Effects of supplementing rumen-protected niacin on fiber composition and metabolism of skeletal muscle in dairy cows during early lactation

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ABSTRACT

Nicotinic acid (NA) has been shown to induce muscle fiber switching toward oxidative type I fibers and a muscle metabolic phenotype that favors fatty acid (FA) utilization in growing rats, pigs, and lambs. The hypothesis of the present study was that supplementation of NA in cows during the periparturient phase also induces muscle fiber switching from type II to type I fibers in skeletal muscle and increases the capacity of the muscle to use free FA, which may help to reduce nonesterified fatty acid (NEFA) flow to the liver, liver triglyceride (TG) accumulation, and ketogenesis. Thirty multiparous Holstein dairy cows were allocated to 2 groups and fed a total mixed ration without (control group) or with ~55 g of rumen-protected NA per cow per day (NA group) from 21 d before expected calving until 3 wk postpartum (p.p.). Blood samples were collected on d -21, -14, -7, 7, 14, 21, 35, and 63 relative to parturition for analysis of TG, NEFA, and β -hydroxybutyrate. Muscle and liver biopsies were collected on d 7 and 21 for gene expression analysis and to determine muscle fiber composition in the musculus semitendinosus, semimembranosus, and longissimus lumborum by immunohistochemistry, and liver TG concentrations. Supplementation of NA did not affect the proportions of type I (oxidative) or the type II: type I ratio in the 3 muscles considered. A slight shift from glycolytic IIx fibers toward oxidative-glycolytic fast-twitch IIa fibers was found in the semitendinosus, and a tendency in the longissimus lumborum, but not in the semimembranosus. The transcript levels of the genes encoding the muscle fiber type isoforms and involved in FA uptake and oxidation, carnitine transport, tricarboxylic acid cycle, oxidative phosphorylation,

and glucose utilization were largely unaffected by NA supplementation in all 3 muscles. Supplementation of NA had no effect on plasma TG and NEFA concentrations, liver TG concentrations, and hepatic expression of genes involved in hepatic FA utilization and lipogenesis. However, it reduced plasma β -hydroxybutyrate concentrations in wk 2 and 3 p.p. by 18 and 26% and reduced hepatic gene expression of fibroblast growth factor 21, a stress hormone involved in the regulation of ketogenesis, by 74 and 56%. In conclusion, a high dosage of rumen-protected NA reduced plasma β -hydroxybutyrate concentrations in cows during early lactation, but failed to cause an alteration in muscle fiber composition and muscle metabolic phenotype.

Key words: niacin, muscle fiber, lactating dairy cow, ketogenesis

INTRODUCTION

During the periparturient phase, high-yielding dairy cows are typically in a strong negative energy balance (NEB) as food intake capacity in this phase is limited and the amount of energy consumed does not meet the high requirement of maintenance and milk production. Negative energy balance leads to mobilization of nonesterified fatty acids (NEFA) from adipose tissue that are released into the blood. The major part of NEFA released from adipose tissue is taken up by the mammary gland and used for the synthesis of milk fat. Approximately 25% of the NEFA passing through hepatic circulation are taken up by the liver (White, 2015). Part of these NEFA is subjected to β -oxidation in the liver. However, as the capacity of β -oxidation in the liver is limited during the periparturient phase, a part of the NEFA is also incorporated into triglycerides (TG), which under physiological conditions are secreted into the blood via very low density lipoproteins (VLDL). However, as the capacity for synthesis and secretion of VLDL in the liver in dairy cows is also limited during this period, TG are accumulating in the liver.

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Consequently, liver TG concentrations increase after parturition, which can impair liver function and result in fatty liver development (Hocquette and Bauchart, 1999; White, 2015). The development of a fatty liver is of high clinical relevance in dairy cows because it not only impairs liver function but also increases the risk of other diseases such as ketosis (Bobe et al., 2004). It has been well established that concentrations of NEFA in plasma are directly linked with the development of fatty liver in dairy cows (Hocquette and Bauchart, 1999). Although NEFA are an important energy source in early lactation, an excessive NEFA supply to the liver will overburden its oxidative capacity, which is frequently found in early-lactating dairy cows (White, 2015). Therefore, nutritional strategies to counteract the development of a fatty liver in dairy cows should consider possibilities to lower plasma NEFA concentrations to a certain degree during the periparturient phase.

Nicotinic acid (NA) is a water-soluble vitamin that is involved in the metabolism of carbohydrates, fats, and proteins. However, NA in high dosages is also known to decrease concentrations of lipids, including TG and low-density lipoprotein, and to increase concentrations of high-density lipoprotein in the plasma of humans (Barter and Rye, 2016; Feingold and Grunfeld, 2017). It is known that NA, when binding to its receptor GPR109A, renamed hydroxy-carboxylic acid receptor 2 (HCAR2), inhibits adipose tissue lipolysis (Wise, 2003; Offermanns, 2006), which reduces the supply of NEFA for hepatic TG synthesis and hepatic VLDL assembly (Wang et al., 2001; Offermanns, 2006). A reduction in plasma NEFA concentrations has been confirmed in feed-restricted dairy cows when NA was infused postparturientally, and the expected NEFA rebound occurred after NA infusion was terminated (Pires and Grummer, 2007). Although oral feeding of non-rumen-protected NA has yielded contradictory data concerning its effects on plasma NEFA concentrations, possibly because too low NA amounts were entering the bloodstream (Niehoff et al., 2009), feeding rumen-protected NA has been shown to reduce plasma NEFA concentrations in early-lactating dairy cows (Yuan et al., 2012). However, NA also has effects that are independent from activation of the HCAR2 receptor, such as, for example, its effect on plasma lipids (Feingold and Grunfeld, 2017). Therefore, the classic view that NA influences hepatic lipid metabolism mainly due to an inhibition of adipose tissue lipolysis has been put into question (Choi et al., 2011).

We have recently observed that high dosages of NA, resembling those required to achieve lipid lowering effects in humans, caused a shift from the glycolytic,

fast-twitch type II fibers to oxidative slow-twitch type I fibers in various skeletal muscles of growing rats, pigs, and sheep (Khan et al., 2013a,b; Ringseis et al., 2013). In line with the fact that type I fibers are rich in mitochondria, exhibit high activities of oxidative enzymes, and mainly use fatty acids for energy production, the animals treated with NA showed an increased expression of genes involved in fatty acid uptake, mitochondrial fatty acid import, and oxidation and oxidative phosphorylation in skeletal muscles (Khan et al., 2013a,b; Ringseis et al., 2013). In parallel with the increased proportion of oxidative type I fibers, NA also induced an upregulation peroxisome proliferator-activated receptor δ (PPAR δ) and PPAR- γ coactivator-1- α (PPARGC1A), transcription factors that are key regulators of muscle fiber composition, mitochondrial biogenesis, fatty acid oxidation, oxidative phosphorylation, and angiogenesis (Wang et al., 2004; Schuler et al., 2006). This finding suggests that muscle fiber transition from type II to type I in animals treated with NA was caused by an induction of genes encoding critical regulators of muscle fiber composition, such as PPAR δ and PPARGC1A. In obese rats, NA treatment caused also a strong reduction of plasma NEFA concentration, associated with lower concentrations of TG in plasma and liver (Ringseis et al., 2013). It is likely that reduced plasma NEFA concentrations in the rats treated with NA were, at least in part, due to increased utilization of fatty acids in skeletal muscle, which significantly contributes to whole-body fatty acid utilization.

In the present study, we investigated the hypothesis that treatment of periparturient cows with high dosages of NA causes a muscle fiber switch in skeletal muscle similar to that observed in rats, pigs, and sheep (i.e., an increase of oxidative type I fibers at the expense of glycolytic type II fibers). Such an effect might be favorable in dairy cows as it is expected that an increase in type I fibers might enhance the uptake of NEFA released from adipose tissue into the skeletal muscle and the utilization of NEFA by β -oxidation. An increased uptake of NEFA into the skeletal muscle might lead to a reduction of plasma NEFA concentration and in turn to a reduced uptake of NEFA into the liver causing a decrease in TG accumulation and ketogenesis.

MATERIALS AND METHODS

The study was carried out at the Educational and Research Centre for Animal Husbandry Hofgut Neumühle (Münchweiler an der Alsenz, Germany). All experimental procedures were approved by the Provincial Government of Coblenz, Germany (23 177-07/G 14-20-050).

Animals and Experimental Design

A total of 30 multiparous Holstein dairy cows kept in a freestall barn equipped with weighing troughs were randomly allocated to 2 groups. One group was fed a TMR (control group). The second group (**NA** group) was fed the same diet and received Niashure (Balchem, New Hampton, NY) at 79 mg/kg of BW (i.e., 55 g of Niashure per 700-kg cow per d), which is equivalent to 47 mg of NA per kg of BW at the duodenum considering an amount of 65% of NA in Niashure (information from the supplier) and a ruminal stability of 92% (own analysis, see below). The selected dosage was comparable to the NA dosage used in earlier studies with rats and sheep where NA supplemented at approximately 30 to 50 mg/kg of BW induced muscle fiber switching in obese Zucker rats (Ringseis et al., 2013), sheep (Khan et al., 2013a), and growing pigs (Khan et al., 2013b). The Niashure dosage was adapted to the cow BW once per week beginning 21 d before expected calving date and until 21 d postpartum (**p.p.**). The Niashure was mixed into 500 g of pressed sugar beet pulp and fed once daily at 0800 h in feed fences to ensure individual and complete intake; cows of the control group received 500 g of pressed sugar beet pulp only. On average, at the start of the trial, the pregnant cows of the control and NA group had a BW of 771 ± 19 and 768 ± 29 kg (mean $\pm$ SEM; $P = 0.94$), a BCS of 3.4 ± 0.06 and 3.3 ± 0.06 ($P = 0.13$), a feed intake of 14.2 ± 0.6 and 13.9 ± 0.5 kg of DM/d ($P = 0.67$), energy balance of 34.2 ± 3.8 and 35.4 ± 3.4 MJ of NE_L/d ($P = 0.82$), and lactation number of 3.2 ± 0.34 and 2.9 ± 0.29 ($P = 0.42$). The cows had free access to feed and water, and feed was provided twice daily at 7 and 14 h. Antepartum and postpartum diets (Table 1) were formulated to meet requirements (GfE, 2001).

Data and Sample Collection

Feed ingredient and TMR samples were collected every month, and silage DM was determined weekly and adjusted in ration formulations. The DMI was recorded with automatic weighing troughs (Roughage Intake Control System, Marknesse, the Netherlands) except during the first 5 to 7 d after calving where cows were kept in small groups separately from the herd. The BW of cows was recorded once per week antepartum (**a.p.**), and twice daily p.p., with an automatic scale (GEA Farm Technologies GmbH, Bönen, Germany). Cows were milked twice daily at 0430 and 1530 h. A composite sample of morning and evening milk was sampled for the first time at 7.2 ± 0.9 d p.p. and afterward once per week, and the bronopol (2-bro-

mo-2-nitropropane-1,3-diol) preservative-treated milk samples were analyzed for milk fat, protein, and lactose by infrared spectrophotometry using a MilkoScan FT+ (Foss Analytical A/S, Hillerød, Denmark) at the laboratory of Landeskontrollverband Rheinland-Pfalz (Bad-Kreuznach, Germany). For milk protein, the reference method for MilkoScan calibration was CP determination by the Kjeldahl method. Approximately 9 mL of blood was collected at 21, 14, and 7 d before expected calving and at wk 1, 2, 3, 5, and 9 p.p. between 0700 and 0800 h (i.e., after milking and before feeding) from the tail vein (vena caudalis mediana) into containers containing EDTA (S-Monovette 9 mL K3E, Sarstedt, Nümbrecht, Germany), kept in ice water for maximum 1 h, centrifuged at $2,000 \times g$ for 10 min at 4°C, and the plasma was harvested and stored at -20°C until analysis. Liver samples were collected 1 and 3 wk p.p. at 0900 h between the 9th and 10th ribs, approximately

Table 1. Ingredient and nutrient composition of the TMR used as experimental diets during the dry period and lactation (% of DM)

Item	Antepartum	Postpartum (1–63 DIM)
Ingredient, % of DM		
Corn silage	34.7	19.3
Grass silage	31.3	18.7
Pressed sugar-beet pulp silage	—	12.5
Grass hay	6.9	7.5
Straw	6.3	3.0
Barley	5.4	8.9
Grain maize	4.7	12.1
Soybean meal	2.8	6.9
Rapeseed meal	5.8	8.9
Cattle salt	0.25	0.43
Urea	0.32	0.25
Limestone	0.24	0.31
Mineral feed ^{1,2}	0.50	0.43
Mineral feed (dry cows) ²	0.80	—
Feed fat	—	0.9
Nutrient, % of DM		
DM	35.6	44.8
Crude ash	6.7	5.9
CP	13.5	16.6
Crude fat	2.9	3.0
Crude fiber	18.3	15.2
Ash-free NDF	40.8	34.9
Ash-free ADF	23.3	19.2
NE _L , MJ	6.4	7.0

¹The mineral feed (Rindamin K11, Schaumann, Pinneberg, Germany) supplied per kilogram of postpartum diet: Ca, P, Na, 0.39 g; Mg, 0.43 g; Zn, 43 mg; Mn, 26 mg; Cu, 6.5 mg; Co, 0.26 mg; J, 0.87 mg; Se, 0.23 mg; vitamin A, 4,343 IU; vitamin D₃, 652 IU; vitamin E, 26 mg.

²The mineral feeds in the antepartum diet, including Rindamin K11 and the mineral feed for dry cows (Rindavital VK C Plus ATG, Schaumann), supplied per kilogram of diet: Ca, P, Na, 0.45 g; Mg, 1.3 g; vitamin A, 12,970 IU; vitamin E, 110 mg; vitamin D₃, 1,228 IU; L-carnitine, 96 mg; biotin, 0.80 mg; Zn, 90 mg; Mn, 46 mg; Cu, 15.5 mg; Co, 0.62 mg; J, 1.80 mg; Se, 0.48 mg; clinoptilolite, 0.8 g; citric acid, 10.0 mg; butylated hydroxytoluene, 6.77 mg; propyl gallate, 2.39 mg; ethoxyquin, 0.96 mg.

15 cm below the transverse processes using a self-made barbed 12-gauge $\times$ 27 cm biopsy needle. The area was shaved, aseptically prepared with 70% ethanol, and the injection site and the peritoneum were anesthetized with 5 mL of Procainidol (20 mg/mL; WDT, Garbsen, Germany). After 5 min, an incision (1 cm) was made with a scalpel and the biopsy needle was inserted cranioventrally toward the liver, and approximately 0.5 to 1 g of tissue was collected. The incision site was treated topically with oxytetracyclin spray (Engemycin, MSD Animal Health GmbH, Unterschleissheim, Germany). Muscle biopsies were taken at 1 and 3 wk p.p., alternating on the left and right body side, from the longissimus lumborum (**LD**), semitendinosus (**ST**), and semimembranosus (**SM**). The muscles were identified by palpation and the biopsy site was shaved, aseptically prepared with 70% ethanol, and anesthetized with 5 mL of Procainidol. After 5 min, an incision (0.5 cm) was made through the skin with a scalpel, and the co-axial cannula (HCC12130, 11 G, 93 mm; BIP Biomed Instrumente und Produkte GmbH, Türkenfeld, Germany) was inserted cranioventrally until about 5 cm depth and approximately 0.5 g of tissue was collected. Liver and muscle tissues were snap-frozen in liquid N₂ and stored at -80°C until analysis. The cows received the anti-inflammatory drug Rifen (3 mL/100 kg of BW; Vetoquinol GmbH, Ravensburg, Germany) for 3 d after biopsy collection.

The BCS was determined every 2 wk by 2 trained investigators on a 1 to 5 scale according to Wildman et al. (1982) and Edmonson et al. (1989).

Determination of Rumen Stability of Niashure

The rumen degradability of Niashure was determined with 8 dry Holstein-Friesian cows fed 6 kg of DM of a diet consisting of grass silage (45%), maize silage (25%), hay (10%), and concentrate (20%), and equipped with ruminal fistulas. The in sacco technique according to Ørskov and McDonald (1979) was used as described in Metwally et al. (2015) and 5 g of Niashure were weighed into the 10 $\times$ 20 cm nylon bags with a pore size of 53 μm , and 2 bags per cow and incubation time were incubated for 2, 4, 6, 9, 12, 24, 48, 72, and 96 h. The NA concentrations in Niashure and in the bag residues was determined by titration with NaOH (AOAC method no. 944.13; AOAC International, 2006). The NA losses after incubation were calculated as the difference between initial weight and residual weight after incubation and the potential ruminal degradability of Niashure as well as the effective ruminal degradability at assumed passage rates of 4, 6, and 8% per h was calculated.

Laboratory Analyses

The feed samples were analyzed by infrared spectrophotometry at the Agricultural Investigation and Research Institute (LUFA; Speyer, Germany) for DM, crude ash, CP, crude fat, ash-free NDF, ash-free ADF, ME, and NE_L.

In plasma of cows, NA and its 2 main metabolites nicotinamide (**NAM**) and nicotinuric acid (**NUA**) were determined by HPLC-MS/MS according to Liu et al. (2012) with modifications as described by Scholz et al. (2014), and nicotinamide-D4 (Cambridge Isotope Laboratories, Tewksbury, MA) was used as internal standard. For NAM, the coefficient of variation was 5.5% for one sample measured on 5 successive days and 3.6% when measured on 1 d. The recovery rate was 92.4 to 97.1%. The limit of detection and the limit of quantification were 0.025 and 0.05 $\mu\text{g/L}$, respectively, for all metabolites.

Plasma concentrations of TG, NEFA, and BHB were determined with the Fluitest TG Kit (Analyticon, Lichtenfels, Germany), the NEFA-HR(2) Kit (Wako, Richmond, VA) and the Autokit 3-HB (Wako). Liver TG were determined with the Fluitest TG Kit as well in liver extracts which were prepared as follows. About 50 mg of liver tissue was homogenized with 0.5 mL of hexane:isopropanol (3:2, vol/vol) at 30 Hz for 3 min with a TissueLyzer (Qiagen, Hilden, Germany) and extracted for 30 min in an ultrasonic bath. After centrifugation (1,000 $\times g$, 15°C , 10 min), the upper fat-containing phase was evaporated to dryness under N₂ at 37°C and resuspended in isopropanol.

Total RNA was isolated from approximately 18 mg of liver and 27 mg of muscle tissue using Trizol reagent (Invitrogen) according to the manufacturer's protocol, and RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively, using an Infinite 200 M microplate reader and a NanoQuant Plate (both from Tecan, Männedorf, Switzerland) and RNA was stored at -80°C . Exemplarily, RNA quality in each 2 liver and muscle RNA samples was determined with an Agilent 2100 Bioanalyzer and the Agilent RNA 6000 Nano Kit (Agilent, Santa Clara, CA) and RNA integrity number values were 7.1 and 7.2 for liver RNA and 7.7 and 7.2 for muscle RNA. The cDNA was synthesized from 1.2 μg of total RNA using oligo(dT) primers according to Zeitz et al. (2016). The cDNA was diluted 1:2 with DNase/RNase free water and stored at -20°C . The quantitative PCR was carried out on a Rotorgene 2000 system (Corbett Research, Mortlake, Australia) using 2 μL of cDNA combined with 8 μL of a mixture composed of 5 μL of KAPA SYBR FAST qPCR Universal Mastermix (Peqlab, Erlangen, Germa-

ny), 0.2 μ L each of 10 μ M forward and reverse primers, and 3.6 μ L of DNase/RNase-free water in 0.1-mL tubes (Ltf Labortechnik, Wasserburg, Germany; Zeitz et al., 2016). Gene-specific primer pairs were from Schlegel et al. (2012a) or were designed using PRIMER3 and BLAST and obtained from Eurofins MWG Operon (Ebersberg, Germany; Supplemental Table S1; <https://doi.org/10.3168/jds.2018-14490>). The amplification of a single product of the expected size was approved using 2% agarose gel electrophoresis stained with GelRed nucleic acid gel stain (Biotium Inc., Hayward, CA). The Ct values of target and reference genes were obtained using Rotorgene Software 5.0 (Corbett Research). All cycle threshold (Ct) values were transformed into relative quantification data using the $2^{-\Delta C_t}$ equation (Livak and Schmittgen, 2001). The highest relative quantities for each gene were set to 1. These expression values of target genes were normalized using the GeNorm normalization factor calculated as the geometric mean of expression data (relative quantities) of the 3 to 4 most stable of the tested reference genes (Vandesompele et al., 2002). The M values were 0.38 (liver) and <1.04 (muscle; target <1.5), and the V values were <0.15 (liver, LD) or 0.16 (ST) and 0.17 (SM; target: <0.15; Vandesompele et al., 2002). The normalized expression values data set was corrected for outliers.

For immunohistochemistry, frozen muscle tissue of LD, SM, and ST was embedded in TissueTek OCT Compound (Hartenstain, Würzburg, Germany) and frozen in liquid N₂, and stored at -20°C until transverse sections of 20 μ m thickness were cut on a cryostat microtome at -18°C (Microme HM 500, Microm International GmbH, Walldorf, Germany). Immunofluorescence analysis of myosin heavy chain (MHC) expression was performed according to Bloemberg and Quadrilatero (2012) with primary antibodies BA-F8 (1:50) specific against bovine MYHC β /slow (MHCI) and BF-35 (1:100) specific for both MHCI and MHCIIa as determined by Duris et al. (2000). Primary antibodies were purchased from the Developmental Studies Hybridoma Bank (University of Iowa), whereas the secondary antibodies Alexa Fluor 350 goat anti-mouse IgG2b (blue) and Alexa Fluor 488 goat anti-mouse IgG1 (green) were purchased from Thermo Fisher Scientific (Waltham, MA). The muscle fibers stained blue by BA-F8 were counted as slow-twitch MHCI fibers, those stained green by MF-35, but not by BA-F8 as the intermediate MHCIIa fibers, and the nonstained muscle fibers were counted as the fast-twitch MHC IIx fibers, the third MHC isoform expressed in muscles of adult cattle (Duris et al., 2000; Toniolo et al., 2005) on the microscope DM 5500 B with camera DFC 340 FX

(Leica, Wetzlar, Germany) with fluorescent light (excitation 320–400 nm and emission 430–510 nm for blue filter, excitation 440–520 nm, and emission 497–557 nm for green filter). The photographs taken with the different filters were combined into one image with Microsoft Image Composite Editor (Microsoft Corp., Redmond, WA), and a mean of 450 but minimum of 100 muscle fibers per muscle and animal were counted and assigned to a fiber type with the software Fiji (Schindelin et al., 2012).

Concentrations of free carnitine and acetyl carnitine in liver were determined with tandem mass spectrometry and deuterated carnitine-d3 (Cambridge Isotope Laboratories, Andover, MA) as an internal standard according to Hirche et al. (2009). For that 100 mg of liver tissue was lyophilized (Christ Gamma 1–20 LMC-2; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and homogenized with 0.5 mL of methanol/water (1:2) at 30 Hz for 4 min, incubated for 20 min in an ultrasonic bath, further incubated at 50°C and 650 U/min for 30 min (ThermoShaker TS1, Biometra, Göttingen, Germany), centrifuged (10°C , 10 min, $21,000 \times g$), and 40 μ L of the supernatant was combined and mixed with methanol/internal standard (200 μ L), incubated at 4°C for 10 min, and centrifuged (15°C , 10 min, $21,000 \times g$), and the supernatant was used for analysis.

Statistical Analysis

Data were statistically analyzed using mixed models with repeated measures over time using the software R version 3.1.1 (R Development Core Team, 2011, Vienna, Austria) and the packages nlme, lme4, lmerTest, and lsmeans. A mixed linear model (function lmer) with unstructured variance-covariance structure was used for accounting for the repeated measures and unbalanced group sizes for the treatments. The following model was applied for the analysis:

$$Y \sim \text{treatment} + \text{week} + \text{treatment:week} + \text{animal},$$

where treatment and week and their interaction were treated as factors with fixed effects, and animal was treated as a factor with random effect to account for the dependency of the repeated measures over time. For time series data, data from wk 1 and 3 or 1 to 3 p.p. were included in the analysis and week was treated as the repeated measure. For analysis of data sets with only one time point, treatment was considered as fixed and animal as random effect and the following model was applied for the analysis:

$$Y \sim \text{treatment} + \text{animal},$$

using the function `lme`, package `nlme`. Pair-wise comparisons were done using the Hochberg test and the function `lsmeans`. Separate standard errors of the mean for each mean are presented because homogeneity of variances was not assumed in the linear model, and sample sizes for the 2 treatments were unbalanced because 1 cow from the niacin treatment became ill and was excluded from the trial.

RESULTS

Rumen Stability of Niashure

The ruminal stability of Niashure was 89.5 ± 0.89 , 91.9 ± 0.64 , and $93.3 \pm 0.52\%$ (mean $\pm$ SE; $n = 8$) at assumed feed passage rates of 4, 6, and 8% per h, respectively.

Animal Performance

Feed intake, milk and milk protein yield, milk urea concentration, and the extent of the NEB were similar in cows treated with NA and control cows (Table 2). Milk fat percentage and milk fat yield in wk 1 to 3 of lactation were lower in cows supplemented with NA than in control cows (Table 2). Correspondingly, milk energy content, and the fat:protein ratio showed a tendency toward decreased values in cows fed NA compared with control cows (Table 2). The BW and BCS were similar in both groups of cows p.p. (Table 2). In addition, feed intake 1 wk a.p. was similar in the control (11.8 ± 0.61 kg of DM) and NA groups (11.6 ± 0.46 kg of DM; $P = 0.85$).

Plasma and Liver Metabolic Parameters

Cows treated with NA had an approximately 4-fold higher concentration of NAM, the main metabolite of NA, in plasma in wk 1 and 3 of lactation compared with control cows (Figure 1), and NA and NUA were below the detection limit. However, NA supplementation neither affected plasma TG and NEFA concentrations nor liver TG concentrations in wk 1 and 3 p.p. (Figures 1 and 2). Liver mRNA concentrations of *HCAR2* were similar in both groups in wk 1 and 3 p.p. as well [data not shown; P (treatment) = 0.74; P (treatment $\times$ week) = 0.80]. Plasma carnitine concentrations in wk 3 a.p. to 9 p.p. also remained unaffected by NA supplementation (2.49 ± 0.17 and 2.56 ± 0.14 $\mu\text{mol/L}$ in control and NA group, respectively, $P = 0.76$). Likewise, liver carnitine concentrations in wk 1 and 3 p.p. were similar in cows of the control group (35.9 ± 3.30) and the NA group (29.4 ± 1.67 ; $P = 0.07$). Plasma BHB concentrations, however, were affected by NA treatment in wk 1 to 3 p.p. (Figure 2). The concentrations of BHB were lower by 18 and 26% at 2 and 3 wk p.p. in cows supplemented with NA than in control cows (Figure 2). After cessation of niacin treatment (i.e., after wk 3 p.p.), plasma TG, NEFA, and BHB concentrations were similar in both groups (Figure 2).

Muscle Fiber Switching

The percentages of type I fibers and the ratio of type II to type I fibers in the LD, ST, and SM muscles in wk 1 and 3 p.p. were not influenced by NA treatment (Table 3, Figure 3). In the SM muscle, the percentage of IIa and IIX fibers (Table 3) and the ratio of IIX to IIa fibers (data not shown) also remained unaffected by

Table 2. Performance of dairy cows fed either a control diet (control) or a diet supplemented with rumen-protected niacin (niacin)¹

Item	1–3 wk p.p.		P-value		
	Control	Niacin	Treatment	Week	Treatment $\times$ week
BW, kg	690 $\pm$ 18	694 $\pm$ 24	0.80	0.73	0.55
BCS	3.17 $\pm$ 0.28	3.09 $\pm$ 0.22	0.32	0.044	0.40
DMI, kg	16.0 $\pm$ 0.8	15.6 $\pm$ 0.6	0.66	<0.001	0.28
Energy balance, MJ of NE _L /d	−72.8 $\pm$ 5.9	−61.5 $\pm$ 8.9	0.21	<0.001	0.72
Milk yield, kg	38.6 $\pm$ 1.6	36.2 $\pm$ 1.9	0.25	<0.001	0.06
Milk fat, %	5.45 $\pm$ 0.29	4.98 $\pm$ 0.27	0.04	<0.001	0.52
Milk protein, %	3.44 $\pm$ 0.14	3.45 $\pm$ 0.12	1.00	<0.001	0.47
Milk fat, kg/d	2.06 $\pm$ 0.13	1.76 $\pm$ 0.13	0.04	0.03	0.36
Milk protein, kg/d	1.29 $\pm$ 0.07	1.22 $\pm$ 0.07	0.28	<0.001	0.03
Milk fat:protein ratio	1.61 $\pm$ 0.09	1.45 $\pm$ 0.08	0.06	0.14	0.58
Milk urea, mg/L	188 $\pm$ 9	177 $\pm$ 8	0.18	0.01	0.50
Milk energy, MJ/d	148 $\pm$ 7	130 $\pm$ 8	0.05	0.08	0.24

¹Mean $\pm$ SEM are displayed ($n = 15$ and 14 animals in the control and niacin group). For BW, DMI, and energy balance, data from wk 1 postpartum (p.p.) were missing, and in these cases, data from wk 2 to 3 p.p. are shown.

NA treatment. However, the percentage of oxidative-glycolytic type IIa fibers was higher and those of glycolytic type IIx fibers as well as the ratio of type IIx to type IIa fibers was lower in the ST muscle of cows supplemented with NA than in control cows (Table 3, Figure 3). In the LD muscle, there were tendencies toward an increase of IIa fibers ($P = 0.05$) and a reduction of IIx fibers ($P = 0.10$; Table 3, Figure 3).

In line with the data on muscle fiber typing, relative mRNA concentrations of the MHC isoform *MYH7*, expressed in type I fibers, in wk 1 and 3 p.p. were unaffected by NA treatment in the LD and SM muscles; however, the relative mRNA concentrations of *MYH7* were higher in the ST muscle in the NA group compared with the control group (Table 4). In addition, relative mRNA concentrations of the 2 fast MHC isoforms *MYH1* and *MYH2* remained unaffected by NA treatment in the SM and ST muscles (Table 4). In the LD muscle, relative mRNA concentration of the isoform *MYH1*, expressed in glycolytic type IIx fibers,

was lower in the NA group compared with the control group (Table 4).

Expression of Key Regulators of Muscle Fiber Transition and Genes Involved in Energy Production in Skeletal Muscle

Relative muscle mRNA concentrations of the *HCAR2* in wk 1 and 3 p.p. were similar in both groups ($P > 0.10$; data not shown). Likewise, relative mRNA concentration of *PPARD*, one of the key regulators of muscle fiber transition, in wk 1 and 3 p.p. was not different between cows supplemented with NA and control cows in all 3 muscles considered (Figure 4A, 4B, 4C). Relative mRNA concentration of *PPARGC1A*, another important regulator of muscle fiber transition, in LD and SM muscles also was not different between the 2 groups of cows (Figure 4A, 4C). In contrast, in ST muscle, relative mRNA concentration of *PPARGC1A* in wk 1 and 3 p.p. was higher in cows treated with NA

Figure 1. Concentrations of nicotinamide (NAM) in the plasma (A) and triglycerides (TG) in the liver (B) of dairy cows fed either a control diet (control) or a diet supplemented with rumen-protected niacin (niacin; n = 15 and 14 animals in the control and niacin group; mean $\pm$ SEM). *Denotes a difference ($P < 0.05$) between the control and niacin group at the respective time point. T = treatment; W = week; T $\times$ W = interaction of treatment and week.

Figure 2. Concentrations of triglycerides (TG; A), nonesterified fatty acids (NEFA; B), and BHB (C) in the plasma of dairy cows fed either a control diet (control) or a diet supplemented with rumen-protected niacin (niacin; $n = 15$ and 14 animals in control and niacin group; mean $\pm$ SEM). The x-axis shows wk 3, 2, and 1 antepartum (-3 to -1) and wk 1, 2, 3, 5, and 9 postpartum (p.p.; 1 to 9). T = treatment; W = week; T $\times$ W = interaction of treatment and week. The statistical analysis included wk 1 to 3 p.p. (1–3) to investigate effects of niacin in the challenging period of early lactation during niacin treatment, and wk 5 to 9 p.p. (5–9) to investigate if effects of niacin persist after cessation of treatment after wk 3 p.p.

than in control cows (Figure 4B). In ST muscle, relative mRNA concentrations of 8 out of 9 genes involved in oxidative energy production including genes involved in FA transport into cell and mitochondrion (*SLC27A1/FATP1*, *CPT1B*, *SLC25A20/CACT*), in mitochondrial FA oxidation (*ACADL*), in the transport of carnitine into the cell (*SLC22A4/OCTN1*, *SLC22A5/OCTN2*) as well as tricarboxylic acid cycle and oxidative phosphorylation (*SDHA*, *COX6A2*) were not different between the NA group and the control group (Table 5). Among genes involved in oxidative energy production, *COX4I1* was the only gene that was upregulated in cows treated with NA compared with control cows (Table 5). Relative mRNA concentrations of genes involved in glucose utilization (*SLC2A4/GLUT4*, *PFKM*) in ST muscle were also similar in both groups of cows (Table 5). In LD and SM muscle, relative mRNA concentrations of all the genes involved in fatty acid uptake and oxidation, carnitine transport, tricarboxylic acid cycle and oxidative phosphorylation, and glucose utilization were not different between cows supplemented with NA and control cows (data not shown).

Expression of Genes Involved in Fatty Acid Utilization, Lipogenesis, Ketogenesis, and the Unfolded Protein Response in the Liver

To assess whether NA treatment had an influence on hepatic lipid metabolism and ketogenesis, we determined mRNA concentrations of several genes involved in fatty acid utilization, lipogenesis, and ketogenesis in liver samples of wk 1 and 3 p.p. Relative mRNA concentrations of *PPARA*, the key regulator of β -oxidation and ketogenesis, and genes involved in fatty acid uptake (*CD36*), fatty acid oxidation (*CPT1A*, *SLC25A20/CACT*, *ACADM*, *ACADL*), carnitine uptake into the cell (*SLC22A5/OCTN2*), and classical genes involved in ketogenesis (*ACAT1*, *HMGCS2*) were not different between cows supplemented with NA and control cows (Table 6). Relative mRNA concentrations of *SREBF1*, the key regulator of lipogenesis, as well as genes involved in fatty acid and TG synthesis (*FASN*, *GPAM*, *DGAT2*) were also not different between the 2 groups of cows (Table 6). However, relative mRNA concentration of fibroblast growth factor 21 (*FGF21*), a regulator of ketogenesis, was reduced in the liver of cows supplemented with NA (Table 6). As *FGF21* is a target gene of the unfolded protein response (UPR), which is triggered by endoplasmic reticulum (ER) stress, we determined several genes of the UPR to find out whether NA affected that pathway. However, mRNA concentrations of various genes of the UPR were not influenced by treatment with NA (Table 6).

Table 3. Muscle fiber type composition in longissimus lumborum, semitendinosus, and semimembranosus of dairy cows fed either a control diet (control) or a diet supplemented with rumen-protected niacin (niacin)¹

Item	1 wk postpartum		3 wk postpartum		<i>P</i> -value		
	Control	Niacin	Control	Niacin	Treatment	Week	Treatment × week
Longissimus lumborum							
Type I, %	32.9 ± 2.06	33.3 ± 1.64	30.6 ± 1.34	31.2 ± 0.97	0.75	0.15	0.97
Type IIa, %	33.0 ± 2.45	34.6 ± 1.38	30.2 ± 1.97	37.1 ± 2.10	0.05	0.61	0.076
Type IIx, %	34.1 ± 3.96	32.1 ± 2.02	39.2 ± 1.99	31.7 ± 2.32	0.10	0.21	0.20
Type II, %	67.1 ± 2.06	66.7 ± 1.64	69.4 ± 1.34	68.8 ± 0.97	0.75	0.15	0.97
Semitendinosus							
Type I, %	21.6 ± 1.68	22.5 ± 0.74	20.9 ± 2.24	21.1 ± 1.60	0.67	0.45	0.81
Type IIa, %	30.3 ± 1.00	36.9 ± 2.93	32.6 ± 2.52	36.5 ± 2.18	0.023	0.68	0.55
Type IIx, %	48.1 ± 1.61	40.6 ± 2.67	46.5 ± 3.71	42.4 ± 2.55	0.028	0.96	0.51
Type II, %	78.4 ± 1.68	77.5 ± 0.74	79.1 ± 2.24	78.9 ± 1.60	0.67	0.45	0.81
Semimembranosus							
Type I, %	28.7 ± 1.63	26.7 ± 1.22	25.8 ± 2.46	28.4 ± 2.17	0.90	0.79	0.21
Type IIa, %	35.2 ± 1.60	35.0 ± 1.43	38.3 ± 2.74	34.4 ± 1.32	0.55	0.29	0.40
Type IIx, %	36.0 ± 1.85	38.3 ± 2.10	35.9 ± 1.78	37.2 ± 2.25	0.69	0.47	0.63
Type II, %	71.3 ± 1.63	73.3 ± 1.22	74.2 ± 2.46	71.6 ± 2.17	0.90	0.79	0.21

¹Mean ± SEM are displayed (n = 10 animals per group).

DISCUSSION

In the present study, we investigated the hypothesis that supplementation of periparturient dairy cows with high dosages of rumen-protected NA induces muscle fiber type switching toward an increase of the oxidative type I fibers in skeletal muscle. The dosage of NA per kg of BW used in this study was comparable with the NA dosages used in recent studies with growing rats, sheep, and pigs in which NA treatment caused a muscle fiber transition (Khan et al., 2013a,b; Ringsseis et al., 2013). Although the intestinal availability of NiaShure has not been reported in the scientific literature and absorbed amounts of NA are unknown, we have confirmed that plasma NAM concentrations rose several-fold in NA-treated cows compared with control cows. Notably, NA dosages used in this study (≥ 55 g/d of NiaShure and ≥ 36 g/d of NA) as well as plasma NAM concentrations achieved in the cows by NA treatment (2–3 μ g of NAM/mL of plasma) were higher than in most other studies performed with dairy cows with rumen-protected NA (24 g/d of NiaShure or 16 g/d of NA, 0.9–1.3 μ g of NAM/mL of plasma in Morey et al., 2011; 12 g/d of NiaShure or 7.8 g/d of NA in Yuan et al., 2012) and non-rumen-protected NA (typically 6–12 g/d of NA and 0.6–1.9 μ g of NAM/mL of plasma in NA-treated cows; reviewed by Niehoff et al., 2009). Because plasma NAM concentrations are elevated during the whole day after a single dose of NiaShure but peak plasma NAM concentrations occur 8 to 10 h after ingestion (Tienken et al., 2015), the concentrations found in the plasma samples collected 23 h after NA supplementation in the present study are

expected to represent only about two-thirds of the peak NAM concentrations. Also, NA and NUA in plasma probably could not be detected because, during the first hours after ingestion, NA is degraded to NAM, and after saturation of the amidation pathway also to NUA, which is itself rapidly degraded (Inamadugu et al., 2010).

The observation that milk yield remained unaffected while milk fat yield was reduced by NA treatment agrees with findings of Morey et al. (2011) and Yuan et al. (2012) in cows supplemented with NA. In addition, milk yield was unaffected in the majority of studies investigating supplementation of non-rumen-protected NA and milk fat yield was unaffected in most of these studies as well (Niehoff et al., 2009). Generally, dietary NA may exert its effects on milk production either by affecting microbial growth and feed degradation in the rumen or by effects on metabolism (Schwab et al., 2005). An effect of NA on microbial growth is unlikely in the present study considering the low ruminal degradability of NiaShure and thus an estimated ruminal NA supply of only 3 to 4 g/d. Low dosages of 6 g of non-rumen-protected NA/d also have not shown effects on production and plasma parameters in a meta-analysis (Schwab et al., 2005). In addition, even 12 g/d of NA had only minimal effects on ruminal fermentation and feed degradation (Schwab et al., 2005). It has been assumed that NA reduces milk fat output due to a decrease in adipose tissue lipolysis and a subsequent decrease in plasma NEFA concentrations (Yuan et al., 2012). Plasma NA concentrations after oral supplementation of 6 to 12 g/d of non-rumen-protected NA were probably not sufficient to activate the HCAR2 receptor and

Figure 3. Ratio of type II:I and type IIx:IIa muscle fibers in the longissimus lumborum (A), semitendinosus (B), and semimembranosus (C) of dairy cows fed either a control diet (control) or a diet supplemented with rumen-protected niacin (niacin; $n = 10$ animals per group; mean $\pm$ SEM) at 1 and 3 wk postpartum (p.p.) *Denotes a difference ($P < 0.05$) between the control and niacin group at the respective time point. T = treatment; W = week; T $\times$ W = interaction of treatment and week. Representative images of cross-sections of the longissimus lumborum (D) at 1 wk p.p., the semitendinosus (E) at 3 wk p.p., and the semimembranosus (F) at 1 wk p.p. incubated with primary antibodies against myosin heavy chain (MHC) I (BA-F8) and MHCIIa (MF-35) followed by incubation with appropriate fluorescent-conjugated secondary antibodies. Fiber types shown include type I (blue), type IIa (green), and type IIx (unstained) fibers. Scale bar = 200 μ m.

thus to affect adipose tissue lipolysis, which may explain why plasma NEFA concentrations were generally not influenced in such studies (Niehoff et al., 2009). In contrast, Yuan et al. (2012) showed that plasma NEFA concentrations were reduced when supplementing 12 g/d of rumen-protected NA. Although, in the present study, plasma NA concentrations in the NA group also should have been high enough to activate the HCAR2 receptor considering the affinity of the receptor for NA (Wise, 2003), neither plasma NEFA nor TG concentrations, which are both sources for milk fat synthesis, were influenced. This also indicates that adipose tissue lipolysis was not influenced. Interestingly, however, plasma concentrations of BHB, the endogenous ligand of the HCAR2 receptor (Offermanns et al., 2011), were higher in the control group, which may have counteracted an effect of NA on HCAR2 and lipolysis. The similar *HCAR2* expression in liver and muscle in both groups also does not contradict the view that HCAR2 receptor activity remained unchanged. To explain the observed effect on milk fat yield in the present study, it may be speculated that NA could have affected uptake into or metabolism of lipids in the mammary gland.

To study the muscle phenotype, we considered 3 muscles of the hindlimb (SM, ST) and back (LD), which are classified as fast-twitch muscles with a high percentage of type II fibers (Schiaffino and Reggiani, 2011). These muscles were expected to have a high potential for fiber switching toward a higher proportion of oxidative fibers. However, we found, in disagreement

with our hypothesis, that NA supplementation did not affect the proportions of type I or type II fibers, or the ratio of type II:type I fibers. The finding of an unchanged ratio of type II:type I fibers was confirmed by largely unchanged transcript levels of *MYH7*, which encodes the myosin heavy chains of type I fibers in the muscle motor units (Schiaffino and Reggiani, 2011). It is noteworthy that, within the class of type II fibers, NA treatment induced a moderate shift toward an increase of type IIa fibers at the expense of type IIx fibers, in LD muscle ($P < 0.10$) and ST muscle ($P < 0.05$). Although both IIx and IIa fibers are abundant in fast-twitch muscles with high levels of glycolytic enzymes, IIa fibers are also equipped with a higher activity of oxidative enzymes (Schiaffino and Reggiani, 2011). In LD muscle, these changes were confirmed by lower transcript levels of *MYH1* (encoding type IIx fibers; $P < 0.05$) and higher transcript levels of *VEGFA* and *VEGFB* ($P < 0.10$) that encode the angiogenic factors VEGFA and VEGFB. It is known that muscle fiber type transformation is accompanied or preceded by angiogenesis, resulting in increased capillary density (Waters et al., 2004). The increased ratios of type IIa: type IIx fibers in LD and ST muscles thus indicate that the oxidative capacity of these muscles could have been slightly enhanced in the NA group compared with the control group. However, gene expression analyses in the muscles showed that relative mRNA concentrations of genes involved in fatty acid uptake and oxidative utilization of fatty acids via β -oxidation, tricarboxylic acid

Table 4. Relative mRNA levels of genes involved in fiber type transformation and angiogenesis in the longissimus lumborum, semitendinosus, and semimembranosus of dairy cows fed either a control diet (control) or a diet supplemented with rumen-protected niacin (niacin)¹

Item	1 wk postpartum		3 wk postpartum		<i>P</i> -value		
	Control	Niacin	Control	Niacin	Treatment	Week	Treatment $\times$ week
Longissimus lumborum							
<i>MYH7</i>	1.00 $\pm$ 0.10	0.73 $\pm$ 0.06	0.89 $\pm$ 0.07	1.00 $\pm$ 0.08	0.26	0.29	0.043
<i>MYH2</i>	1.00 $\pm$ 0.27	1.43 $\pm$ 0.30	1.12 $\pm$ 0.27	1.79 $\pm$ 0.43	0.13	0.80	0.96
<i>MYH1</i>	1.00 $\pm$ 0.09	0.74 $\pm$ 0.07	1.71 $\pm$ 0.15	1.33 $\pm$ 0.13	0.004	<0.001	0.86
<i>VEGFA</i>	1.00 $\pm$ 0.18	1.36 $\pm$ 0.27	0.98 $\pm$ 0.20	1.46 $\pm$ 0.25	0.05	0.69	0.31
<i>VEGFB</i>	1.00 $\pm$ 0.12	1.29 $\pm$ 0.14	0.90 $\pm$ 0.15	1.28 $\pm$ 0.25	0.08	0.13	0.78
Semitendinosus							
<i>MYH7</i>	1.00 $\pm$ 0.16	1.52 $\pm$ 0.20	0.95 $\pm$ 0.15	1.44 $\pm$ 0.21	<0.001	0.74	0.73
<i>MYH2</i>	1.00 $\pm$ 0.37	1.33 $\pm$ 0.41	0.95 $\pm$ 0.27	1.36 $\pm$ 0.42	0.38	0.87	0.77
<i>MYH1</i>	1.00 $\pm$ 0.13	1.00 $\pm$ 0.12	1.27 $\pm$ 0.11	1.17 $\pm$ 0.16	0.41	0.16	0.34
<i>VEGFA</i>	1.00 $\pm$ 0.24	1.07 $\pm$ 0.19	0.49 $\pm$ 0.12	0.66 $\pm$ 0.16	0.12	0.010	0.86
<i>VEGFB</i>	1.00 $\pm$ 0.17	0.92 $\pm$ 0.14	0.54 $\pm$ 0.09	0.83 $\pm$ 0.19	0.62	0.030	0.46
Semimembranosus							
<i>MYH7</i>	1.00 $\pm$ 0.14	0.91 $\pm$ 0.09	0.68 $\pm$ 0.09	1.06 $\pm$ 0.12	0.14	0.053	0.001
<i>MYH2</i>	1.00 $\pm$ 0.50	0.79 $\pm$ 0.28	1.04 $\pm$ 0.33	0.23 $\pm$ 0.08	0.30	0.18	0.033
<i>MYH1</i>	1.00 $\pm$ 0.15	0.78 $\pm$ 0.08	0.89 $\pm$ 0.12	1.19 $\pm$ 0.12	0.5	0.28	0.05
<i>VEGFA</i>	1.00 $\pm$ 0.21	1.09 $\pm$ 0.24	1.19 $\pm$ 0.24	0.86 $\pm$ 0.14	0.77	0.62	0.4
<i>VEGFB</i>	1.00 $\pm$ 0.23	1.28 $\pm$ 0.22	1.15 $\pm$ 0.26	0.71 $\pm$ 0.09	0.92	0.32	0.25

¹Mean $\pm$ SEM are displayed (n = 15 and 14 animals in the control and niacin group).

Figure 4. Relative mRNA abundance (fold of control) of peroxisome proliferator activated receptor δ (*PPARD*) and PPAR- γ coactivator-1- α (*PPARGC1A*) in the longissimus lumborum (A), semitendinosus (B), and semimembranosus (C) of dairy cows fed either a control diet (control) or a diet supplemented with rumen-protected niacin (niacin; $n = 15$ and 14 animals in the control and niacin group; mean $\pm$ SEM) at 1 and 3 wk postpartum (p.p.). *Denotes a difference ($P < 0.05$) between the control and niacin group at the respective time point. T = treatment; W = week; T $\times$ W = interaction of treatment and week.

cycle, and oxidative phosphorylation were not influenced by NA treatment. Obviously, even the observed increase of the ratio of IIa:IIx fibers in ST and LD muscle induced by NA failed to cause any alteration in the metabolic phenotype of those muscles. The analyses performed in skeletal muscle overall indicate that NA treatment did not influence FA uptake and utilization in skeletal muscle.

The finding that NA treatment did not influence muscle fiber composition is in contrast to our recent studies in growing rats, pigs, and sheep. A possible reason for these diverging results between the present study and the studies performed in nonlactating animals (Khan et al., 2013a,b; Ringseis et al., 2013) may be that growing and lactating animals differ profoundly in metabolism and energy balance (Allen et al., 1994). The metabolic phenotype of skeletal muscle is mainly regulated by *PPARD*, the main PPAR isoform occurring in muscle, and *PPARGC1A*, a multifunctional co-regulator. Activation of both of these transcription factors in skeletal muscle enhances the formation of type I fibers, stimulates mitochondrial biogenesis, and induces the transcription of a broad spectrum of enzymes involved in oxidative energy production (i.e., the utilization of fatty acids as oxidative fuels; Ehrenborg and Krook, 2009; Liu and Lin, 2011). In studies with the nonlactating animals, NA treatment caused a strong upregulation of both *PPARD* and *PPARGC1A* in skeletal muscles, which might provide the molecular basis for the muscle fiber transition and the induction of the oxidative muscle phenotype observed in these animals (Khan et al., 2013a,b; Ringseis et al., 2013). However, both of these transcription factors in skeletal muscle are also regulated by nutritional factors. In this respect, it has been shown that energy deprivation leads to an upregulation of both *PPARD* and *PPARGC1A* in skeletal muscles, resulting in an increased energy production from fatty acids (de Lange et al., 2006; Ehrenborg and Krook, 2009). It is likely that upregulation of these transcription by energy deprivation is caused by an increased amount of NEFA delivered from adipose tissue to skeletal muscle in the state of a NEB (Ehrenborg and Krook, 2009). Fatty acids are acting as activators of *PPARD*, and an increased activity of *PPARD* is associated with an upregulation of *PPARGC1A* (de Lange et al., 2006). Dairy cows in early lactation are typically in NEB, and it is expected that increased NEFA plasma concentrations resulting from lipolysis in adipose might activate *PPARD* in skeletal muscle. Indeed, it has been recently shown that *PPARD* in skeletal is upregulated in dairy cows during the transition from pregnancy. It has been assumed that an upregulation of *PPARD* causes an increased transport of NEFA into muscle and subsequent oxidation, which helps to protect the liver

Table 5. Relative mRNA levels of genes involved in the utilization of fatty acids and glucose in the semitendinosus of dairy cows fed either a control diet (control) or a diet supplemented with rumen-protected niacin (niacin)¹

Item	1 wk postpartum		3 wk postpartum		P-value		
	Control	Niacin	Control	Niacin	Treatment	Week	Treatment × week
Fatty acid uptake and oxidation							
<i>SLC27A1/FATP1</i>	1.00 ± 0.20	1.15 ± 0.29	0.64 ± 0.17	1.13 ± 0.28	0.09	0.21	0.16
<i>CPT1B</i>	1.00 ± 0.08	1.08 ± 0.08	0.97 ± 0.09	0.95 ± 0.09	0.82	0.23	0.51
<i>ACADL</i>	1.00 ± 0.09	1.02 ± 0.12	1.27 ± 0.09	1.31 ± 0.13	0.94	0.001	0.97
<i>SLC25A20/CACT</i>	1.00 ± 0.15	0.96 ± 0.21	0.93 ± 0.16	0.84 ± 0.18	0.50	0.28	0.98
Carnitine transport							
<i>SLC22A4/OCTN1</i>	1.00 ± 0.13	1.04 ± 0.15	1.70 ± 0.32	1.25 ± 0.24	0.39	0.17	0.29
<i>SLC22A5/OCTN2</i>	1.00 ± 0.12	1.13 ± 0.08	0.81 ± 0.09	1.00 ± 0.11	0.052	0.037	0.037
Tricarboxylic acid cycle and oxidative phosphorylation							
<i>SDHA</i>	1.00 ± 0.27	1.24 ± 0.38	1.64 ± 0.46	1.93 ± 0.61	0.89	0.14	0.45
<i>COX4I1</i>	1.00 ± 0.07	1.38 ± 0.10	1.10 ± 0.07	1.38 ± 0.11	<0.001	0.58	0.42
<i>COX6A2</i>	1.00 ± 0.15	1.12 ± 0.10	1.19 ± 0.20	1.08 ± 0.09	0.63	0.82	0.43
Glucose utilization							
<i>SLC2A4/GLUT4</i>	1.00 ± 0.32	0.80 ± 0.18	0.57 ± 0.14	1.38 ± 0.45	0.39	0.390	0.06
<i>PFKM</i>	1.00 ± 0.32	1.22 ± 0.28	0.66 ± 0.18	1.12 ± 0.34	0.47	0.305	0.65

¹Mean ± SEM are displayed (n = 15 and 14 animals in the control and niacin group).

from excess fat load during the first days after parturition (Schäff et al., 2013). This assumption, however, could provide an explanation for the lack of effect of NA treatment on muscle fiber composition and phe-

notype in this study. If PPARD in skeletal muscle was already activated by high plasma NEFA concentrations occurring during early lactation it seems plausible that NA treatment did not further enhance biochemical ef-

Table 6. Relative mRNA levels in the liver of dairy cows fed either a control diet (control) or a diet supplemented with rumen-protected niacin (niacin)¹

Item	1 wk postpartum		3 wk postpartum		P-value		
	Control	Niacin	Control	Niacin	Treatment	Week	Treatment × week
Fatty acid uptake and oxidation							
<i>PPARA</i>	1.00 ± 0.15	1.13 ± 0.19	1.08 ± 0.13	1.21 ± 0.12	0.35	0.08	0.53
<i>SLC25A20/CACT</i>	1.00 ± 0.09	1.07 ± 0.09	0.84 ± 0.04	0.81 ± 0.06	0.92	<0.001	0.48
<i>CD36</i>	1.00 ± 0.15	1.70 ± 0.22	1.44 ± 0.20	1.77 ± 0.31	0.06	0.28	0.004
<i>CPT1A</i>	1.00 ± 0.19	1.17 ± 0.20	1.04 ± 0.17	1.05 ± 0.16	0.63	0.74	0.40
<i>ACADL</i>	1.00 ± 0.10	1.18 ± 0.15	0.90 ± 0.09	1.09 ± 0.09	0.18	0.10	0.34
<i>ACADM</i>	1.00 ± 0.11	1.11 ± 0.13	0.94 ± 0.10	1.09 ± 0.10	0.32	0.21	0.33
<i>SLC22A5/OCTN2</i>	1.00 ± 0.15	1.08 ± 0.13	1.28 ± 0.21	1.08 ± 0.16	0.93	0.72	0.40
Lipogenesis							
<i>SREBF1</i>	1.00 ± 0.15	1.16 ± 0.18	1.20 ± 0.20	1.62 ± 0.32	0.23	0.06	0.66
<i>ACACA</i>	1.00 ± 0.18	1.23 ± 0.22	0.90 ± 0.15	2.15 ± 0.58	0.03	0.72	0.18
<i>FASN</i>	1.00 ± 0.20	1.38 ± 0.31	0.81 ± 0.12	1.70 ± 0.39	0.09	0.90	0.53
<i>GPAM</i>	1.00 ± 0.21	1.36 ± 0.25	0.94 ± 0.14	1.31 ± 0.25	0.13	0.41	0.81
<i>DGAT2</i>	1.00 ± 0.08	0.94 ± 0.06	0.84 ± 0.05	0.85 ± 0.08	0.75	0.067	0.88
Ketogenesis							
<i>ACAT1</i>	1.00 ± 0.06	0.93 ± 0.04	0.94 ± 0.06	0.87 ± 0.06	0.27	0.13	0.88
<i>FGF21</i>	1.00 ± 0.34	0.26 ± 0.06	0.44 ± 0.06	0.19 ± 0.06	<0.001	0.08	0.94
<i>HMGCS2</i>	1.00 ± 0.07	1.17 ± 0.12	1.00 ± 0.07	1.02 ± 0.09	0.50	0.26	0.42
Unfolded protein response							
<i>ATF4</i>	1.00 ± 0.13	1.18 ± 0.12	0.89 ± 0.12	0.93 ± 0.08	0.34	0.017	0.77
<i>XBP1</i>	1.00 ± 0.19	0.96 ± 0.15	0.80 ± 0.10	0.88 ± 0.11	0.60	0.30	0.70
<i>EDEM1</i>	1.00 ± 0.13	1.32 ± 0.10	1.15 ± 0.12	1.22 ± 0.11	0.05	0.83	0.19
<i>DNJAC3</i>	1.00 ± 0.22	1.19 ± 0.21	1.19 ± 0.18	1.20 ± 0.21	0.40	0.94	0.15
<i>HSPA5</i>	1.00 ± 0.26	1.14 ± 0.15	1.02 ± 0.19	0.97 ± 0.17	0.43	0.28	0.33
<i>HERPUD1</i>	1.00 ± 0.14	0.92 ± 0.08	0.87 ± 0.12	0.90 ± 0.10	0.77	0.32	0.60

¹Mean ± SEM are displayed (n = 15 and 14 animals in the control and niacin group).

fects triggered by PPARD, and by PPARGC1A, which is closely related to PPARD.

One hypothesis underlying this study was that an increased uptake of NEFA into skeletal muscle by NA treatment could reduce the amounts of NEFA delivered to the liver. However, as FA uptake into skeletal muscle, according to the results of gene expression analysis, obviously was not enhanced, the amount of NEFA delivered to the liver was probably also unchanged. This suggestion agrees with the observation that expression of hepatic genes in fatty acid utilization, lipogenesis, and ketogenesis as well as hepatic TG concentration were not affected by the NA treatment. The NEFA taken up into the liver are activators of PPARD, the key regulator of the transcription of genes involved in fatty acid uptake, β -oxidation of fatty acids, carnitine homeostasis, and ketogenesis (Kersten et al., 1999). Therefore, a reduced uptake of NEFA into the liver would be expected to lower the expression of genes involved in these pathways. The finding that hepatic TG concentration, which is another parameter that is closely related to NEFA uptake into the liver (Grum et al., 1996; Hocquette and Bauchart, 1999), was also not different between control cows and cows supplemented with NA, is another indication that NEFA uptake into the liver was not influenced by NA treatment. The finding that NA treatment did not influence genes of fatty acid oxidation agrees with a previous study of Grum et al. (2002), which showed that supplemental NA does not influence palmitate oxidation in liver homogenates of dairy cows in early lactation. Moreover, the finding that hepatic TG concentration was not influenced by NA treatment agrees with previous studies, which also did not observe an effect of rumen-protected NA on hepatic TG concentration in early-lactating dairy cows (Morey et al., 2011; Yuan et al., 2012). The finding that liver carnitine concentration as well as the expression of hepatic *SLC22A5/OCTN2*, the most important carnitine transporter, was not influenced by NA treatment fits into this picture. Carnitine homeostasis (carnitine synthesis, carnitine uptake into cells) is regulated by PPARA (Ringseis and Eder, 2009), and it has been shown that conditions leading to an activation of PPARA by an increased flow of NEFA into the liver, including fasting or energy restriction, are inducing an upregulation of carnitine synthesis and carnitine uptake in the liver, associated with an increase of carnitine concentration in the liver (Luci et al., 2008; Ringseis et al., 2009; Schlegel et al., 2012b). Hence, the finding that liver carnitine concentration remained unchanged is another indication that NA did not influence the flow of NEFA into the liver.

In the present study, the observed reduction of plasma BHB in wk 2 and 3 in the cows treated with NA in comparison to control cows was surprising for several reasons. First, this finding is in contrast to almost all studies dealing with NA supplementation in dairy cows which did not find an effect of rumen-protected (Yuan et al., 2012) or non-rumen-protected NA (e.g., Driver et al., 1990; Christensen et al., 1996; Drackley et al., 1998) on plasma BHB concentration in dairy cows during early lactation. However, in some cases, a reduction in plasma BHB concentrations due to NA treatment has been reported as well (Fronk and Schultz, 1979; Erickson et al., 1992; Morey et al., 2011). Second, plasma concentrations of NEFA, the key metabolite for the production of ketone bodies in the liver, were not different between control cows and cows supplemented with NA. Third, expression of *HMGCS2*, the key enzyme of ketogenesis in the liver (Hegardt, 1999), was unchanged in the cows treated with NA. However, we observed that expression of *FGF21* in the liver was decreased in cows treated with NA. The FGF21 has been identified as a novel stress hormone that is induced by a variety of environmental and metabolic stressors (Kim and Lee, 2015). Recent findings suggest that FGF21 plays an adaptive role in maintaining energy homeostasis during stressful conditions (Kim and Lee, 2014). Besides other metabolic changes induced by FGF21 such as an increase of lipolysis, glucose uptake, or fatty acid oxidation, FGF21 is regarded as a key regulator of ketogenesis (Badman et al., 2007). Interestingly, *FGF21* is strongly induced in the liver in dairy cows during the transition from pregnancy to lactation (Carriquiry et al., 2009; Schoenberg et al., 2011; Schlegel et al., 2013). It has been suggested that the induction of *FGF21* in the liver of dairy cows is due to metabolic stress occurring in the peripartum phase, which aims to maintain energy homeostasis during this phase (Schoenberg et al., 2011; Kim and Lee, 2015). For example, it has been shown that energy deficits caused by fasting induce hepatic FGF21 expression via *PPARA* (Badman et al., 2007) and that protein and AA restriction increase hepatic FGF21 expression as well (Kim and Lee, 2015). However, neither energy deficit nor *PPARA* expression differed between groups in the present study. Moreover, it has been found that *FGF21* is particularly induced by ER stress (Schaap et al., 2013), a condition that has been observed in the liver of cows during early lactation (Gessner et al., 2014). To find out whether NA treatment could have attenuated ER stress in the liver of the cows, we determined mRNA concentrations of several genes of the UPR, which is triggered by the occurrence of ER stress. The finding that the expression of these

genes in the liver remained unchanged indicates that NA treatment did not influence the occurrence of ER stress in the liver in wk 1 and 3. Thus, it is possible that NA treatment improved stress conditions, other than ER stress, which in turn led to a reduced expression of *FGF21* in the liver. For example, NA has been reported to strengthen the intestinal barrier and reduce passage of bacterial endotoxins (Zhong et al., 2015), which are increased under rumen acidosis conditions that frequently occur in high-yielding transition cows (Gozho et al., 2007). The reduced expression of *FGF21* in the liver could be involved in the reduced plasma BHB concentrations observed in cows treated with NA in wk 2 and 3 p.p. However, further research is required to establish a potential interaction between NA treatment, *FGF21*, and ketogenesis in dairy cows. The disagreement of our study with many other studies dealing with the effects of NA treatment on ketogenesis in dairy cows could be due to the fact that the NA dosage used in our study was 2 to 10 times higher and plasma NAM concentrations were 2 to 4 times higher than in most other studies (e.g., Niehoff et al., 2009; Morey et al., 2011; Yuan et al., 2012), suggesting that effects of NA on ketogenesis are possibly occurring only at very high plasma levels. In addition, it has to be noted that plasma BHB concentrations were below the threshold for subclinical ketosis in both groups.

CONCLUSIONS

Data of the present study show that high dosages of rumen-protected NA do not influence muscle fiber composition and the metabolic phenotype of skeletal muscle in dairy cows during early lactation. This finding indicates that NA treatment does not increase fatty acid utilization in skeletal muscle of cows. Gene expression analyses, moreover, indicate that NA treatment does not alter lipid metabolism (fatty acid oxidation, lipogenesis) in the liver during early lactation. Moreover, we observed that plasma concentration of BHB was reduced by NA treatment during early lactation, an effect that could be due to a reduced expression of *FGF21*, a regulator of ketogenesis, observed in the liver of cows supplemented with NA.

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