immune and stress response. Genes up regulated by SNEB also displayed increased expression when analysed using real time RT-PCR (eg. ACADVL) and likewise genes down regulated had reduced expression levels when examined using real time RT-PCR (eg. IGFBP-ALS).

It has been shown in previous studies that the IGF-1 hormone plays a critical role in regulating follicle function and ultimately pregnancy outcome. Similarly, NEB during the early postpartum period has significant effects on the expression of genes of the GH-IGF axis. In terms of the IGF

system, we found a dramatic difference in IGF-1 mRNA expression between EB groups, where SNEB animals displayed decreased expression for this gene. The reduced expression of IGFBP-ALS in SNEB cows is likely to reduce the amount of IGF-I circulating in the ternary complex (half-life ~12 h) and increase the amount of IGF-I circulating in low molecular weight BPs (half life ~30 mins) in these cows. This premise is supported by the SNEB cows having lower circulating IGF-I at this time.

The physiological action of GH is initiated when GH binds to GH receptors (GH-R), the majority of which are located in liver and adipose tissues. While NEB persists, GH levels remain high and adipose tissue sensitivity to insulin is reduced, allowing tissue mobilization. Once EB improves, GH function is re-established as it binds the GHR-1A in the liver, ultimately leading to IGF-1 synthesis. The reduction in GHR-1A mRNA levels seen here in SNEB cows is likely to reduce GHR signalling and attenuate IGF-1 synthesis. The comparison between energy balance groups is of great interest as the mechanisms by which nutrient partitioning is altered during NEB, may arise due to altered homeorhetic set-points, possibly due to changes in the GH-IGF axis.

As expected, SNEB predominantly affected the expression of genes involved in catabolic processes such as lipid and glucose metabolism. Pyruvate carboxylase (PC) which converts pyruvate, bicarbonate ions and ATP into oxaloacetate, ADP and phosphate, is used in gluconeogenesis as a stage in the interconversion of pyruvate to phosphoenolpyruvate. PC expression is up-regulated in SNEB animals. Pyruvate dehydrogenase [lipoamide] kinase isozyme 4 (PDK4) inhibits the mitochondrial pyruvate dehydrogenase complex by phosphorylation of the E1 alpha subunit. Pyruvate dehydrogenase (Opposite to PC), is an allosteric enzyme that transforms pyruvate into acetyl-CoA by a process called Oxidative decarboxylation, which is then used in the citric acid cycle. PDK4 expression was shown to be up regulated in the SNEB animals when analysed by Real Time PCR (Table 2), suggesting a

decrease in pyruvate dehydrogenase activity. Thiamine pyrophosphokinase (TPK) is a cellular enzyme involved in the regulation of thiamine metabolism. TPK catalyzes the conversion of thiamine, a form of vitamin B1, to thiamine pyrophosphate (TDP) which is an active cofactor for enzymes involved in glycolysis and energy production, including transketolase, pyruvate dehydrogenase, and alpha-ketoglutarate dehydrogenase. TPK expression was shown to be down regulated in the SNEB animals, therefore reducing the availability of the cofactor and providing further evidence for decreased pyruvate dehydrogenase activity. Expression patterns of these three genes suggests that gluconeogenesis is the dominant pathway in SNEB cows.

The transfer of the fatty acid moiety across the inner mitochondrial membrane involves carnitine. From the Real Time PCR analysis of CPT1B, its expression was shown to be up regulated four fold in the SNEB animals. The carnitine palmitoyl transferases catalyze transfer of a fatty acid between the thiol of Coenzyme A and the hydroxyl on carnitine. The acyl-coenzyme A dehydrogenase, very long chain (ACADVL) enzyme which was found to be upregulated in SNEB animals is a key enzyme, catalysing the dehydrogenation of long-chain fatty acids in mitochondrial beta-oxidation. The enzyme catalyzes the major part of mitochondrial palmitoyl-CoA dehydrogenation in liver.

The FABPs roles include fatty acid uptake, transport, and metabolism. FABP5, functions as an antioxidant protein by scavenging reactive lipids (i.e., fatty acids) such as 4-hydroxynonenal and leukotriene A_4. In addition, FABP5 also plays a role in basal and hormone-stimulated lipolysis in adipose tissue. The mRNA expression levels for FABP5 were almost two fold greater in the SNEB animals as compared to MNEB animals on Real Time analysis. Therefore, SNEB resulted in the upregulation of keys genes involved fatty acid oxidation and gluconeogenesis which might partly explain the increased plasma concentrations of BHBs and NEFAs in this energy balance group.

Such trends in gene expression were supported by blood concentrations of NEFAs and BHB, which were higher in SNEB compared to MNEB animals. Excessive accumulation of lipids is known to induce cellular trauma and this may account for the up regulation of immune and stress response genes in the SNEB cow while there was a down regulation of genes involved in cellular growth and proliferation in this group. High levels of lipid by products are also known to inhibit cellular proliferation and this may explain the decreased expression of cell growth associated genes in SNEB cows.

Pathway analysis using IPA revealed a number of interacting gene networks; LXR/RXR activation, which was found to be the top listed canonical pathway in IPA which further evidence for altered metabolic gene expression during NEB. LXRs form functional heterodimers with the RXRs and are involved in the regulation of cholesterol, lipid and glucose metabolism. It has been shown in other cell types that increased LXR/RXR signalling inhibits cellular proliferation and may induce apoptotic activity.

Conclusions

- Cows in SNEB show altered hepatic gene expression in metabolic processes as well as a down regulation of the IGF system suggesting that these cows are metabolically stressed and are likely to have impaired fertility.

- Cows in SNEB showed elevated expression of key genes involved in the inflammatory response and reduced expression of genes involved in cellular proliferation which is likely to compromise adaptation and recovery from negative energy balance.

- It is possible that reducing hepatic lipid accumulation during the early post partum period may help reduce inflammatory response and improve cellular proliferation thereby hastening recovery from NEB.

- Finally, EB in lactating dairy cows has a major impact on liver function, affecting a range of biological functions and processes which are likely to have significant effects on subsequent cow fertility.

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