Nutrition-induced ketosis alters metabolic and signaling gene networks in liver of periparturient dairy cows

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Loor JJ, Everts RE, Bionaz M, Dann HM, Morin DE, Oliveira R, Rodriguez-Zas SL, Drackley JK, Lewin HA. Nutrition-induced ketosis alters metabolic and signaling gene networks in liver of periparturient dairy cows. Physiol Genomics 32: 105–116, 2007. First published October 9, 2007; doi:10.1152/physiolgenomics.00188.2007.—Dairy cows are highly susceptible after parturition to developing liver lipidosis and ketosis, which are costly diseases to farmers. A bovine microarray platform consisting of 13,257-annotated oligonucleotides was used to study hepatic gene networks underlying nutrition-induced ketosis. On day 5 postpartum, 14 Holstein cows were randomly assigned to ketosis-induction (n = 7) or control (n = 7) groups. Cows in the ketosis-induction group were fed at 50% of day 4 intake until they developed signs of clinical ketosis, and cows in the control group were fed ad libitum throughout the treatment period. Liver was biopsied at 10–14 (ketosis) or 14 days postpartum (controls). Feed restriction increased blood concentrations of nonesterified fatty acids and β-hydroxybutyrate, but decreased glucose. Liver triacylglycerol concentration also increased. A total of 2,415 genes were altered by ketosis (false discovery rate = 0.05). Ingenuity Pathway Analysis revealed downregulation of genes associated with oxidative phosphorylation, protein ubiquitination, and ubiquinone biosynthesis with ketosis. Other molecular adaptations included upregulation of genes and nuclear receptors associated with cytokine signaling, fatty acid uptake/transport, and fatty acid oxidation. Genes downregulated during ketosis included several associated with cholesterol metabolism, growth hormone signaling, proton transport, and fatty acid desaturation. Feed restriction and ketosis resulted in previously unrecognized alterations in gene network expression underlying key cellular functions and discrete metabolic events. These responses might help explain well-documented physiological adaptations to reduced feed intake in early postpartum cows and, thus, provide molecular targets that might be useful in prevention and treatment of liver lipidosis and ketosis.

bovine; microarray; lactation; hepatic lipidosis

NEGATIVE ENERGY BALANCE and inappetence, which are typical during the periparturient period (e.g., Ref. 16), are prerequisites for the onset of ketosis soon after parturition. Adaptations of intermediary metabolism in liver from cows undergoing nutrition-induced ketosis were first described over 40 yr ago (e.g., Refs. 4, 8). Ketosis is a metabolic disorder closely associated with liver lipidosis. Despite advances made over the last decades to understand the pathology and etiology of liver lipidosis and ketosis in dairy cattle (7), the molecular events associated with these diseases remain largely unknown. Liver lipidosis develops when the hepatic uptake of nonesterified fatty acids (NEFA) exceeds their oxidation and secretion as triacylglycerols (TAG) in very-low-density lipoproteins (VLDL) by the liver (17). Liver lipidosis occurs primarily during the first 4 wk postpartum (7). This disorder is associated with decreased health status, well-being, productivity, and reproductive performance of cows (7). Costs to the US dairy industry for treating primary ketosis are over $60 million (7).

Application of cattle cDNA microarray technology has greatly increased the understanding of hepatic molecular adaptations occurring from late pregnancy through early lactation due to physiological state and plane of nutrition prepartum. A 7,872 cattle cDNA microarray (18) was initially used to complement measurements of liver tissue metabolism, blood metabolite concentrations, and overall animal performance (37). This complementary approach allowed development of integrative models linking the transcriptome and whole animal measurements of tissue function. Evidence was presented to suggest that hepatic inflammatory responses (e.g., cytokines, acute-phase proteins) occur around parturition and, thus, serve as signals to initiate or augment adipose and/or muscle tissue catabolism. Furthermore, those studies showed that moderate overfeeding of energy in the dry period results in transcriptional changes predisposing cows to hepatic lipidosis and compromising overall liver health early postpartum (38). In the present study, a bovine microarray platform consisting of 13,257 annotated oligonucleotides was used to aid in our studies of dairy cattle biology. This technology was used to test the hypothesis that hepatic metabolic and signaling gene networks are coordinately altered by feed restriction and ketosis partly to accommodate increased NEFA delivery into liver as well as metabolic demands for milk synthesis.

MATERIALS AND METHODS

Microarray Construction and Annotation

Details of the development of the 13,257 oligonucleotide microarray are described in the Suppl. Materials and Methods (1). Briefly, 38,732 high-quality expressed sequence tag (EST) sequences based on the 7,872 cDNA array [National Center for Biotechnology Information (NCBI) GEO: GPL2864; Ref. 18] and an embryonic cDNA library were filtered for repeats and contaminating sequences of viral, bacterial, or mitochondrial origin using RepeatMasker (52), and used for CAP3 clustering (26). ArrayOligoSelector version 3.8.1 (58) was used to design 70-mer oligos from unique cluster and singlet sequences. All designed oligos were aligned by BLASTN similarity.

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1 The online version of this article contains supplemental material.
sied tissue (1.0–1.5 g) was weighed and stored in liquid N2 prior to biopsy under local anesthesia at the onset of clinical ketosis (9–14 days postpartum) to allow outdoor exercise for 3 days and treated, if necessary (12). Cows in the control group remained healthy throughout the dietary treatment period, as determined by daily physical examinations (12). Clinical ketosis developed on day 4 or 5 after parturition, at which time cows were treated (12) and returned to ad libitum feeding. Data on Fig. 1 and Suppl. Fig. S1 reflect data from qPCR was analyzed as described elsewhere (38) using the right-tailed Fisher’s Exact Test. Data generated from these analyses were used for development of an integrative model linking gene networks and physiological events in various metabolic tissues (see Fig. 4).

**Data Analyses**

Data from a total of 28 microarrays were normalized for dye and array effects (i.e., Loess normalization and array centering) and used for statistical analysis. A mixed effects model was then fitted to the adjusted ratios (liver/reference standard) using Proc MIXED (SAS; SAS Inst., Cary, NC). The model consisted of status (i.e., ketosis or healthy) and dye as fixed effect, and cow as a random variable. Probability values for fixed effects were adjusted for the number of comparisons using Benjamini and Hochberg’s false discovery rate (FDR; Ref. 46). Differences in relative expression were considered significant at an FDR-adjusted P ≤ 0.05 (i.e., unadjusted P ≤ 0.01). Data from qPCR were analyzed as described elsewhere (38) using the same statistical model described above. Differences were considered significant at P ≤ 0.05. Differences in concentrations of serum metabolites and liver chemical composition were assessed using a similar statistical model as above but also included the fixed effects of time and the interaction of time × treatment. Differences were considered significant at P ≤ 0.05. Gene expression network and pathway analysis was performed using Ingenuity Pathway Analysis (IPA) using genes with FDR-adjusted P = 0.06 (Ingenuity Systems, Mountain View, CA).

**Data Mining**

The entire microarray and qPCR data set with associated statistical P values were imported into IPA. GenBank IDs were used to identify individual sequences. This set of genes was used as the reference set for function and canonical pathway analysis statistical calculations in IPA. The significance value associated with functions and pathways is a measure of the likelihood that the distribution of differentially expressed genes (DEG) in these pathways and functions is due to chance. The significance is expressed as a P value, which is calculated using the right-tailed Fisher’s Exact Test. Data generated from these analyses were used for development of an integrative model linking gene networks and physiological events in various metabolic tissues (see Fig. 4).

**RESULTS**

**Animal Performance, Serum Parameters, and Liver Composition**

Cows in the control group remained healthy throughout the dietary treatment period, as determined by daily physical examinations (12). Clinical ketosis developed on days 9–14 postpartum, at which time cows were treated (12) and returned to ad libitum feeding. Data on Fig. 1 and Suppl. Fig. S1 reflect only the subset of cows used for transcript profiling, which responded in a similar fashion as the entire group reported by Dann et al. (Ref. 12; e.g., Table 3 and 7). Despite feed restriction and lower milk production during the test period, cows that developed ketosis were able to return to statistically significant at an FDR-adjusted P ≤ 0.05 (i.e., unadjusted P ≤ 0.01).
similar levels of feed intake and milk production (Fig. 1). However, it is important to note that cows developing ketosis had numerically lower milk production. Serum concentrations of NEFA, BHBA, glucose, and insulin in healthy cows or cows induced to develop ketosis by feed restriction early postpartum are shown in Fig. 1. Values prior to feed restriction and ketosis are shown for comparison. Serum NEFA and BHBA concentrations increased steadily from 1 wk prior to parturition through the end of week 1 postpartum. The numerical increases in serum NEFA on days 0 and +1 for cows in the ketosis group were due to a single cow that was fed ad libitum during the dry period. Feed restriction resulted in a nearly twofold increase in serum NEFA concentration and threefold increase in serum BHBA concentration. Serum glucose concentration decreased during feed restriction and ketosis, but insulin concentration did not change. Total lipid, TAG, and glycogen contents in liver on the various sampling days are shown in Suppl. Fig. S1. Liver total lipid and TAG contents at the onset of clinical ketosis or day 14 postpartum were markedly greater in cows in the ketosis-induction group, with differences persisting until at least day 28 postpartum. In contrast, liver glycogen content was lower in ketotic cows. These temporal changes in blood metabolites and liver composition were typical of those previously reported for periparturient dairy cows (37, 38).

Fig. 1. Feed intake (dry matter basis), milk production, and serum concentrations of NEFA, BHBA, glucose, and insulin in healthy cows or cows induced to develop ketosis by feed restriction early postpartum. Health status effect *P = 0.05. Feed intake (P = 0.18), milk production (P = 0.15), and serum concentrations of metabolites and hormones did not differ statistically after the feed restriction period (i.e., from day 19 to 44 postpartum). NEFA, nonesterified fatty acids; BHBA, β-hydroxybutyrate.
Table 2. qPCR results

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene Name</th>
<th>Microarray</th>
<th>Trend</th>
<th>qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACAA1</td>
<td>acetyl-coenzyme A acyltransferase 1</td>
<td>1.3</td>
<td>↑↑keto</td>
<td>1.8*</td>
</tr>
<tr>
<td>ACHATסק</td>
<td>acetyl-coenzyme A acyltransferase 1</td>
<td>1.0</td>
<td>↑↑keto</td>
<td>1.8*</td>
</tr>
<tr>
<td>ACAA1</td>
<td>acetyl-coenzyme A carboxylase</td>
<td>-1.5</td>
<td>↓↓keto</td>
<td>-1.6**</td>
</tr>
<tr>
<td>ACOX1</td>
<td>acyl-CoA oxidase 1, palmitoyl</td>
<td>1.4</td>
<td>↑↑keto</td>
<td>2.2*</td>
</tr>
<tr>
<td>ALBסק</td>
<td>albumin</td>
<td>4.4</td>
<td>↓keto</td>
<td>1.0</td>
</tr>
<tr>
<td>APOA1</td>
<td>apolipoprotein A-1</td>
<td>1.9</td>
<td>↑↑keto</td>
<td>3.4*</td>
</tr>
<tr>
<td>APORסק</td>
<td>apolipoprotein B</td>
<td>1.4</td>
<td>↑↑keto</td>
<td>2.0*</td>
</tr>
<tr>
<td>BDH1סק</td>
<td>3-hydroxybutyrate dehydrogenase, type 1</td>
<td>1.3</td>
<td>↑↑keto</td>
<td>1.5*</td>
</tr>
<tr>
<td>CPT1Aסק</td>
<td>carnitine palmitoyltransferase 1A (liver)</td>
<td>-3.2</td>
<td>↓↓keto</td>
<td>-1.4*</td>
</tr>
<tr>
<td>DBI</td>
<td>diazepam-binding inhibitor</td>
<td>-2.6</td>
<td>↓↓keto</td>
<td>1.6*</td>
</tr>
<tr>
<td>FABP3</td>
<td>fatty acid binding protein 3</td>
<td>-10.4</td>
<td>↓↓keto</td>
<td>-4.6*</td>
</tr>
<tr>
<td>FADS2</td>
<td>fatty acid desaturase 2</td>
<td>-2.4</td>
<td>↓↓keto</td>
<td>-2.0*</td>
</tr>
<tr>
<td>GSTA4</td>
<td>glutathione S-transferase, alpha 4</td>
<td>-1.7</td>
<td>↓↓keto</td>
<td>1.0</td>
</tr>
<tr>
<td>HMGCSEסק</td>
<td>3-hydroxy-3-methylglutaryl-coenzyme A synthase</td>
<td>-1.9</td>
<td>↓↓keto</td>
<td>-2.1*</td>
</tr>
<tr>
<td>HMGCR</td>
<td>3-hydroxy-3-methylglutaryl-coenzyme A reductase</td>
<td>-1.1</td>
<td>↓↓keto</td>
<td>2.1**</td>
</tr>
<tr>
<td>LPIN1סק</td>
<td>lipin 1</td>
<td>-1.3</td>
<td>↓↓keto</td>
<td>2.1*</td>
</tr>
<tr>
<td>LPIN3סק</td>
<td>lipin 3</td>
<td>-1.1</td>
<td>↓↓keto</td>
<td>1.3</td>
</tr>
<tr>
<td>OXCT1סק</td>
<td>3-oxoacid CoA transferase 1</td>
<td>-6.1</td>
<td>↓↓keto</td>
<td>-4.2*</td>
</tr>
<tr>
<td>PON1סק</td>
<td>paraoxonase 1</td>
<td>-2.1</td>
<td>↓↓keto</td>
<td>-1.9*</td>
</tr>
<tr>
<td>SCD</td>
<td>stearyl-CoA desaturase</td>
<td>2.9</td>
<td>↑↑keto</td>
<td>1.4*</td>
</tr>
<tr>
<td>SCOM1スク</td>
<td>sterol-C4-methyl oxidase-like</td>
<td>-3.5</td>
<td>↓↓keto</td>
<td>-1.2*</td>
</tr>
<tr>
<td>SLC27A2スク</td>
<td>solute carrier family 27 (fatty acid transporter), member 2</td>
<td>2.9</td>
<td>↑↑keto</td>
<td>1.4*</td>
</tr>
<tr>
<td>SREBF2スク</td>
<td>sterol regulatory element binding transcription factor 2</td>
<td>-1.5</td>
<td>↓↓keto</td>
<td>-1.4*</td>
</tr>
<tr>
<td>FOXC2スク</td>
<td>forkhead box C2</td>
<td>2.1</td>
<td>↑↑keto</td>
<td>2.2*</td>
</tr>
<tr>
<td>HNF4Aスク</td>
<td>hepatocyte nuclear factor 4, alpha</td>
<td>1.3</td>
<td>↑↑keto</td>
<td>1.6*</td>
</tr>
<tr>
<td>PPARGスク</td>
<td>peroxisome proliferative activated receptor, alpha</td>
<td>-1.2</td>
<td>↓↓keto</td>
<td>1.4*</td>
</tr>
<tr>
<td>PPARDスク</td>
<td>peroxisome proliferative activated receptor, deltal</td>
<td>1.4</td>
<td>↑↑keto</td>
<td>1.3*</td>
</tr>
<tr>
<td>PPARGC1A钪</td>
<td>peroxisome proliferative activated receptor, gamma coactivator-1 alpha</td>
<td>1.3</td>
<td>↑↑keto</td>
<td>2.7*</td>
</tr>
<tr>
<td>TP53钪</td>
<td>TP53 tumor protein p53 (Li-Fraumeni syndrome)</td>
<td>-1.3</td>
<td>↓↓keto</td>
<td>1.4*</td>
</tr>
<tr>
<td>NR4A1スク</td>
<td>nuclear receptor subfamily 4, group A, member 1</td>
<td>1.5</td>
<td>↑↑keto</td>
<td>2.4*</td>
</tr>
<tr>
<td>NR1A3钪</td>
<td>constitutive androstan receptor</td>
<td>-3.5</td>
<td>↓↓keto</td>
<td>-1.2*</td>
</tr>
</tbody>
</table>

**Lipid metabolism/fatty acid transport/metabolic process**

- ATP synthesis-coupled proton transport
- Mitochondrial electron transport, NADH to ubiquinone
- Urea cycle
- Gluconeogenesis
- Protein binding/transport

*Not present in differentially expressed genes (DEG), false discovery rate \( P > 0.05 \); ‡gene not present on the microarray platform; ‡quantitative PCR (qPCR) did not correspond to results obtained by microarray; \* \( P = 0.05 \); ** \( P = 0.10 \).
Table 3. Subset of DEG (microarray or qPCR) with empirical ≥1.8-fold expression level due to ketosis

<table>
<thead>
<tr>
<th>Molecular Function</th>
<th>Gene Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell death</td>
<td>A2M*, A1EM1, BSG, C6, CCNG1, CX3CL1, DAG1, DIRAS3, FN1, FTH1, GALNT2, GHR*, HSPA8, IFNB1, IGF2R, TMR2B, LDLR, NUA1I, PCNA, PDZK1, PLAC8, PRKCB1, PTPrC, SFRP1, SH3GLB1, SIRPA, SOD1, TEGT, TMEM123, TNFSF15P1, TOP2A, TYSM, CD55, CD79B, CDC6, CDC7, EPO*, FER, IL6*, MYB, NEU3, NR4A1*, NTRK2, PLG, PLK3, PMP22, PRKCE, RNASE1, SPN, SST, TNFRSF25*, TRPC1, VDR</td>
</tr>
<tr>
<td>Molecular transport</td>
<td>LDLR, PCK1*, SCD4, SOD1, CA3, FTH1, IFNB1, A2M*, BSG, CX3CL1, FCGR3B, FN1, ADCYAP1R1, CCKBR, EPO*, IL6*, PLG, SST, TRPC1, VDR, ANGPTL4*, LNIP1*, FABP3*, SLC27A*, CATI*, APOAI*, ARG1*, SLC6A9</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>ACS52, DB1*, FADS1, FADS2*, LDLR, PCK1*, SC4MOL*, SCD4, SH3GLB1, HMGC4*, ADH5, RHDI1, FN1, ELOVL6, GHR*</td>
</tr>
<tr>
<td>Cell signaling</td>
<td>ACAA1*, ACX1*, ANGPTL4*, FABP3*, IL6*, LPIN1*, NEU3, SLC27A2, PLG, VDR, AGRP, APOAI*, EPO*, ADH4, HSD3B1</td>
</tr>
<tr>
<td>Cell growth and proliferation</td>
<td>ADCYAP1R1, CCKBR, EPO*, IL6*, PLG, SST, TRPC1, VDR, KIF23</td>
</tr>
<tr>
<td>Immune response</td>
<td>DIRAS3, NEME2, GHR*, LDLR, PCNA, SOD1, FN1, EPO*, NR4A1*, PLG, PRKCE, TNFRSF25*, ARG1*, MYB, ADAM12, HSD3B1, IL6*, L3MBTL</td>
</tr>
<tr>
<td>Enriched Functions and Canonical</td>
<td>ACG1, ACF1, ANGPTL4*, FABP3*, IL6*, LPIN1*, NEU3, SLC27A2, PLG, VDR, AGRP, APOAI*, EPO*, ADH4, HSD3B1</td>
</tr>
<tr>
<td>Metabolic and Signaling Pathways</td>
<td>ADCYAP1R1, CCKBR, EPO*, IL6*, PLG, SST, TRPC1, VDR, KIF23</td>
</tr>
</tbody>
</table>

*Expression level verified or measured by qPCR (Table 2).

Differential Expression of Genes in Liver Tissue

A total of 8,831 elements representing 8,369 unique genes on the microarray were expressed in liver (7,346 genes with unique UniGene identification and 1,023 nonredundant unannotated sequences). ANOVA at a cutoff FDR P value ≤ 0.05 (unadjusted P ≤ 0.01) resulted in a total of 2,415 unique DEG due to feed restriction-induced ketosis (Suppl. Excel File).

Enriched Functions and Canonical Metabolic and Signaling Pathways

A total of 8,373 genes from the array platform could be mapped to the IPA database. Of the 2,415 DEG, 1,399 could be mapped to a known molecular function or pathway and 1,411 were eligible for generating networks using IPA. Among network-eligible genes, there were 811 downregulated and 600 upregulated genes. A total of 437 downregulated and 248 upregulated genes were assigned to one or more biological function, disease, and/or developmental process (Suppl. Fig. S2). Biological categories with the largest number of downregulated genes were protein synthesis, cell death, small molecule biochemistry, and lipid metabolism. Canonical metabolic pathway analysis showed that oxidative phosphorylation, ubiquinone biosynthesis, pyruvate metabolism, and butanoate metabolism contained the largest number of downregulated genes; canonical signaling pathways analysis showed that estrogen receptor and protein ubiquitination contained the largest number of downregulated genes (Fig. 2). Fewer numbers of upregulated genes were associated with metabolic and/or signaling pathways. Among these, estrogen receptor signaling, GABA receptor signaling, glycerolipid metabolism, and fatty acid metabolism contained the largest number of upregulated genes (Fig. 2).

Selected Networks Affected by Ketosis

We identified 17 networks each with a total of 30 to 35 focus genes among DEG using IPA. Analysis of these networks revealed links between IL6 (upregulated with ketosis) and fatty acid metabolism, cholesterol synthesis, oxidative stress, lipoprotein metabolism, amino acid metabolism, cellular development, and immune function (Fig. 3). Networks containing IL6 incorporated genes such as GSTA4, GHR, and ARG1, all of which were also verified by qPCR (see section below). IPA also revealed a network of genes containing the transcription factor SREBF2 (downregulated with ketosis) as a central regulator of the expression of lipid metabolism genes such as SC4MOL, HMGC4, SCD4, FADS2, and HMGCR, all of which were markedly downregulated due to ketosis (Table 2, Fig. 3). Another key network revealed regulatory loops between the nuclear receptors HNF4A, PPARA, PPARGC1A, and PPARD (all upregulated by ketosis), several upregulated genes associated with regulation of transcription (FOXC2), fatty acid oxidation (ACOX1, ACA1A1, ACAT1), fatty acid transport (FABP3), and other aspects of lipid metabolism (LPIN1, LPIN3) (Fig. 3). IPA also highlighted links between palmitic acid and oleic acid, the major NEFA reaching bovine liver, and the nuclear receptors PPARA, PPARGC1A, and PPARD, as well as fatty acid oxidation genes like ACA1A1 and the proinflammatory gene IL6. Most of these genes were verified by qPCR (Table 2).
logs by microarray or qPCR. Eight selected genes (ACAT1, LPIN1, PPARA, PPARGC1A, SOCS2, IL1R2, IGF1, PCK1) with FDR $P < 0.05$ were found to change significantly when measured by qPCR (Table 2). Among these, IL1R2 and IGF1 had microarray expression values $>2$-fold (see Table 2).

**DISCUSSION**

**Effects of Feed Restriction on Energy Balance and Metabolism**

The main focus of our study was to test the hypothesis that metabolic and signaling gene networks are coordinately altered in liver during feed restriction and ketosis in periparturient cows. Biological parameters in blood and liver tissue of cows in the ketosis-induction group were consistent with previous data (e.g., Ref. 7) showing decreased serum glucose concentration and increased serum NEFA, serum BHBA, and liver TAG concentrations. This suggests that our feed restriction model was appropriate for mimicking adaptations observed in postpartum dairy cows in the field.

**Liver and the Growth Hormone/IGF-1 Axis**

Recent studies of liver function in periparturient dairy cows have focused on understanding aspects of macronutrient me-
data that this enzyme is not closely associated with the etiology of ketosis. Oxidation might not have been affected and supporting other evidence (15).

ACOX1 is involved in the regulation of growth hormone signaling via GHR as well as IGF-1 signaling (54). Our microarray platform contains most of the currently known genes involved in IGF-1 signaling (Suppl. Fig. S4). Despite IGFI downregulation and IGF1R upregulation in cows in the ketosis-induction group, downstream signaling events were essentially downregulated (Suppl. Fig. S4).

Intermediate Metabolism Responses Inferred by Gene Expression

Lipid. A recent microarray study provided evidence for upregulation of lipid catabolism-associated genes in mouse liver after starvation (2 days; Ref. 5). Our data revealed upregulation of ACAT1, BDH1, ACOX1, SLC27A2, FABP3, and ACAA1 (Table 2). In contrast, expression of DBI, which codes for a long-chain fatty acyl-CoA-binding protein (25), was drastically downregulated by ketosis. Peroxisomal oxidation (ACOX1) results in the production of short-chain fatty acyl-CoA intermediates that are completely oxidized in mitochondria (15). CPT1A expression did not change with ketosis, suggesting that mitochondrial capacity for long-chain fatty acid oxidation might not have been affected and supporting other data that this enzyme is not closely associated with the etiology of ketosis (11). Along with SLC27A2, upregulation of FABP3 might allow liver cells to cope with the large influx of NEFA that occurs during feed restriction. We speculate that, in bovine liver, upregulation of FABP3 during ketosis might channel fatty acids (e.g., palmitic and oleic acid; Fig. 3) toward β-oxidation (↑PPARA, ↑ACAAI, ↑ACOX1), as has been observed in exercising muscle (50). Overall, these results provide evidence of both increased transport and channeling of long-chain fatty acids derived from the circulation towards peroxisomal β-oxidation and increased mitochondrial synthesis of ketone bodies.

Our data indicated that feed restriction and ketosis are associated with downregulation of cholesterol synthesis (HMGCR, SC4MOL), de novo fatty acid synthesis (FASN, ACACA), and fatty acid desaturation (SCD, FADS2) (Table 2, Fig. 3). Most of these genes are under the control of the transcription factor SREBF2 (24), which also was substantially downregulated (Table 2, Fig. 3). In starved mice, lipid synthesis-associated genes were downregulated (5). Downregulation of FADS1 (Suppl. Excel File) and FADS2 in liver of cows with ketosis suggests a substantial impairment of polyunsaturated fatty acid synthesis, which could result in modifications of hepatic concentrations of cholesterol esters and/or phospholipids as seen in mice (53). Similarly, SCD downregulation (≥4-fold; Table 2) is suggestive of impaired de novo synthesis of oleic acid, which is the major monounsaturated fatty acid in cellular membranes. Mice with targeted disruption in SCD (Sdcd−/−) have significant reductions in tissue content of TAG, suggesting that monounsaturated fatty acids synthesized endogenously are important for normal TAG synthesis (e.g., 39). In fact, both SCD and DGAT2 are colocalized in the membranes of the endoplasmic reticulum and mitochondria, which might increase channeling of fatty acids toward esterification (39).

Expression of SCD also has been strongly correlated with control of cell proliferation and survival, all of which are positively correlated with oleic acid concentration, cholesterol synthesis, and phospholipid synthesis in vitro (49).

Cows with hepatic lipidosis have reduced circulating concentrations of VLDL (7). In nonruminant animals (rodents, humans, chicken), there appears to be an absolute need for endogenous synthesis of oleic acid for synthesis and export of VLDL from liver (e.g., Ref. 34), which implies that SCD downregulation in postpartum cows indirectly might lead to liver lipidosis. Sustained downregulation of SCD expression in mice prevents diet-induced hepatic insulin resistance, obesity, and liver lipidosis (21, 23). Our results suggest that downregulation of SCD also may play an indirect role in bovine liver lipidosis accompanying feed restriction by further impairing VLDL synthesis and secretion. In fact, it has been demonstrated that oleic acid increases hepatocyte VLDL synthesis (29). Interestingly, DBI, which codes for a long-chain fatty acyl-CoA-binding protein (25), was drastically downregulated by ketosis (Table 2). In mice overexpressing DBI, the ratio of unsaturated to saturated fatty acids in liver increased significantly (25). Jolly et al. (28) demonstrated a critical role for DBI-mediated channeling of unsaturated fatty acids for phosphatidic acid biosynthesis in liver tissue.

It has been speculated that decreased serum concentrations of ApoB, ApoAI, and LCAT, and induction of haptoglobin and SAA1 synthesis are intimately related to development of fatty liver and ketosis (1, 7, 31). Along with microsomal triglyceride transfer protein (MTP), APOB is necessary for the formation of TAG-rich VLDL particles in liver that are subsequently exported to peripheral tissues. There was no change in expression of MTP in cows with ketosis (data not shown). Furthermore, our data showed upregulation of APOB and APOAI (Table 2, Fig. 3), and no changes in haptoglobin (HP, Table 2) or SAA1 (data not shown) in cows with ketosis. Liver from ketotic cows was characterized by substantial downregulation of genes involved in protein synthesis, protein trafficking, protein ubiquitination, and molecular transport (Fig. 2, Suppl. Fig. S2). The fact that APOB was upregulated along with downregulation of genes associated with protein ubiquitination/degradation and TAG accumulation (Suppl. Fig. S1) points at other mechanisms limiting VLDL synthesis and export, e.g., lower de novo synthesis of cholesterol and oleic acid, rather than deficient ApoB synthesis.

Carbohydrate. We observed that ketosis resulted in downregulation of ENO1, PDHB, PGK1, TP1, GPI, and PGAM1 (Suppl. Excel File) all of which play roles in glycolysis, TCA cycle, and gluconeogenesis (Fig. 3). Among gluconeogenic genes, PCK1 was dramatically downregulated and PC and FBP1 were upregulated (Table 2, Suppl. Excel File). Velez and Donkin (55), using a feed restriction model with midlactation cows, also found that development of ketosis resulted in lower liver expression of PCK1 (Table 2). PCK1 has long been considered to catalyze the rate limiting step for gluconeogenesis (47). However, recent data have shown that changes in...
acute-phase proteins (22) such as prevented an IL-6-mediated increase in mRNA of positive responses such as enhanced fatty acid oxidation and, in the case of fatty acid oxidation (20). In fact, $PPAR_{A}$ and $TNF-$ receptor that in nonruminants mediates the effects of NEFA metabolism and occupies the proximal promoter region of several hundred genes in human liver (44). $PPARA$ is a nuclear receptor that in nonruminants mediates the effects of NEFA and derivatives on transcription of genes commonly called PPAR-target genes (41, 42). Genes under partial control of $PPARA$ include those associated with peroxisomal and mitochondrial fatty acid oxidation, lipoprotein, bile and amino acid metabolism, glucose homeostasis, and inflammation (41). We suggest that palmitate and/or oleate in NEFA reaching liver from adipose tissue at supraphysiological concentrations affect expression of nuclear receptors and metabolic genes. Consequently, these fatty acids can then elicit physiological responses such as enhanced fatty acid oxidation and, in the case of palmitate, inflammation (Fig. 3).

With regard to inflammation, our data (Table 2, Fig. 3) seem to indicate that upregulation of $PPARA$ with ketosis might have prevented an IL-6-mediated increase in mRNA of positive acute-phase proteins (22) such as $HP$, $SAA_{1}$, and $A2M$ as well as downregulation of the negative acute-phase protein $ALB$. Optimal $PPARA$ function in liver during the periparturient period (e.g., through nutrition and environment) might be of practical importance in terms of delaying or blocking systemic responses to proinflammatory cytokines such as IL-6, IL-1β, and TNF-α that might be elevated in blood.

$LPIN_{1}$ seems to play a crucial role in activating the overall process of fatty acid oxidation (20). In fact, $LPIN_{1}$ activates a subset of $PPARGC_{1A}$-target genes (through $PPARA$) including those involved in fatty acid oxidation and mitochondrial oxidative phosphorylation. In the bovine liver, however, the role of $LPIN_{1}$ might be more closely associated with fatty acid oxidation because our data showed a substantial downregulation of oxidative phosphorylation in feed restricted cows with ketosis (Fig. 2).

Liver Inflammation and Functional Consequences Inferred by Gene Expression

Our data demonstrate genomic adaptations of a number of inflammation-related genes and acute-phase proteins, likely as a result of effects on both hepatocytes and Kupffer cells (Fig. 2). We observed differential expression of $PTPN_{1}$, $IL6$, $A2M$, and $IL1R_{2}$ (Table 2). There was a remarkable increase in $IL6$ (30) in cows with ketosis (Table 2). Network analysis (Fig. 3) suggests that IL-6 might have a central role in a wide range of liver-specific functions in cattle such as lipoprotein metabolism ($APOB$ and $APOA_{1}$; Refs. 30, 57), fatty acid oxidation ($ACOX_{1}$; Ref. 22), urea cycle ($ARG1$; Ref. 35), oxidative stress ($GSTA_{4}$; Ref. 14), transcription regulation ($SREBF_{2}$; Ref. 24), and protein degradation through proteasomes (Fig. 2, Suppl. Figs. S2 and S3). Alterations in all the above-cited functions might contribute to development of liver lipidosis, ketosis, and insulin resistance, thus impairing normal liver function.

The potential link between $IL6$, $ACOX_{1}$, and $ARG1$ is particularly relevant to postparturient infection in cows because it might influence the ability of the liver to cope with the increased influx of NEFA as well as maintaining flux through the urea cycle. $ARG1$ in hepatocytes is downregulated by acute exposure to proinflammatory cytokines (35). However, $PPARA$ activation results in total suppression of the IL-6-induced acute-phase response in mouse hepatocytes and increases $ACOX_{1}$ expression (22). Thus, $PPARA$ up-regulation or activation in our study might have been sufficient to attenuate potential negative effects of IL-6 on $ARG1$ and $ACOX_{1}$.

Hepatocytes possess IL-6R, which allows IL-6 to initiate downstream signaling cascades that govern, among other responses, the resolution of acute innate immune responses and the transition to an acquired immune response (48). The physiological action of IL-6 signaling on resolution of acute inflammation is closely linked with STAT1, IFN-γ, TGF-β, GATA-3, and NF-κB action (48). These interactions appear to influence disease outcome significantly. A common feature arising among recent studies is the role of IL-6 signaling in orchestrating leukocyte recruitment, activation, and apoptotic clearance (48). Gene expression signatures from our study, which relied on whole liver tissue, highlight the likely importance of both hepatocytes and Kupffer cells in mediating bovine liver adaptations to feed restriction and ketosis. Despite upregulation of $IL6$, feed restriction and ketosis did not result in upregulation of other genes that are signatures of a proinflammatory state (Tables 2 and 3). It remains to be determined whether IL-6 upregulation is a cause or an effect of liver lipidosis and ketosis, or whether it only represents a generalized response by hepatocytes and immune cells in liver to disturbance of biological homeostasis.

Transcription Factors and Their Regulatory Circuits in Liver

Our findings underscore the central roles of $HNF4_{A}$, $PPARA$, $FOXC_{2}$, $PPARGC_{1A}$, and $PPARD$ in the molecular adaptations necessary to cope with altered metabolism due to feed restriction and ketosis in bovine liver (Fig. 3, Table 2). We present some of the first data in the bovine that reveal regulatory loops between these transcription factors and genes involved in fatty acid transport (e.g., $FABP_{3}$), fatty acid oxidation (e.g., $ACOX_{1}$, $ACAA_{1}$), and ketone body metabolism (e.g., $ACAT_{1}$). $HNF4_{A}$ plays a pivotal role in glucose and fatty acid metabolism and occupies the proximal promoter region of several hundred genes in human liver (44). $PPARA$ is a nuclear receptor that in nonruminants mediates the effects of NEFA and derivatives on transcription of genes commonly called PPAR-target genes (41, 42). Genes under partial control of $PPARA$ include those associated with peroxisomal and mitochondrial fatty acid oxidation, lipoprotein, bile and amino acid metabolism, glucose homeostasis, and inflammation (41). We suggest that palmitate and/or oleate in NEFA reaching liver from adipose tissue at supraphysiological concentrations affect expression of nuclear receptors and metabolic genes. Consequently, these fatty acids can then elicit physiological responses such as enhanced fatty acid oxidation and, in the case of palmitate, inflammation (Fig. 3).

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**Intracellular Energy Status During Feed Restriction and Ketosis**

The pathways most affected by feed restriction and ketosis were oxidative phosphorylation, protein ubiquitination, and ubiquinone synthesis (Fig. 2, Suppl. Fig. S2). Our data set contained 97, 125, and 41 genes of those that compose the entire pathways of oxidative phosphorylation (162 genes total), protein ubiquitination (200 genes total), and ubiquinone biosynthesis (104 genes total), respectively. Oxidative phosphorylation and ubiquinone biosynthesis are tightly connected because ubiquinone is an important electron transporter during oxidative phosphorylation. In our study, feed restriction and ketosis likely decreased liver ubiquinone biosynthesis through downregulation of 14 genes that are components of the NADH-quinone oxidoreductase enzyme complex, which is composed of at least 42 genes in mammals (2). This enzyme is essential for the oxidation of NADH (2). The potential reduction in ubiquinone availability, along with the downregulation of genes coding for enzymes of oxidative phosphorylation (e.g., NDUFb6), clearly suggest that during feed restriction, and perhaps as a consequence of ketosis, the liver reduced overall metabolic activity and thus energy utilization. The extracellular factors and molecular adaptations involved remain elusive. Our data confirm observations found in 2 day-starved mice (5).

Ubiquitination is an energy-requiring step for proteasomal-dependent degradation of proteins, which represents a key regulatory step of cell activity and function (10). Regulated protein turnover via the ubiquitin-proteasome system underlies

![Fig. 4. Interrelationships between nutritional status and mammary demands for milk synthesis on physiological adaptations in adipose tissue and liver early postpartum. Copious milk production by the postpartum cow takes precedence in the utilization of the animal’s resources, i.e., demands for milk synthesis place a large toll on carbohydrate resources. Metabolic changes underlying onset of ketosis are associated primarily with a shortage of carbohydrate precursors and, as a consequence, rates of gluconeogenesis (4). Clearly, management and/or disease incidence are likely to influence drastically the nutritional status of cows early postpartum (e.g., Refs. 15, 16). Thus, if either of these factors prevents the animal from achieving expected rates of feed intake the likelihood of developing ketosis increases. Upon withdrawal of feed, metabolic signals (e.g., glucocorticoids, catecholamines) enhance adipose tissue lipolysis and NEFA delivery to liver. Resident macrophages in adipose could contribute to influx of cytokines and other proinflammatory mediators into liver. It is also likely that both hepatocytes and Kupffer cells can directly respond to influx of NEFA (e.g., palmitic acid, oleic acid) and/or adipokines, which might lead to inflammation (e.g., IL6 upregulation). The excessive NEFA, cytokine, and BHBA accumulation in liver could account for responses observed for metabolism and cell signaling pathways at the mRNA level. The ketogenic liver reduces energy production and metabolism, perhaps due to decreased milk production, but maintains fatty acid oxidation through upregulation of peroxisome proliferator-activated receptor (PPAR) signaling pathways. Reduced energy production also would ensure that generation of reactive oxygen species is lower (32), thus lessening tissue damage during ketosis. Nutrition-induced ketosis early postpartum could affect animal health and well-being and in the long term have negative effects on reproduction and productive life. Data from this study indicated a tendency for cows developing ketosis for synthesizing less milk well after they had returned to normal feed intake (Fig. 1).
a wide variety of signaling pathways, from cell-cycle control and transcription to development (43). We found that feed restriction and ketosis resulted in severe downregulation of genes associated with ubiquitination (Fig. 2). The functional consequences of impaired intracellular protein degradation in liver might include alterations in cellular turnover, effects on gene regulation, modulation of cell signaling, induction of apoptosis and necrosis, release of reactive oxygen species, and loss of gene/protein function (3). Such changes could be a primary factor involved in the impairment of hepatic function that contributes to the onset of clinical ketosis in dairy cows.

**ANGPTL4, a Novel Gene in Bovine Periparturient Metabolic Adaptations**

Recent studies have indicated that **ANGPTL4** codes for a protein that regulates TAG, lipoprotein lipase (LPL) activity, and NEFA metabolism as well as insulin resistance in rodents (36). The expression of **ANGPTL4** is under nutritional control, with its blood concentration increased by fasting in rodents. Treatment with PPAR agonists results in sustained upregulation of **ANGPTL4** (32), which in turn appears to downregulate tissue LPL activity (e.g., heart, adipose) and VLDL-TAG utilization (40), as well as to increase lipolysis (32). Cows with fatty liver and ketosis have greater adipocyte lipolysis rates (7). Thus, increased **ANGPTL4** production from the bovine liver during periods of reduced feed intake might serve as a signal for lipolysis and contribute to the sustained release of NEFA and lipid accumulation in the liver (Fig. 4). The negative correlation between insulin and adipose **ANGPTL4** (56) suggests that this hormone participates in the control of **ANGPTL4** production, particularly during periods of positive energy balance. Ketotic cows in our study had numerically lower insulin concentrations than control cows (Fig. 1). Further studies need to be conducted to evaluate the biological significance of liver-derived proteins such as **ANGPTL4** in the metabolic adaptations that encompass metabolically active tissues such as liver and adipose during the periparturient period. The possible presence of such a mechanism(s) has been proposed previously (17) but not identified.

**Summary and Conclusions**

Our study is one of the first to uncover alterations in metabolic and signaling gene network expression signatures in liver that are associated with negative energy balance and ketosis in periparturient cows. A number of these gene expression changes (Fig. 3) can be readily linked to well-documented metabolic adaptations underlying the disease including fatty acid oxidation, ketogenesis, and cholesterol synthesis. We present evidence that liver tissue responds to negative energy balance and ketosis by shutting down energy-generating processes, resulting in lower protein synthesis. As a result, a large number of genes associated with cell death are downregulated and only a few upregulated genes appear to be essential for maintaining basal cellular growth and proliferation. The proinflammatory cytokine IL-6 appears linked to transcriptional regulation of nitrogen metabolism (e.g., amino acid transport, urea synthesis), fatty acid oxidation, and cholesterol synthesis during ketosis through its effects on key enzymes (e.g., **ACOX1, ARGI**) or transcription factors (e.g., **SREBF2**). More importantly, however, coordinated mRNA expression of nuclear receptors and transcription factors (e.g., **PPARA, PPARGC1A, HNF4A**) in liver appears essential to sustain fatty acid oxidation and ketogenesis. Our findings also reveal potentially novel genes (e.g., **LPIN1, LPIN3, ANGPTL4**) that might play key roles in hepatic metabolic adaptations to negative energy balance and the changing physiological state near the time of parturition, including adipose tissue lipolysis and hepatic fatty acid oxidation (Fig. 4).

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