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Transcript profiling of the ruminant liver indicates a unique program of transcriptional regulation of ketogenic enzymes during food restriction

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ABSTRACT

Ruminants absorb little glucose and rely on hepatic gluconeogenesis and ketogenesis in the fed state to convert short-chain fatty acids produced during digestion into glucose and ketone bodies, respectively. In contrast to the non-ruminant response, fluxes through gluconeogenic and ketogenic pathways decrease during food restriction. Transcriptional regulation responsible for these unique food restriction responses has not been established. To determine the hepatic transcriptional response of ruminants to an acute drop in dietary nutrient supply, 102 yearling heifers were assigned to either ad libitum feeding or 24 h of food withdrawal in a randomized block design. Liver biopsies were obtained for microarray and quantitative real-time PCR analyses of gene expression. Plasma concentrations of non-esterified fatty acids were higher in food restricted heifers, while levels of β-hydroxybutyrate, triacylglycerol, and glucose were decreased. Despite a decline in substrate supply and a lower hepatic production of glucose, expression of the key gluconeogenic enzymes pyruvate carboxylase, phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase was upregulated as in non-ruminants. Downregulation of cholesterolgenic genes and upregulation of fatty acid oxidative genes were consistent with SREBP-2 and PPARα control, respectively. Ketogenesis from short-chain fatty acids was downregulated, contrary to the non-ruminant response to food restriction. Short-chain fatty acids may exert transcriptional control in the ruminant liver similar to that demonstrated in the large intestine of non-ruminants.

1. Introduction

As the organ central to the energy distribution network, the ruminant liver is responsible for processing incoming digestion products and managing nutrient supply to accommodate the nutritional requirements of the rest of the body. When food intake is restricted, the liver will contribute to the glucose flux by gluconeogenesis from lactate, amino acids and other gluconeogenic precursors delivered from peripheral tissues. During food restriction, the liver oxidizes fatty acids instead of glucose for energy, and through ketogenesis produces beta-hydroxybutyrate as an alternative fuel for the central nervous system and periphery (Frayn, 1999). Subsequently, hepatic energy expenditure on lipid, protein and glycogen synthesis is curtailed.

Many of the non-ruminant hepatic responses to food restriction are mediated by the activation and repression of key signaling and transcription factors due to changes in circulating concentrations of nutrients and hormones. During the restriction of food intake or fasting, insulin concentrations fall while glucagon and fatty acid concentrations rise. Glucagon activates transcription of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), which then acts as a coactivator in gluconeogenic and urea cycle gene expression (Desvergne et al., 2006; Sugden et al., 2010). Insulin counteracts these glucagon effects through Akt-mediated phosphorylation of transcription factors, including PGC-1α, leading to their exclusion from the nucleus and accelerated degradation within the cytoplasm (Li et al., 2007). Elevated fatty acid concentrations during food restriction allosterically activate hepatic peroxisome proliferator-activated receptor α (PPARα), which stimulates expression of enzymes involved in fatty acid oxidation and ketogenesis (Crestani et al., 2004; Martinez-Jimenez et al., 2010). Hepatic expression of lipogenic enzymes is under the control of sterol regulatory element binding protein-1c (SREBP-1c), whose expression and nuclear activity is also regulated by insulin and glucagon signals (Yamamoto et al., 2007; Li et al., 2010). Through these transcriptional effects, capacities for hepatic amino acid catabolism, gluconeogenesis, fatty

Abbreviations: α-AN, α-amino N; ADH, alcohol dehydrogenase; BHBA, β-hydroxybutyrate; CYP, cytochrome P450; DEG, differentially expressed genes; FDR, false discovery rate; NEFA, non-esterified fatty acids; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; PPARα, peroxisome proliferator-activated receptor α; PPRE, peroxisome proliferator response element; qPCR, quantitative real-time PCR; SREBP-1c, sterol regulatory element binding protein-1c; TAG, triacylglycerol.

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acid oxidation and ketogenesis are increased during food restriction, while capacity for lipogenesis declines.

Due to the presence of fermentative microbes in the foregut, the bulk of carbohydrate ingested by a ruminant is degraded to the short-chain fatty acids acetate, propionate and butyrate, and essentially all of the remaining glucose is removed by cells of the gastrointestinal tract prior to entry into the liver (Reynolds et al., 1991b). The liver is thus responsible for glucose production in the ruminant even in the fed state. In contrast to the non-ruminant response, food restriction decreases hepatic gluconeogenesis and ketogenesis in non-pregnant, non-lactating ruminants, primarily because of the lower influx of propionate and butyrate from the rumen (Katz and Bergman, 1969; Reynolds et al., 1991a, 1991b, 1992). Characteristics of transcriptional regulation in the ruminant liver that produce these unique responses to food restriction have not been established.

The purpose of this research was to evaluate the hepatic transcriptional response to food restriction in ruminants. Previous studies have evaluated food restriction responses of ruminants during pregnancy or lactation (Loor et al., 2006, 2007; McCarthy et al., 2010), when there is a homeorhetic drive to maintain nutrient supply to reproductive tissues. To avoid confounding effects of the physiological state, we used animals that were neither pregnant nor lactating. A cDNA microarray consisting of 8329 oligonucleotide probes was used to identify changes in gene expression as a result of a 24-h food withdrawal. Because of the large mass of rumen contents that passes distally at 3 to 5%/h (Leupp et al., 2009), 24 h without food does not produce a complete loss of nutrient absorption from the gut but reduces it to approximately 35% of the fed rate. The changes observed and reported here are therefore indicative of a transcriptional response to an acute drop in dietary nutrient supply.

2. Material and methods

2.1. Animals and experimental design

The University of Guelph Animal Care Committee approved all experimental procedures and ensured that the trial was conducted in accordance with guidelines of the Canadian Council on Animal Care. As part of a large study to evaluate relationships between hepatic gene expression in juveniles and lactational performance as adults, 102 postpubertal Holstein heifers (*Bos taurus*) at 367 ± 19 days of age and 362 ± 26 kg body mass were randomly assigned to either ad libitum feeding or 24 h of food withdrawal in a randomized block design. At approximately 10 months of age, blocks of 10 heifers were randomly split into two pens of 5 animals each. Heifers were offered ad libitum access to water and a total mixed ration of alfalfa and grass silage, corn silage, dry hay and mineral to provide 1.53 Mcal net energy/kg and 16.25% crude protein, on a dry matter basis. The ration was provided fresh every day at 10:00 h. The day before collecting liver biopsies, feed was not offered to one of the two pens of heifers in each block.

2.2. Liver biopsy and blood sampling

Liver tissue was extracted between 08:00 and 12:00 h via biopsy as previously described (Greenwood et al., 2009). Briefly, the site of incision was shaved and disinfected using chlorhexidine solution (0.5% chlorhexidine gluconate) and local anesthesia (approx. 10 mL per heifer; Xylocaine†, containing 2% lidocaine hydrochloride injection USP, AstraZeneca, Canada Inc.) was administered. An incision was made in the intercostal space and 2 g liver tissue were extracted via biopsy using a trocar and cannula. Liver biopsies were snap frozen in liquid N_2 and stored at −80 °C until total RNA isolation.

Blood samples were collected from a coccygeal vein using sodium heparin and K-EDTA Vacutainers (Becton-Dickinson). Samples were immediately centrifuged and plasma withdrawn and frozen at −20 °C. Spectrophotometric assays were used to analyze glucose (Sigma kit no. 510-A; Raabo and Terkildsen, 1960), triacylglycerol (TAG; Sigma kit no. 336; McGowan et al., 1983), non-esterified fatty acids (NEFA C kit; Wako Chemicals GmbH, Neuss, Germany; Johnson and Peters, 1993), β-hydroxybutyrate (BHBA; Cant et al., 1993) and α-amino nitrogen (αAN; Evans et al., 1993).

2.3. Total RNA extraction, cDNA synthesis and labeling

Total RNA from liver tissue and reference sample tissue was isolated using both TriReagent (Ambion Inc., Austin, TX, USA) and RNeasy Midi Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. The reference sample was derived from bovine liver, spleen, and placental tissue collected from mature, lactating cows at slaughter and stored at −80 °C until use. All RNA samples used for microarrays (n = 102) and quantitative real-time PCR (qPCR; n = 24) were evaluated using the Agilent 2000 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA). The average RNA integrity number (Schroeder et al., 2006) for all samples was 8.1 ± 0.72. cDNA was synthesized from ~20 μg RNA and labeled with Alexa Fluor 555 or Alexa Fluor 647 using SuperScript Indirect cDNA Labeling System (Invitrogen, Burlington, ON, Canada).

2.4. Microarrays and quantitative real-time PCR

The BLO 8400 bovine long oligonucleotide array (http://ncbi.nlm.nih.gov/geo/ accession no. GPL8564) consisting of 8329 70-mer oligonucleotide probes spotted in duplicate was used to identify hepatic transcript profiles. The oligonucleotides were selected from a collection of 13,914 ESTs from 15 bovine tissues assembled by the National Bovine Functional Genomics Consortium. The array also included 10 positive bovine control sequences and 20 negative control sequences spotted multiple times. The complete list of annotated genes and all microarray data have been deposited in the National Centre for Biotechnology Information Gene Expression Omnibus database (http://ncbi.nlm.nih.gov/geo/ accession no. GSE16154).

Alexa Fluor 555 labeled reference cDNA and Alexa Fluor 647 labeled sample cDNA, generated from 5 to 20 μg mRNA, were mixed and hybridized to the array. All hybridizations were performed with a GeneTAC HybStation (Genomic Solutions, Ann Arbor, MI, USA) in sealed chambers using an 18-hour step-down hybridization protocol consisting of 3 six-hour periods of 42 °C, 35 °C and 30 °C, respectively. Post hybridization, arrays were subject to 3 different buffer washes while in the HybStation, and were then dried by centrifugation.

Microarrays were scanned and quantified using a Genepix 4200A axon scanner (Axon Instruments, Union City, USA) and processed using Gene Pix Pro 6.0 (Axon Instruments). Gene Spring GX (Agilent Technologies, Santa Rosa, CA, USA) analysis software was used for LOWESS normalization.

For microarray validation purposes, qPCR was used to measure transcript abundance of 15 genes that were identified as either statistically significantly differentially expressed or as genes of interest. Primers were designed for target genes using Primer Express v3.0 (Applied Biosystems, Streetsville, ON, Canada). Specific *Bos taurus* mRNA sequences for target genes were identified using sequential Basic Local Alignment Search Tool against human and mouse transcript sequences found in the UniGene database. Target gene sequences were then imported into Primer Express v3.0. Forward and reverse primers were optimally designed to cover exon–exon junctions to account for alternative splicing when possible (Table 1). Total liver RNA from 24 heifers (n = 12 fed and n = 12 restricted), randomly selected equally from each treatment group, was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied BioSystems) and subjected to DNase treatment (Qiagen) for removal of any genomic contamination according to manufacturer's instructions.