Hepatic transcript profiling in early-lactation dairy cows fed rumen-protected niacin during the transition from late pregnancy to lactation

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ABSTRACT

In dairy cows, administration of high dosages of niacin (nicotinic acid, NA) was found to cause antilipolytic effects, which are mediated by the NA receptor hydroxyl-carboxylic acid receptor 2 (HCAR2) in white adipose tissue (WAT), and thereby an altered hepatic lipid metabolism. However, almost no attention has been paid to possible direct effects of NA in cattle liver, despite evidence that HCAR2 is also expressed in the liver and is even more abundant than in WAT. Because of this, we hypothesized that feeding a high dosage of rumen-protected NA to dairy cows influences critical metabolic or signaling pathways in the liver by inducing changes in the hepatic transcriptome. To identify these pathways, we applied genome-wide transcript profiling in liver biopsies obtained at d 7 postpartum (p.p.) from dairy cows used in our recent study; cows received either no NA (control group, $n = 9$) or 79 mg of rumen-protected NA/kg of body weight daily (NA group, $n = 9$) from 21 d before calving until 3 wk p.p. Hepatic transcript profiling revealed that 487 transcripts were differentially expressed (filter criteria: fold change >1.2 or <-1.2 and $P < 0.05$) in the liver at d 7 p.p. between cows fed NA and control cows. Substantially more transcripts were downregulated ($n = 338$), whereas only 149 transcripts were upregulated by NA in the liver of cows. Gene set enrichment analysis for the upregulated transcripts revealed that the most-enriched gene ontology biological process terms were exclusively related to immune processes, such as leukocyte differentiation, immune system process, activation of immune response, and acute inflammatory response. Gene set enrichment analysis of the downregulated transcripts showed that the most-enriched biological process terms

were related to metabolic processes, such as cellular metabolic process, small molecule metabolic process, lipid catabolic process, organic cyclic compound metabolic process, small molecule biosynthetic process, and cellular lipid catabolic process. In conclusion, hepatic transcriptome analysis showed that rumen-protected NA induces genes that are involved mainly in immune processes, including acute phase response and stress response, in dairy cows at d 7 p.p. Thus, supplementation of a high dosage of rumen-protected NA to dairy cows in the periparturient period may induce or amplify the systemic inflammation-like condition that is typically observed in the liver of high-yielding dairy cows in the p.p. period.

Key words: niacin, liver, lactating dairy cow, acute phase response

INTRODUCTION

Niacin or nicotinic acid (NA) is a water-soluble vitamin that is provided to animals from different dietary sources and in ruminants from microbial synthesis in the rumen (Brent and Bartley, 1984). It is required in the animal's tissues as a precursor of nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$), which are important coenzymes in numerous enzymatic reactions in intermediary metabolism. Apart from serving as a precursor for coenzymes, NA at pharmacologic dosages exerts pronounced plasma lipid-modulating effects, such as decreasing triacylglycerols (TAG) and very low density lipoproteins (VLDL); indeed, NA has been in clinical use as an effective drug for the treatment of different forms of dyslipidemia in humans for more than 5 decades (Carlson, 2005). The plasma lipid-modulating effects of NA have been largely ascribed to its antilipolytic effect, which is mediated by activation of the white adipose tissue (WAT) NA receptor hydroxyl-carboxylic acid receptor 2 (HCAR2), thereby decreasing plasma levels of nonesterified fatty acids (NEFA) and availability of NEFA for TAG synthesis and VLDL assembly in the liver (Gille et al., 2008).

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In dairy cows, administration of high dosages of NA either in rumen-protected form or via the abomasum to reduce or avoid ruminal degradation of NA was also found to cause antilipolytic effects (Pires et al., 2007; Pires and Grummer, 2007; Pescara et al., 2010; Morey et al., 2011; Yuan et al., 2012). Such effects might be helpful in high-yielding dairy cows during the postpartum (**p.p.**) period because dairy cows are typically in strong negative energy balance during this phase, leading to an excessive mobilization of NEFA from WAT into the circulation. High circulating levels of NEFA, together with a hepatic inflammation-like condition typically arising in the p.p. period in high-yielding dairy cows (Bradford et al., 2015), are considered major challenges for the liver, predisposing p.p. dairy cows to the development of hepatic lipid-related disorders such as fatty liver and ketosis. In line with this, 2 studies reported at least a tendency for reduced liver TAG concentrations in dairy cows supplemented with rumen-protected NA in the p.p. period (Morey et al., 2011; Yuan et al., 2012). In addition, it was shown that rumen-protected NA (Morey et al., 2011) and even unprotected NA (Erickson et al., 1992; Fronk and Schultz, 1979) decrease the plasma concentration of BHB in p.p. dairy cows. We have also recently demonstrated that plasma concentrations of BHB in dairy cows fed a TMR without (control) or with rumen-protected NA from 21 d before calving until 3 wk p.p. were decreased in the early p.p. period (Zeitz et al., 2018). However, in our study, rumen-protected NA failed to lower plasma concentrations of NEFA and liver concentrations of TAG (Zeitz et al., 2018). This indicates that the antilipolytic effect of NA mediated via HCAR2 in WAT alone cannot convincingly explain the reduced plasma levels of BHB; additional effects of NA in other tissues might be involved. In this regard, it is noteworthy that NA receptor HCAR2 is also expressed in the liver of cattle, where it is even more abundant than in WAT (Titgemeyer et al., 2011), whereas in humans and mice *HCAR2* mRNA is absent in the liver and predominantly found in WAT and immune cells (Tunaru et al., 2003; Wise et al., 2003). Moreover, HCAR2 expression in the liver of cattle was found not only in tissue-resident immune cells of the liver (Kupffer cells), but also in parenchymal cells (Titgemeyer et al., 2011). This suggests that NA in cattle may cause direct effects in the liver that are mediated independently of its antilipolytic effects. Because of the abundant HCAR2 expression in cattle liver, we hypothesized that feeding rumen-protected NA to dairy cows influences critical metabolic or signaling pathways in the liver by inducing changes in the hepatic transcriptome. To identify these pathways, we applied genome-wide transcript profiling

in liver biopsies obtained at d 7 p.p. from dairy cows that received either no NA (control group) or a daily rumen-protected NA dosage (NA group) from 21 d before calving until 3 wk p.p.

MATERIALS AND METHODS

Animal Experiment

A total of 22 multiparous Holstein dairy cows were used, which were part of a study carried out at the Educational and Research Centre for Animal Husbandry, Hofgut Neumühle (Münchweiler an der Alsenz, Germany) and approved by the Provincial Government of Koblenz, Germany (23 177-07/G 14-20-050). The experimental period lasted from 3 wk before expected calving until 1 wk p.p. Details on feeding and husbandry conditions are given in Zeitz et al. (2018). In brief, cows were assigned to 2 groups (control group and NA group, each $n = 11$) and kept in a freestall barn equipped with weighing troughs. Clinical symptoms of infectious and other diseases that were treated by a veterinarian were found in 3 cows of the control group (1 with mastitis, 1 with anorexia and diarrhea, 1 with milk fever) and 3 cows of the NA group (1 with metritis, 1 with mastitis, 1 with milk fever). The cases of mastitis were treated locally with cefquinom (Cobactan, MSD Tiergesundheit, Unterschleißheim, Germany), whereas the cows with metritis were treated with tetracycline (Bela-Pharm, Vechta, Germany). Cows with milk fever and anorexia and diarrhea received an infusion therapy. Both groups of cows received the same corn and grass silage-based antepartum and p.p. TMR during pregnancy and lactation, respectively. The diets were calculated to meet the energy and protein requirements of dairy cows following the recommendations of the German Society of Nutrition Physiology (GfE, 2001). The cows had free access to the TMR, which was supplied twice daily at 0700 and 1400 h. In addition, cows of the NA group were given 79 mg of rumen-protected NA (NiaShure; Balchem, New Hampton, NY) per kg of BW daily. Considering the rumen degradability of NiaShure (Zeitz et al., 2018), the applied NA dosage was equivalent to 47 mg of NA at the duodenum and estimated to be equivalent to 31 mg of absorbable NA per kg of BW based on information given by the supplier. This NA dosage was close to that required for pharmacological use as an antilipidemic drug in humans (10–30 mg of NA/kg of BW; Offermanns, 2006). The NA dosage was adapted to the BW of the cows once per week starting 3 wk before expected calving until 1 wk p.p. The individual NA dosage was mixed into a 500-g pressed sugar beet pulp portion, which was supplied to each cow once

daily at 0800 h in feed fences, ensuring complete intake. Cows of the control group were given 500 g of pressed sugar beet pulp without supplemented NA.

Sample Collection

Blood samples were collected on d 7 p.p. between 0700 and 0800 h (after milking and before feeding) from tail vein into EDTA-containing vacutainers (S-Monovette 9 mL K3E, Sarstedt, Nümbrecht, Germany) and kept on ice for <1 h. Plasma was obtained by centrifugation at $2,000 \times g$ for 10 min at 4°C and stored at -20°C. Biopsies of the liver were also taken on d 7 p.p. at 0900 h between the 9th and 10th ribs approximately 15 cm below the transverse processes by means of a self-made barbed 12-gauge $\times$ 27 cm biopsy needle under local anesthesia, as described in detail (Zeitz et al., 2018). Liver samples were immediately snap-frozen in liquid N₂ and stored at -80°C.

RNA Isolation

Total RNA isolation from liver samples was carried out using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and stored at -80°C. The concentration and purity of the RNA was estimated from the optical density at 260 and 280 nm, respectively, using a NanoQuant plate (Infinite 200, Tecan, Mainz, Germany). Before sample processing at the Centre of Excellence for Fluorescent Bioanalytics (University of Regensburg, Regensburg, Germany), the concentration and integrity of RNA was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany). The total RNA concentration, optical density A260:A280 ratios, and RNA integrity number (RIN) values of all samples were $0.22 \pm 0.08 \mu\text{g}/\mu\text{L}$, 2.00 ± 0.03 , and 7.2 ± 0.5 (mean $\pm$ SD, $n = 18$), respectively. From the 11 available RNA samples per group, the 2 samples with the lowest RIN values were excluded.

Microarray Hybridization and Analysis

For microarray analysis, 9 total RNA samples per group were used. Total RNA samples were processed according to the GeneChip WT Plus Reagent Kit (Affymetrix, High Wycombe, UK) as recently described in detail (Gessner et al., 2017). The Bovine Gene 1.0 Sense Target array (Affymetrix), representing approximately 23,000 bovine transcripts, was used. The microarray data have been submitted in MIAME (minimum information about a microarray experiment)-compliant format to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO)

public repository (Edgar et al., 2002; GEO accession no. GSE116058). Microarray analysis was carried out using the Robust Multichip Analysis (RMA) algorithm (Irizarry et al., 2003). Annotation of microarrays was performed with the Affymetrix BovGene-1_0-st-v1_Probeset_Release 36 annotation file. Transcripts were defined as differentially expressed when the fold change (FC) between the NA group and control group was >1.2 or <-1.2 and the P -value of the unpaired Student's t -test (2-tailed distribution, 2-sample equal variance) for each transcript was <0.05 . Filtering of differentially expressed transcripts using the GEO2R tool (<http://www.ncbi.nlm.nih.gov/geo/geo2r/>) with Benjamini and Hochberg false discovery rate adjustment method failed, because the adjusted P -values for all transcripts were >0.05 .

Bioinformatic Prediction of mRNA Targets of the Differentially Expressed miRNAs

The mRNA targets of differentially regulated microRNAs (miRNA) were predicted *in silico* using TargetScan release version 7.1 (<http://www.targetscan.org/>) based on the presence of conserved 6 to 8mer sites matching the seed region of each miRNA (Lewis et al., 2005). A cumulative weighted "context++" score, which is a measure of predicted efficacy of targeting of the sites (Agarwal et al., 2015), of <-0.50 was used as cut-off criterion for predicting mRNA targets.

Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) using gene ontology (GO) terms within GO categories (biological process, cellular component, molecular function) was applied to extract biological meaning from the identified differentially expressed transcripts and predicted mRNA targets; GO terms with a P -value <0.05 were considered as enriched. The GSEA was carried out using the Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.8 bioinformatic resource (Huang et al., 2009a,b).

Quantitative PCR Analysis

Quantitative PCR (qPCR) analysis in liver tissue was carried out as described in our recent paper (Zeitz et al., 2018). Gene-specific primer pairs for the reference genes were the same as used by Zeitz et al. (2018). Primer pairs for target genes were designed using PRIMER3 and BLAST and synthesized by Eurofins MWG Operon (Ebersberg, Germany). Primer characteristics are shown in Supplemental Table S1 (<https://doi.org/10.3168/jds.2018-15232>).

Statistical Analysis

All data were evaluated by Student's *t*-test using the Minitab statistical software (release 13, Minitab Inc., State College, PA).

RESULTS

Performance of Dairy Cows

Feed intake, milk and milk protein yields, milk urea concentration, BW, BCS, and the extent of negative energy balance did not differ between cows fed rumen-protected NA and control cows at wk 1 to 3 p.p., as reported recently (Zeitz et al., 2018). Milk fat percentage and milk fat yield at wk 1 to 3 of lactation were lower in cows fed rumen-protected NA than in control cows (Zeitz et al., 2018).

Identification of NA-Regulated Transcripts in the Liver

To investigate the effect of NA on the hepatic transcriptome of early-lactating dairy cows, we used a

bovine microarray representing approximately 23,000 *Bos taurus* transcripts. Taking into account the filter criteria, 487 transcripts were found to be differentially expressed in the liver between cows fed NA and control cows. Only 149 transcripts were upregulated by NA in the liver of cows; substantially more transcripts were downregulated ($n = 338$). The 30 most strongly up- and downregulated transcripts are presented in Table 1 and Table 2, respectively. Although all of the upregulated transcripts were protein-coding mRNAs, the downregulated transcripts consisted of 319 protein-coding mRNAs and 19 non-protein-coding miRNAs. The latter are summarized in Table 3.

Validation of Microarray Data for Selected NA-Regulated mRNAs by qPCR

Microarray data were validated by qPCR analysis of 12 differentially regulated mRNAs, which were randomly selected from the most strongly up- and down-regulated mRNAs. Table 4 shows that in the case of all mRNAs to be validated, the effect direction was the same between qPCR and microarray data. Because

Table 1. The 30 most strongly upregulated transcripts in the liver of cows fed rumen-protected niacin (NA) compared with control cows at 1 wk postpartum

Gene symbol	Gene description	Fold change ¹ NA vs. control
<i>HP</i>	Haptoglobin	2.85
<i>ECEL1</i>	Endothelin converting enzyme-like 1	2.67
<i>NNAT</i>	Neuronatin	2.40
<i>S100A12</i>	S100 calcium binding protein A12	1.92
<i>LOC782061</i>	Aldo-keto reductase family 1, member C1-like	1.89
<i>LOC100296421</i>	UDP-glucuronosyltransferase 2B4	1.74
<i>FOLR3</i>	Folate receptor 3 (gamma)	1.66
<i>C5H12orf75</i>	Chromosome 5 open reading frame, human c12orf75	1.65
<i>BOLA-DQA5</i>	Major histocompatibility complex, class II, DQ α 5	1.57
<i>APCS</i>	Amyloid P component, serum	1.56
<i>TRGC6</i>	T-cell receptor gamma chain TRGC6	1.50
<i>CD28</i>	CD28 molecule	1.48
<i>SPATA9</i>	Spermatogenesis associated 9	1.47
<i>GNMT</i>	Glycine N-methyltransferase	1.45
<i>C2H2orf88</i>	Chromosome 2 open reading frame, human c2orf88	1.42
<i>TYROBP</i>	TYRO protein tyrosine kinase binding protein	1.41
<i>NCALD</i>	Neurocalcin delta	1.41
<i>LOC789690</i>	Olfactory receptor 12D2	1.39
<i>LOC613345</i>	H3 histone, family 3A-like	1.39
<i>MAP2</i>	Microtubule-associated protein 2	1.38
<i>LOC100335322</i>	60S ribosomal protein L22-like 1	1.37
<i>GPX4</i>	Glutathione peroxidase 4	1.36
<i>LOC511657</i>	Olfactory receptor 8B3	1.36
<i>C20H5orf49</i>	Chromosome 20 open reading frame, human c5orf49	1.35
<i>SRXN1</i>	Sulfiredoxin 1	1.35
<i>TGM5</i>	Transglutaminase 5	1.35
<i>CIAPIN1</i>	Cytokine induced apoptosis inhibitor 1	1.34
<i>TRD</i>	T-cell receptor delta chain	1.32
<i>LY86</i>	Lymphocyte antigen 86	1.32
<i>ALDOA</i>	Aldolase A, fructose-bisphosphate	1.32

¹Fold change was calculated from the signal log ratios as follows: $2^{\text{Signal log ratio}}$ if signal log ratio ≥ 0 and $(-1) \times 2^{-(\text{Signal log ratio})}$ if signal log ratio < 0 . Signal log ratios were calculated from $n = 9$ microarrays per group.

Table 2. The 30 most strongly downregulated transcripts in the liver of cows fed rumen-protected niacin (NA) compared with control cows at 1 wk postpartum

Gene symbol	Gene description	Fold change ¹ NA vs. control
<i>LOC540627</i>	Cytochrome p450 2c42	-2.06
<i>CABYR</i>	Calcium binding tyrosine-(y)-phosphorylation regulated	-2.04
<i>RBP5</i>	Retinol binding protein 5, cellular	-1.94
<i>RAD21L1</i>	RAD21-like 1 (S. pombe)	-1.90
<i>MFSD2A</i>	Major facilitator superfamily domain containing 2a	-1.79
<i>LOC782865</i>	Olfactory receptor 481-like	-1.78
<i>SEC14L3</i>	SEC14-like 3 (S. cerevisiae)	-1.78
<i>MIR2305</i>	MicroRNA mir-2305	-1.75
<i>MIR186</i>	MicroRNA mir-186	-1.71
<i>KIF23</i>	Kinesin family member 23	-1.71
<i>ELOVL2</i>	ELOVL fatty acid elongase 2	-1.69
<i>LOC100299811</i>	Non-histone chromosomal protein hmg-14	-1.69
<i>SLC17A3</i>	Solute carrier family 17 (organic anion transporter), member 3	-1.65
<i>TXNDC16</i>	Thioredoxin domain containing 16	-1.65
<i>BREH1</i>	Retinyl ester hydrolase type 1	-1.62
<i>LOC104974345</i>	Glycine N-acyltransferase-like	-1.62
<i>OAS1Y</i>	2,5-Oligoadenylate synthetase 1, 40/46kDa	-1.62
<i>ATP8B3</i>	ATPase, aminophospholipid transporter, class I, type 8B, member 3	-1.61
<i>DDX58</i>	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	-1.59
<i>LOC505468</i>	Cytochrome p450 2c19	-1.58
<i>LOC790431</i>	Zinc finger protein 347-like	-1.58
<i>LOC100300095</i>	Zinc finger protein 665	-1.56
<i>TTC39C</i>	Tetratricopeptide repeat domain 39c	-1.54
<i>MIR101-2</i>	MicroRNA mir-101-2	-1.54
<i>LOC516494</i>	Arylsulfatase D	-1.53
<i>LOC100336599</i>	Arylsulfatase D	-1.51
<i>RALGPS2</i>	Ral GEF with PH domain and SH3 binding motif 2	-1.51
<i>GSAP</i>	Gamma-secretase activating protein	-1.51
<i>CYP2C87</i>	Cytochrome P450, family 2, subfamily C, polypeptide 87	-1.50
<i>ABCC5</i>	ATP-binding cassette, sub-family C (CFTR/MRP), member 5	-1.49

¹Fold change was calculated from the signal log ratios as follows: $2^{\text{Signal log ratio}}$ if signal log ratio ≥ 0 and $(-1) \times 2^{-(\text{Signal log ratio})}$ if signal log ratio < 0 . Signal log ratios were calculated from $n = 9$ microarrays per group.

Table 3. MicroRNAs downregulated in the liver of cows fed rumen-protected niacin (NA) compared with control cows at 1 wk postpartum

Gene symbol	Gene description	Fold change ¹ NA vs. control
<i>MIR2305</i>	MicroRNA mir-2305	-1.75
<i>MIR186</i>	MicroRNA mir-186	-1.71
<i>MIR101-2</i>	MicroRNA mir-101-2	-1.54
<i>MIR30F</i>	MicroRNA mir-30f	-1.41
<i>MIR2475</i>	MicroRNA mir-2475	-1.41
<i>MIR23B</i>	MicroRNA mir-23b	-1.38
<i>MIR2303</i>	MicroRNA mir-2303	-1.37
<i>MIR2483</i>	MicroRNA mir-2483	-1.37
<i>MIR27B</i>	MicroRNA mir-27b	-1.36
<i>MIR2461</i>	MicroRNA mir-2461	-1.36
<i>MIR2465</i>	MicroRNA mir-2465	-1.35
<i>MIR33B</i>	MicroRNA mir-33b	-1.33
<i>MIR103-2</i>	MicroRNA mir-103-2	-1.33
<i>MIR2473</i>	MicroRNA mir-2473	-1.31
<i>MIR365-2</i>	MicroRNA mir-365-2	-1.30
<i>MIR664A</i>	MicroRNA mir-664a	-1.25
<i>MIR23A</i>	MicroRNA mir-23a	-1.24
<i>MIR660</i>	MicroRNA mir-660	-1.22
<i>MIR2291</i>	MicroRNA mir-2291	-1.22

¹Fold change was calculated from the signal log ratios as follows: $2^{\text{Signal log ratio}}$ if signal log ratio ≥ 0 and $(-1) \times 2^{-(\text{Signal log ratio})}$ if signal log ratio < 0 . Signal log ratios were calculated from $n = 9$ microarrays per group.

of the different number of biological replicates and the different detection principle of transcript abundance between microarray and qPCR, the FC and *P*-values differed more or less. Statistical analysis revealed that 5 of the 12 mRNAs analyzed by qPCR were regulated by NA treatment at a significance level of $P < 0.05$ (*HP*, *TYROBP*, *RBP5*, *MFSD2A*, *BREH1*) and 4 at a significance level of $P < 0.1$ (*FOLR3*, *LOC540627*, *ATP8B3*, *DDX58*), whereas 3 mRNAs were not differentially regulated (*APCS*, *GNMT*, *LOC505468*).

Identification of Biological Processes Affected by NA-Regulated Protein-Coding Transcripts in the Liver

To identify biological processes affected by the NA-regulated protein-coding transcripts, GSEA was performed using GO biological process terms. The GSEA of the upregulated mRNAs revealed that the most-enriched biological process terms (lowest *P*-value) were exclusively related to immune processes. Examples of the most-enriched terms are regulation of leukocyte differentiation, immune system process, leukocyte dif-

Table 4. Real-time quantitative (q)PCR validation of selected microarray data

Gene symbol	Fold change		<i>P</i> -value	
	Microarray ¹	qPCR ²	Microarray	qPCR
<i>HP</i>	2.85	6.05	0.032	0.044
<i>FOLR3</i>	1.66	1.58	0.011	0.082
<i>APCS</i>	1.56	1.68	0.046	0.158
<i>GNMT</i>	1.45	1.72	0.042	0.200
<i>TYROBP</i>	1.41	1.81	0.012	0.036
<i>LOC540627 (CYP2C42)</i>	−2.06	−1.85	0.014	0.073
<i>RBP5</i>	−1.94	−2.87	0.001	0.024
<i>MFSD2A</i>	−1.79	−1.73	0.031	0.007
<i>BREH1</i>	−1.62	−1.49	0.003	0.042
<i>ATP8B3</i>	−1.61	−1.90	0.020	0.064
<i>DDX58</i>	−1.59	−1.90	0.023	0.051
<i>LOC505468 (CYP2C19)</i>	−1.58	−1.54	0.045	0.292

¹The microarray fold change (FC) was calculated from the signal log ratios as follows: $2^{\text{Signal log ratio}}$ if signal log ratio ≥ 0 and $(-1) \times 2^{-(\text{Signal log ratio})}$ if signal log ratio < 0 . Signal log ratios were calculated from $n = 9$ microarrays per group.

²The qPCR FC was calculated analogously from normalized $2^{-\Delta Ct}$ ratios. Normalized $2^{-\Delta Ct}$ expression was calculated from $n = 11$ samples per group.

ferentiation, hematopoiesis, immune system development, regulation of immune system process, activation of immune response, and acute inflammatory response. Table 5 shows all biological process terms with *P*-values < 0.01 , including the number of genes assigned to these terms.

The GSEA of the downregulated mRNAs showed that the most-enriched biological process terms were related to metabolic processes, such as cellular metabolic process, small molecule metabolic process, lipid catabolic process, organic cyclic compound metabolic process, small molecule biosynthetic process, and cellular lipid catabolic process. Table 6 shows all biological process terms with *P*-values < 0.01 , including the number of genes assigned to these terms.

Identification of Cellular Components of Proteins Encoded by the NA-Regulated Protein-Coding Transcripts in the Liver

To further identify cellular components of proteins encoded by the NA-regulated protein-coding transcripts, GSEA was performed using GO cellular component terms separately for the up- and downregulated mRNAs. The GSEA of the upregulated mRNAs showed that the most-enriched cellular component terms were blood microparticle, extracellular exosome, and extracellular vesicle. All cellular component terms with *P*-values < 0.01 including the number of genes assigned to these terms, are listed in Table 7.

The GSEA of the downregulated mRNAs showed that the most enriched cellular component terms were dealing with lipoprotein particles: chylomicron, triglyceride-rich lipoprotein particle, and VLDL particle. All cellular component terms with *P*-values < 0.01 , includ-

ing the number of genes assigned to these terms, are listed in Table 8.

Prediction of Target mRNAs of the NA-Regulated miRNAs and Functional Analysis

To identify further protein-coding transcripts that are regulated by rumen-protected NA in the liver of cows, we performed bioinformatic target mRNA prediction for the top 10 regulated miRNAs. Considering a cumulative weighted context++ score < -0.50 , 831 mRNA targets were identified. Data including gene names, total and 8mer, 7mer, and 6mer sites and cumulative weighted context++ score are shown in Supplemental Table S2 (<https://doi.org/10.3168/jds.2018-15232>). To clarify the biological relevance, we carried out GSEA using the GO category “molecular function” with the predicted mRNA targets. As shown in Table 9, enriched GO “molecular function” terms associated with the mRNA targets of the top 10 downregulated miRNAs were mainly involved in RNA polymerase II-dependent transcriptional activator activity.

DISCUSSION

Hepatic transcript profiling at d 7 p.p. in dairy cows fed without or with rumen-protected NA during the transition from late pregnancy to lactation revealed that approximately 490 transcripts are regulated by rumen-protected NA. Considering that only 5 transcripts were regulated > 2 -fold or < -2 -fold indicates that the influence of rumen-protected NA on the hepatic transcriptome of p.p. dairy cows is moderate. However, we can exclude the possibility that low availability of NA from the rumen-protected NA source (NiaShure) ac-

Table 5. Enriched gene ontology (GO) biological process terms¹ associated with upregulated genes in the liver of cows fed rumen-protected niacin (NA) compared with control cows at 1 wk postpartum

GO biological process term	Count	<i>P</i> -value	Fold enrichment
Regulation of leukocyte differentiation	8	0.0002	6.38
Immune system process	25	0.0004	2.13
Leukocyte differentiation	11	0.0005	3.90
Hematopoiesis	13	0.0012	2.99
Regulation of hematopoiesis	8	0.0018	4.57
Hematopoietic or lymphoid organ development	13	0.0019	2.83
Regulation of myeloid leukocyte differentiation	5	0.0024	8.82
Immune system development	13	0.0029	2.68
Regulation of immune system process	15	0.0030	2.42
Myeloid leukocyte differentiation	6	0.0038	5.72
Activation of immune response	7	0.0044	4.51
Positive regulation of immune system process	11	0.0054	2.81
Acute inflammatory response	4	0.0090	9.25

¹The GO terms are sorted by their enrichment *P*-values [Expression Analysis Systematic Explorer (EASE) score] (top: lowest *P*-value, bottom: highest *P*-value). Only GO terms with *P*-values <0.01 are shown.

counts for the moderate influence on hepatic transcriptome because we have recently shown (for the cows of this study) that plasma concentration of nicotinamide,

which is rapidly formed from NA in the liver, was markedly higher at d 7 p.p. in the NA-supplemented cows than in control cows (Zeitz et al., 2018). In addition,

Table 6. Enriched gene ontology (GO) biological process terms¹ associated with downregulated genes in the liver of cows fed rumen-protected niacin (NA) compared with control cows at 1 wk postpartum

GO biological process term	Count	<i>P</i> -value	Fold enrichment
Cellular metabolic process	129	0.0001	1.26
Small molecule metabolic process	36	0.0001	1.98
Metabolic process	134	0.0006	1.20
Lipid catabolic process	11	0.0009	3.65
Organic cyclic compound metabolic process	78	0.0013	1.36
Small molecule biosynthetic process	13	0.0023	2.82
Single-organism biosynthetic process	24	0.0023	1.97
Cellular lipid catabolic process	8	0.0025	4.31
Triglyceride catabolic process	4	0.0028	13.86
Single-organism catabolic process	17	0.0033	2.27
Positive regulation of lipoprotein lipase activity	3	0.0037	31.18
Positive regulation of triglyceride catabolic process	3	0.0037	31.18
Catabolic process	32	0.0038	1.69
Cellular catabolic process	27	0.0047	1.77
Positive regulation of triglyceride lipase activity	3	0.0049	27.28
Monocarboxylic acid metabolic process	13	0.0049	2.56
Neutral lipid catabolic process	4	0.0052	11.19
Acylglycerol catabolic process	4	0.0052	11.19
Plasma lipoprotein particle organization	4	0.0052	11.19
Primary metabolic process	119	0.0058	1.18
Fatty acid metabolic process	10	0.0058	3.03
Lipid transport	9	0.0059	3.31
Protein-lipid complex subunit organization	4	0.0064	10.39
Glycerolipid catabolic process	4	0.0071	10.04
Organic substance catabolic process	30	0.0075	1.65
Organic substance metabolic process	124	0.0076	1.16
Steroid esterification	3	0.0078	21.83
Cholesterol esterification	3	0.0078	21.83
Sterol esterification	3	0.0078	21.83
Regulation of triglyceride catabolic process	3	0.0078	21.83
Single-organism metabolic process	53	0.0083	1.39
Cellular aromatic compound metabolic process	72	0.0094	1.29
Positive regulation of fatty acid biosynthetic process	3	0.0094	19.84
Regulation of lipoprotein lipase activity	3	0.0094	19.84

¹The GO terms are sorted by their enrichment *P*-values [Expression Analysis Systematic Explorer (EASE) score] (top: lowest *P*-value, bottom: highest *P*-value). Only GO terms with *P*-values <0.01 are shown.

Table 7. Enriched gene ontology (GO) cellular component terms¹ associated with upregulated genes in the liver of cows fed rumen-protected niacin (NA) compared with control cows at 1 wk postpartum

GO cellular component term	Count	P-value	Fold enrichment
Blood microparticle	6	0.00014	11.97
Extracellular exosome	29	0.00022	2.03
Extracellular vesicle	29	0.00024	2.02
Extracellular organelle	29	0.00024	2.02
Membrane-bounded vesicle	33	0.00027	1.88
Extracellular space	18	0.00036	2.64
Vesicle	33	0.00056	1.81
Extracellular region	37	0.00105	1.66
Extracellular region part	34	0.00108	1.72
Cell surface	10	0.00754	2.88
Collagen trimer	4	0.00998	8.92

¹The GO terms are sorted by their enrichment *P*-values [Expression Analysis Systematic Explorer (EASE) score] (top: lowest *P*-value, bottom: highest *P*-value). Only GO terms with *P*-values <0.01 are shown.

the plasma nicotinamide concentration at d 7 p.p. in NA-supplemented cows was significantly higher than in most other studies with dairy cows fed either rumen-protected NA (Morey et al., 2011; Yuan et al., 2012) or unprotected NA (Niehoff et al., 2009). The marked elevation of plasma nicotinamide is probably the result of the high daily NA dosage provided to the cows of the NA group, which was markedly higher than in the abovementioned studies (Niehoff et al., 2009; Morey et al., 2011; Yuan et al., 2012). Despite the overall moderate influence on hepatic gene expression, a significant finding of this study was that genes upregulated by rumen-protected NA were almost exclusively involved in immune processes. In fact, the most-enriched biological process terms related to upregulated genes were regulation of leukocyte differentiation, immune system process, immune system development, regulation of immune system process, activation of immune response, and acute inflammatory response. The involvement of

the upregulated genes in immune processes was reflected by the identification of numerous genes dealing with immune processes among the list of upregulated genes such as *HP*, *NNAT*, *S100A12*, *BOLA-DQA5*, *APCS*, *TRGC6*, *CD28*, *NCALD*, *GPX4*, *SRXN1*, *CIAPIN1*, and *TRD*. Of note, among these genes, we identified 2 acute phase proteins (**APP**)—*HP* (the most strongly upregulated gene in the liver of NA-treated dairy cows) and *APCS*—suggesting that rumen-protected NA induces or amplifies (considering that a systemic inflammation-like condition typically occurs in the liver of high-yielding dairy cows in the p.p. period) an acute phase response in the liver of dairy cows in the early p.p. period. This assumption was substantiated by our observation that induction of hepatic *HP* in NA-treated dairy cows could be confirmed by qPCR. Because cows with clinical symptoms of infectious (mastitis, metritis) and noninfectious diseases (milk fever), which are known to induce an acute phase response, were simi-

Table 8. Enriched gene ontology (GO) cellular component terms¹ associated with downregulated genes in the liver of cows fed rumen-protected niacin (NA) compared with control cows at 1 wk postpartum

GO cellular component term	Count	P-value	Fold enrichment
Chylomicron	4	0.0002	31.8
Triglyceride-rich lipoprotein particle	4	0.0007	22.0
Very low density lipoprotein particle	4	0.0007	22.0
RNA polymerase I transcription factor complex	3	0.0011	53.7
Intracellular	173	0.0019	1.1
Nucleoplasm	42	0.0019	1.6
Nuclear lumen	52	0.0041	1.5
Intracellular membrane-bounded organelle	134	0.0053	1.2
Plasma lipoprotein particle	4	0.0054	11.0
Lipoprotein particle	4	0.0054	11.0
Protein-lipid complex	4	0.0067	10.2
Intracellular part	164	0.0081	1.1
Intracellular organelle lumen	55	0.0086	1.4
Organelle lumen	55	0.0087	1.4

¹The GO terms are sorted by their enrichment *P*-values [Expression Analysis Systematic Explorer (EASE) score] (top: lowest *P*-value, bottom: highest *P*-value). Only GO terms with *P*-values <0.01 are shown.

Table 9. Enriched gene ontology (GO) molecular function terms¹ associated with the target mRNAs of the top 10 downregulated microRNAs in the liver of cows fed rumen-protected niacin (NA) compared with control cows at 1 wk postpartum

GO molecular function term	Count	P-value	Fold enrichment
RNA polymerase II transcription factor activity, sequence-specific DNA binding	52	0.000004	1.99
Protein binding	474	0.000057	1.11
Transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding	29	0.000100	2.25
Transcription factor activity, sequence-specific DNA binding	76	0.000105	1.56
Nucleic acid binding transcription factor activity	76	0.000108	1.56
Transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	23	0.000173	2.47
Sequence-specific DNA binding	67	0.000255	1.57
Sequence-specific double-stranded DNA binding	49	0.000318	1.71
Binding	598	0.000367	1.05
Protein kinase regulator activity	18	0.000468	2.65
RNA polymerase II regulatory region DNA binding	42	0.000537	1.76
Macromolecular complex binding	76	0.000604	1.47
Transcription regulatory region sequence-specific DNA binding	46	0.000714	1.68
RNA polymerase II regulatory region sequence-specific DNA binding	41	0.000906	1.73

¹The GO terms are sorted by their enrichment *P*-values [Expression Analysis Systematic Explorer (EASE) score] (top: lowest *P*-value, bottom: highest *P*-value). Only GO terms with *P*-values <0.001 are shown.

larly distributed among the 2 groups, we are confident that the effect of NA supplementation was not biased by the illness history of the cows. However, future studies should investigate whether our observation from transcriptome analysis of an amplification of an acute phase response in dairy cows fed rumen-protected NA is accompanied by increased plasma concentrations of APP. In the present study, we did not consider the plasma concentrations of APP, because APP, like many other blood parameters, are known to exhibit marked time-dependent fluctuations in dairy cows, which might have biased the effect of NA supplementation, and the plasma concentrations of APP respond to an inflammatory or stress signal with a certain time shift. In addition, future studies are needed to clarify whether our observation at the transcriptome level can be attributed to the relatively high daily NA dosage.

Apart from induction of APP-encoding genes, an interesting finding was that several stress-responsive genes were identified as upregulated, such as antioxidant proteins (*SRXN1*, *GPX4*), calcium binding proteins (*S100A12*, *NCALD*) and drug-metabolizing/detoxifying enzymes (*AKR1C1*, *UGT2B4*), genes that are known to be regulated by the stress-responsive transcription factors nuclear factor- κ B (NF- κ B) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2). This observation indicates that rumen-protected NA induces a stress-response in the liver of p.p. dairy cows. A further indication of the initiation of stress response in the liver of cows fed rumen-protected NA is the identification of *ATF3* and *E2F4* as upregulated genes. The *ATF3* protein belongs to the ATF/CREB family of transcription factors (Hai and Hartman, 2001), encoded by an immediate-early gene that is rapidly

induced by a wide spectrum of stress signals (Hai et al., 1999). As a transcription factor, *ATF3* directly or indirectly alters the expression of a variety of genes, and one common characteristic for many of its target genes is their ability to regulate inflammation (Hai et al., 2010). The *E2F4* gene is also a stress-responsive gene (Crosby et al., 2007), encoding a member of the family of E2F transcription factors that play a key role in the G₁/S cell cycle checkpoint and the control of cell proliferation (Bertoli et al., 2013). Interestingly, members of the E2F transcription factors regulate inflammatory signaling by physically interacting with NF- κ B (Ankers et al., 2016). The exact reason for the induction of a stress response in the liver of cows fed rumen-protected NA is unclear. However, a stress response is typically initiated to cope with the presence of stress-inducing stimuli. Important stress-inducing stimuli, which are known to activate redox-sensitive transcription factors such as NF- κ B and Nrf2, are reactive oxygen species (ROS). At least in studies with rats, it was shown that NA supplementation increases hydrogen peroxide levels in plasma (Sun et al., 2012; Li et al., 2013a) and causes a dosage-dependent increase in oxidative DNA damage in the liver (Li et al., 2013b). In addition, a study with Jurkat cells demonstrated that NA stimulates ROS production in vitro (Crowley et al., 2000). In dairy cows, at least indirect evidence for the stimulation of ROS production by NA supplemented during the periparturient period was provided in a recent study showing that the ferric-reducing ability of plasma was decreased, indicating a decreased plasma antioxidative capacity in cows at 2 wk p.p. (Bühler et al., 2018b). Bühler et al. (2018b) attributed the effect to an improved energy status of NA-supplemented cows due to higher

NAD⁺ and NADP⁺ levels, leading to an increased cell respiration and activity of ROS-generating superoxide dismutase. One might speculate that the NA-induced oxidative stress in p.p. dairy cows is responsible for the observation that the number of early-apoptotic polymorphonuclear leukocytes and late-apoptotic peripheral blood mononuclear cells is increased in dairy cows receiving supplemental NA during the periparturient period (Bühler et al., 2016), because oxidative stress is known to stimulate different signaling pathways in immune cells, leading to apoptotic cell death (Wedi et al., 1999). In any case, the findings from Bühler et al. (2016) and those from another study from Bühler et al. (2018a), in which the phagocytotic activity of blood immune cells was investigated, show that NA supplementation in periparturient dairy cows affects immune function in the p.p. period.

With regard to the genes downregulated in cows fed rumen-protected NA, these genes were mainly involved in different metabolic processes, such as small molecule metabolic process, lipid catabolic process, organic cyclic compound metabolic process, small molecule biosynthetic process, and cellular lipid catabolic process. This was reflected by the identification of many genes encoding metabolic enzymes and proteins, such as *CYP2C42*, *RBP5*, *ELOVL2*, *SLC17A3*, *ATP8B3*, *BREH1*, and *CYP2C19*. The induction of genes encoding certain enzymes of the cytochrome P450 family (*CYP2C42*, *CYP2C19*) may indicate that drug metabolism and catabolism of steroid hormones is influenced by NA supplementation in the liver of cows in the early p.p. period. For instance, progesterone is inactivated primarily in the liver of cows by different CYP enzymes, including those of the 2C subfamily (Murray, 1991, 1992). In line with this, it was found that feeding dairy cows a high-cornstarch diet reduces hepatic activities of CYP2C and CYP3A and increases progesterone half-life compared with feeding a high-fiber diet (Lemley et al., 2010). From the literature, no data are available to demonstrate that rumen-protected NA influences circulating levels of progesterone. However, future studies should address this issue because increased hepatic progesterone catabolism decreases circulating levels of progesterone, and insufficient circulating progesterone levels have been implicated as a major cause of poor fertility in lactating dairy cows (Lonergan, 2011). It is also interesting that several genes encoding apolipoproteins, such as apolipoprotein (*APO*)A1, *APOA5*, *APOB*, and *APOC2*, were among the genes downregulated by rumen-protected NA. As important constituents of lipoproteins, apolipoproteins are formed in the liver to be assembled with hepatic lipids, such as TAG and phospholipids, and are secreted as VLDL particles into the circulation (Shelness and

Sellers, 2001). Our observation from transcript profiling therefore suggests that rumen-protected NA affects hepatic lipoprotein assembly and secretion in dairy cows in the early p.p. period. In line with this, bioinformatic analysis of downregulated genes revealed that the most enriched cellular component terms were chylomicron, triglyceride-rich lipoprotein particle, and VLDL particle. Impaired hepatic production of apolipoproteins is in agreement with the above-described indications of induction of an acute phase response in the liver of cows fed rumen-protected NA. It is well known that in the course of the acute phase response, the liver increases the production of so-called positive APP, such as HP, APCS, and many others, which play major roles in restoring tissue homeostasis during the early phase of inflammation, injury, trauma, and infection (Venteclef et al., 2011; Ceciliani et al., 2012). In contrast, the production of several other proteins normally synthesized by the liver at high rates, such as apolipoproteins, RBP, transferrin, and albumin, is reduced during the acute phase response; these proteins are called negative APP. Consequently, the pool of free amino acids can be preferentially utilized for the production of positive APP and de novo synthesis of glucose (Ceciliani et al., 2012). This means that immune function has priority over anabolic function. Despite indications from the hepatic transcriptome signature that rumen-protected NA impairs hepatic lipoprotein assembly and secretion in dairy cows, no evidence for this has been found at the phenotypic level. In fact, in our recently published study, we showed that TAG concentration in the liver did not differ between NA-supplemented and control cows at 1 and 3 wk p.p. (Zeitz et al., 2018). Also, the TAG concentration in plasma of these cows did not differ between NA-supplemented and control cows in the p.p. period (Zeitz et al., 2018). One explanation for this discrepancy between hepatic transcriptome signature and phenotype may be that the effects of rumen-protected NA on hepatic gene expression were only moderate overall. For instance, the genes encoding apolipoproteins (*APOA1*, *APOA5*, *APOB*, *APOC2*) were downregulated in the NA-treated cows by only −1.45- to −1.21-fold. Obviously, this effect is not biologically relevant, at least in this specific case. This clearly indicates that data from transcript profiling may provide valuable information about metabolic and signaling pathways affected by a specific treatment. However, transcriptomic data must be cautiously interpreted and it is important to confirm, by determining suitable phenotypic parameters, whether these changes in gene expression are of biological significance.

A further interesting finding of the present study was that rumen-protected NA causes downregulation of several miRNAs in the liver of dairy cows. MicroRNAs

are small non-protein-coding RNAs that regulate gene expression mainly at the posttranscriptional level by binding to complementary mRNA sequences, leading to degradation of the specific mRNAs or repression of protein translation (Hutvagner and Zamore, 2002). Interestingly, a single miRNA can regulate hundreds of protein-encoding target mRNAs, indicating the great regulatory potential of miRNAs (Bartel, 2004). We recently demonstrated in a study with rats that NA at a pharmacologic dosage alters the expression of miRNAs in skeletal muscle, and *in silico* target gene prediction indicated that more than 1,800 mRNAs are putative targets of NA-regulated miRNAs (Couturier et al., 2014). Moreover, we showed that many of the predicted target mRNAs from the most strongly downregulated miRNAs are involved in transcription regulator activity and regulation of transcription from RNA polymerase, indicating that processes dealing with gene transcription are activated by NA treatment because the mRNAs from downregulated miRNAs are less targeted and, thus, less degraded. Interestingly, the recent rat study also showed that several stress-responsive transcription factors including *ATF3*, *ATF1*, and one member of the E2F family, *E2F7*, are targets of the miRNAs downregulated by NA (Couturier et al., 2014). In the liver of cows fed rumen-protected NA, 831 mRNAs were predicted as targets of the 10 most strongly downregulated miRNAs. As in skeletal muscle of rats, we found, in the liver of cows, that processes dealing with gene transcription (e.g., RNA polymerase II transcription factor activity, sequence-specific DNA binding, transcriptional activator activity) are the most enriched molecular function terms associated with these miRNA-regulated mRNA targets. In addition, as in skeletal muscle of rats, the stress-sensitive transcription factor E2F7 could be identified as a target of the miRNAs downregulated by rumen-protected NA in the liver of cows. Thus, based on these similar findings between rats and dairy cows, we propose that miRNA-mRNA interactions are generally involved in NA-dependent regulation of gene expression and that these interactions may play a specific role in the activation of the stress response in the liver of p.p. dairy cows.

CONCLUSIONS

Hepatic transcriptome analysis showed that rumen-protected NA induces genes that are involved mainly in immune processes, including acute phase response and stress response in dairy cows at d 7 p.p. These findings indicate that supplementation of rumen-protected NA to dairy cows in the periparturient period may induce or amplify the systemic inflammation-like condition that is typically observed in the liver of high-yielding dairy

cows in the p.p. period. Because the daily dosage of NA was markedly higher than in recent studies dealing with rumen-protected NA, future studies have to clarify whether the relatively high NA dosage is responsible for the indications of an inflammation-like condition.

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