Acylcarnitine profiles in serum and muscle of dairy cows receiving conjugated linoleic acids or a control fat supplement during early lactation

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ABSTRACT

Acylcarnitines (ACC) are formed when fatty acid (FA)-coenzyme A enters the mitochondria for β -oxidation and the tricarboxylic acid cycle through the carnitine shuttle. Concentrations of ACC may vary depending on the metabolic conditions, but can accumulate when rates of β -oxidation exceed those of tricarboxylic acid. This study aimed to characterize muscle and blood serum acylcarnitine profiles, to determine the mRNA abundance of muscle carnitine acyltransferases, and to test whether dietary supplementation (from d 1 in milk) with conjugated linoleic acids (CLA; 100 g/d; each 12% of *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA; $n = 11$) altered these compared with control fat-supplemented cows (CTR; $n = 10$). Blood samples and biopsies from the semitendinosus muscle were collected on d -21 , 1, 21, and 70 relative to parturition. Serum and muscle ACC profiles were quantified using a targeted metabolomics approach. The CLA supplement did not affect the variables examined. The serum concentration of free carnitine decreased with the onset of lactation. The concentrations of acetylcarnitine, hydroxybutyrylcarnitine, and the sum of short-chain ACC in serum were greater from d -21 to 21 than thereafter. The serum concentrations of long-chain ACC tetradecenoylcarnitine (C14:1) and octadecenoylcarnitine (C18:1) concentrations were greater on d 1 and 21 compared with d -21 . Muscle carnitine remained unchanged, whereas short- and medium-chain ACC, including propenoylcarnitine (C3:1), hydroxybutyrylcarnitine, hydroxyhexanoylcarnitine,

hexanoylcarnitine (C6:1), and pimelylcarnitine were increased on d 21 compared with d -21 and decreased thereafter. In muscle, the concentrations of long-chain ACC (from C14 to C18) were elevated on d 1. The mRNA abundance of carnitine palmitoyltransferase 1, muscle isoform (*CPT1B*) increased 2.8-fold from d -21 to 1, followed by a decline to nearly prepartum values by d 70, whereas that of *CPT2* did not change over time. The majority of serum and muscle short- and long-chain ACC were positively correlated with the FA concentrations in serum, whereas serum carnitine and C5 were negatively correlated with FA. Time-related changes in the serum and muscle ACC profiles were demonstrated that were not affected by the CLA supplement at the dosage used in the present study. The elevated concentrations of long-chain ACC species in muscle and of serum acetylcarnitine around parturition point to incomplete FA oxidation were likely due to insufficient metabolic adaptation in response to the load of FA around parturition.

Key words: acylcarnitine, carnitine shuttle, skeletal muscle, conjugated linoleic acid, early lactation

INTRODUCTION

The transition from late gestation through early lactation in dairy cows is associated with a substantial mobilization of body reserves, in particular fat, leading to a marked increase in circulating concentrations of fatty acids (FA), which are oxidized by hepatic and extrahepatic tissues as an energy source (Grummer, 1993). However, in the liver, the oxidative capacity for FA and for exporting FA via very low density lipoproteins is limited; thus, fatty liver may result from increased lipolysis (Grummer, 2008). Peroxisomal β -oxidation, as an auxiliary pathway for oxidizing FA during extensive FA mobilization, helps to dampen accumulation of fat in the liver (Grum et al., 1994, 1996, 2002). In addition,

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the oxidative capacity for FA in other tissues, such as skeletal muscle, may also contribute in reducing the metabolic load of FA on the liver (Kuhla et al., 2011; Schäff et al., 2013), though the quantitative contribution of skeletal muscle to FA metabolism in dairy cows during the periparturient is not known.

In dairy cows, plasma FA mainly comprise SFA, including palmitic acid (C16:0) and stearic acid (C18:0), and oleic acid (C18:1n9c) as a MUFA (Leroy et al., 2005; Tyburczy et al., 2008). For generating energy from long-chain FA, they need to be transported from the cytoplasm into the mitochondrial matrix across the mitochondrial membranes through a carnitine-dependent transport shuttle. This transport system is regulated by carnitine acyltransferases [i.e., carnitine palmitoyltransferase 1 (**CPT1**; present in the mitochondrial outer membrane) and 2 (**CPT2**; located on the matrix side of the inner membrane); Flanagan et al., 2010; Schooneman et al., 2014]. Once inside the mitochondria, carnitine and long-chain acyl-CoA are regenerated by CPT2, which can then be further oxidized via the tricarboxylic acid (**TCA**) cycle and respiratory chain to provide ATP (Schooneman et al., 2013). Deficiencies in these enzymes or impaired functions, or depletion of TCA cycle intermediates, may lead to incomplete mitochondrial FA oxidation, resulting in accumulation of acylcarnitines (**ACC**), which may be associated with development of insulin resistance, as documented in human studies (Adams et al., 2009; Mihalik et al., 2010; Sun et al., 2016).

Serum ACC undergo time-related changes in dairy cows during the transition from late pregnancy to early lactation (Kenéz et al., 2016) and differ between cows experiencing excessive versus low lipolysis, as classified via the serum FA concentrations postpartum (Humer et al., 2016). The plasma ACC profile may reflect the intramitochondrial acyl-CoA pattern; however, it is not clear to what extent circulating levels of ACC reflect tissue ACC metabolism, as plasma ACC represent the sum from different tissues, mainly skeletal muscle and liver (Schooneman et al., 2014; Xu et al., 2016).

Supplementation with *trans*-10,*cis*-12 CLA is used to reduce milk fat content in early-lactation dairy cows as a dietary strategy to improve energy status to counteract the physiological negative energy balance (Sippel et al., 2009; Schlegel et al., 2012). The *trans*-10,*cis*-12 isomer is referred as the most effective isomer lowering milk fat content mainly through inhibition of de novo FA synthesis in the mammary gland, accompanied by a reduction of FA uptake from triacylglycerol rich lipoproteins due to inhibition of lipoprotein lipase activity (Bauman et al., 2011). A number of studies have examined the effects of CLA on hepatic lipid metabolism in growing beef cattle (Shibani et al., 2012) or dairy cows

(Schlegel et al., 2012) through assessing expression of genes involved in lipid metabolism; but to our knowledge no evaluation exists of the effects of CLA on lipid metabolism in ruminant skeletal muscle. Considering skeletal muscle to be principal contributor to the serum ACC pool (Koves et al., 2008), we tested the hypothesis that dietary supplementation with CLA may alter the expression of muscle carnitine acyltransferases in conjunct with free carnitine and ACC profiles in both serum and muscle of dairy cows. We compared CLA supplementation with control fat-supplemented cows to address potential changes in capacity for mitochondrial β -oxidation of FA in skeletal muscle in the context of the negative energy balance typical for early lactation. Using serum samples and biopsies from semitendinosus muscle, we aimed to determine changes in serum and muscle concentrations of ACC and muscle expression of *CPT1B* and *CPT2* mRNA related to treatment and time from late pregnancy to lactation.

MATERIALS AND METHODS

Animals, Treatment, and Experimental Design

All animal experiments were in accordance with the European Community regulations concerning the protection of experimental animals and the guidelines of the LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, Germany, File Number 33.14.42502-04-071/07). The experimental design and zootechnical data were reported previously (Pappritz et al., 2011a). A subset of animals and samples from that previous study (i.e., only multiparous cows) was considered for the current study. Briefly, 21 Holstein cows, housed in a freestall barn were fed ad libitum with a partial mixed ration according to the recommendations of the German Society of Nutrition Physiology (GfE, 2001). The partial mixed ration (6.8 MJ of NE_L/kg of DM) consisted of 37.8% corn silage, 25.2% grass silage, and 37% concentrate (DM basis). At 1 DIM, cows were allotted to either the CLA group (n = 11) or the control group (**CTR**; n = 10). The animals in the CLA group received 100 g/d of encapsulated rumen-protected CLA (Lutrell Pure, BASF SE, Ludwigshafen, Germany) supplying 7.6 g of *cis*-9,*trans*-11 CLA and 7.6 g of *trans*-10,*cis*-12 CLA per day. The animals in the CTR group received 100 g/d of rumen-protected control fat supplement (Silafat, BASF), in which CLA was substituted by stearic acid to form an isoenergetic control diet using a fatty acid with the same number of carbon atoms as in CLA. The supplements were provided with 4 kg of additional concentrate (8.8 MJ of NE_L/kg DM) from DIM 1 throughout the observation period.

Blood and Muscle Tissue Sampling

Blood samples were taken from the jugular vein on d -21, 1, 21, and 70 relative to parturition using evacuated tubes. Cows were sampled after the morning milking before they had access to the new fresh ration. Blood samples were allowed to clot and centrifuged at $1,500 \times g$ at 4°C for 20 min. Serum was recovered and frozen (-80°C) until analysis. Muscle biopsies (semitendinosus muscle) were collected on d -21, 1, 21, and 70 relative to parturition, snap-frozen in liquid nitrogen, and stored at -80°C until analysis.

Estimation of Insulin Sensitivity

Data needed for the estimation of insulin sensitivity were reported elsewhere (Pappritz et al., 2011a). Insulin sensitivity was estimated by calculating the revised quantitative insulin sensitivity check index (**RQUICKI**) from the data of blood glucose, insulin, and FA (Holtenius and Holtenius, 2007).

Acylcarnitine Profiling

The acylcarnitine profiles in muscle and serum were determined by flow-injection electrospray ionization MS/MS profiling through targeted metabolomics using the AbsoluteIDQ p180 Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria). Free carnitine and 40 acylcarnitines were simultaneously quantified. The abbreviations, written as Cx:y, are used to describe the total number of carbons and double bonds of all chains, respectively. All analyses were performed in the Helmholtz Zentrum München, German Research Center for Environmental Health, Genome Analysis Center. For serum, 10 µL of the thawed sample were applied directly to the assay. For muscle, 25 mg of frozen samples were homogenized and extracted using homogenization tubes with ceramic beads (1.4 mm) and a Precellys 24 homogenizer with an integrated cooling unit (PEQLAB Biotechnology GmbH, Darmstadt, Germany). We added 3 µL of a dry ice cooled mixture of ethanol/phosphate buffer (85/15 vol/vol) to each milligram of frozen muscle tissue. After centrifugation, 10 µL of the homogenate supernatant were applied to the well plate of the p180 kit. The assay procedures of the AbsoluteIDQ p180 Kit, the detailed description of the tissue preparation, and the metabolite nomenclature have been described in detail previously (Zukunft et al., 2013, 2018). Sample handling was performed by a Hamilton Microlab STAR robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and an Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, UK) along with standard laboratory equipment. Mass spectromet-

ric analyses were done on an API 4000 triple quadrupole system (Sciex Deutschland GmbH, Darmstadt, Germany) equipped with a 1200 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and a HTC PAL auto sampler (CTC Analytics, Zwingen, Switzerland) controlled by the software Analyst 1.6.1. Data evaluation for quantification of metabolite concentrations and quality assessment was performed with the MetIDQ software package, which is an integral part of the AbsoluteIDQ kit. Internal standards were used as reference for the calculation of metabolite concentrations. The concentrations of the plasma samples were given in micromoles per liter, the concentrations of the tissue samples in picomoles per milligram of tissue, and the concentrations of tissue homogenate in micromoles per liter.

RNA Extraction and Quantitative Real-Time Reverse Transcription-PCR

The preparation of the samples, including RNA extraction and cDNA synthesis, was described in detail previously (Saremi et al., 2012a,b; Sadri et al., 2015). Quantification of the mRNA of the targeted genes was performed in an Mx3000P cycler (Agilent, Santa Clara, CA) and in accordance with MIQE guidelines (Bustin et al., 2009). Primer sequences and the real-time PCR conditions are shown in Table 1.

The reaction was performed in triplicate in a total volume of 10 µL consisting of 2 µL of cDNA (diluted 1:4) as template, 1 µL of primer mix, 2 µL of water, and 5 µL of the DyNAmo ColorFlash SYBR Green qPCR Kit master mix (Thermo Scientific, Germany). For each PCR run, a negative-template control for quantitative PCR as well as a negative-template control and no-reverse transcriptase control of cDNA were included. A standard curve was generated using serial dilutions of cDNA to calculate efficiency-corrected relative quantities of the targets (run-specific target amplification efficiency). A set of 2 inter-run calibrators was used for each PCR plate to correct for run-to-run variation. The mRNA abundance of the target genes was normalized using the 4 most stable reference genes (Saremi et al., 2012a); namely, emerin (*EMD*), RNA polymerase II (*POLR2A*), eukaryotic translation initiation factor 3 (*EIF3K*), and low-density lipoprotein receptor-related protein 10 (*LRP10*) using qBase^{PLUS} 2.0 (Biogazelle, Ghent, Belgium).

Statistical Analysis

Statistical analysis of the data was carried out using SAS software (version 9.2; SAS Institute Inc., Cary, NC). The data were tested for normality before analy-

Table 1. Characteristics of the primers and the real-time PCR conditions

Gene ¹	Sequence (5'-3')	NCBI accession no.	Length (bp)	Annealing condition (s/°C)	Mean efficiency
<i>CPT1B</i>					
Forward	GCAGATGATGGCTATGGA	NM_001034349.2	78	20/61	90.2
Reverse	GGAGAACTTGCTGGAGAC				
<i>CPT2</i>					
Forward	GTAGCCAGTAAGCACTATTC	NM_001045889.2	180	60/59	97.0
Reverse	CCAAGTCTTACCTCCTGATA				
<i>EMD</i>					
Forward	GCCCTCAGCTTCACTCTCAGA	NM_203361	100	45/59	95.5
Reverse	GAGGCGTTCCCGATCCTT				
<i>POLR2A</i>					
Forward	GAAGGGGGAGAGACAAACTG	X63564	86	60/60	100.4
Reverse	GGGAGGAAGAAGAAAAAGGG				
<i>EIF3K</i>					
Forward	CCAGGCCCAACCAAGAAGAA	NM_001034489	180	60/59	89.8
Reverse	TTATACCTTCCAGGAGGTCCATGT				
<i>LRP10</i>					
Forward	CCAGAGGATGAGGACGATGT	BC149232	125	45/59	98.4
Reverse	ATAGGGTTGCTGTCCCTGTG				

¹*CPT1B* = carnitine palmitoyltransferase 1, muscle isoform; *CPT2* = carnitine palmitoyltransferase 2; *EMD* = emerin; *POLR2A* = RNA polymerase II; *EIF3K* = eukaryotic translation initiation factor 3; *LRP10* = low-density lipoprotein receptor-related protein 10.

sis using the UNIVARIATE procedure. When the data were not normally distributed (BW, BCS, DMI, and blood glucose, insulin, FA, and RQUICKI), they were transformed using a $\log_{10}$ transformation before analysis. Body weight, BCS, DMI, blood metabolites, and mRNA data were analyzed using repeated measures in the MIXED procedure of SAS. The model included treatment, time, and interaction of treatment $\times$ time as the fixed effects and cow as the random effect. No significant effect of treatment or interaction of treatment $\times$ time were observed on the tested variables. Therefore, data from the 2 feeding groups were merged for the final statistical analysis of the data. The appropriate covariance structure for all repeated statements was determined according to the Akaike information criterion and Bayesian information criterion. The Tukey-Kramer adjustment was applied to account for multiple comparisons. The threshold of significance was set at $P \leq 0.05$; trends were declared at $0.05 < P \leq 0.10$.

Serum and muscle ACC data were analyzed with MetaboAnalyst 3.0 (Xia et al., 2015). A preliminary statistical analysis of the data showed no significant effect of treatment or interaction of treatment $\times$ time. Thus, data from the 2 feeding groups were merged for the final statistical analysis. The *k*-nearest neighbors algorithm was used to estimate the values of missing data. Metabolites with more than 50% of missing values (i.e., values lower than limit of detection) were omitted. Data were generalized log-transformed and Pareto-scaled to correct for heteroscedasticity, to reduce the skewness

of the data, and to reduce mask effects (van den Berg et al., 2006). Principal component analysis (**PCA**), partial least squares discriminant analysis (**PLS-DA**), and variable importance of projection (**VIP**) were conducted to identify those metabolites showing significant differences among the 4 time points. The PLS-DA models were validated by 10-fold cross validation and 2,000 permutation tests (Figures 3 and 4; Szymanska et al., 2012). The VIP score was used to rank the metabolites based on their importance in discriminating different time points. In addition, a one-way ANOVA followed by Tukey's HSD test was performed on the data to further confirm the significance of important metabolites identified from PCA and PLS-DA. The threshold of significance was set at false discovery rate (**FDR**) ≤ 0.10 .

The Spearman rank-order correlation was used to reveal correlations between ACC profiles, mRNA data, and RQUICKI using PROC CORR. The *P*-values were adjusted for multiple comparisons by calculating FDR using PROC MULTTEST. The cutoff condition of correlation analyses was set as $|\rho| \geq 0.20$ and $\text{FDR} \leq 0.10$.

RESULTS

BW, BCS, and DMI

Neither BW nor BCS and DMI were different between groups. As shown in Figure 1 for the merged groups, both BW and BCS decreased with time ($P < 0.0001$), whereas DMI increased with time ($P = 0.0002$).

RQUICKI

For RQUICKI, no treatment effects were observed, but RQUICKI tended to decrease by about 4% from d 1 to 21 (Figure 2; $P = 0.10$).

Figure 1. Time course of BW (A), BCS (B), and DMI (C) in dairy cows during late gestation and early lactation. Data are means $\pm$ SEM. Time effect: $P < 0.0001$ (BW and BCS) and 0.0002 (DMI).

Acylcarnitine Profiles in Serum

The serum ACC concentrations were not influenced by CLA supplementation, but changes related to time were observed. With unsupervised and supervised pattern recognition analyses, clear separations between d -21 and postpartum time points in serum were observed (Figures 3A and 3B). Moreover, the top 15 ACC that contributed most significantly to the separation between the 4 time points were identified by the respective validated PLS-DA model and VIP (Figures 3C and 3D). To further confirm the specificity and significance of important metabolites identified from PCA and PLS-DA, we performed univariate analysis using one-way ANOVA and Tukey's HSD test on each metabolite. In total, 7 serum ACC and 3 related indices changed over time ($VIP \geq 1.0$, $FDR < 0.10$; Figure 4). The serum concentrations of free carnitine decreased with the onset of lactation. The serum concentrations of acetylcarnitine (C2), butyrylcarnitine (C4), and sum of the short-chain ACC concentrations (C2-C5) were elevated around parturition compared with d 70. The serum concentrations of hydroxybutyrylcarnitine (C4-OH) and octadecenoylcarnitine (C18:1) increased from d -21 to 21 and then remained unchanged. The CPT1 ratio, the ratio of free carnitine to the sum of palmitoylcarnitine and stearoylcarnitine [carnitine/(C16:1+C18:0)] decreased with the onset of lactation. The CPT2 ratio [(C16:0+C18:1)/C2] was higher after parturition compared with prepartum values.

Acylcarnitine Profiles in Skeletal Muscle

The muscle ACC concentrations did not differ between the groups, but changes related to time were

Figure 2. Time course of the estimated insulin sensitivity as measured by the revised quantitative insulin sensitivity check index (RQUICKI) in dairy cows during late gestation and early lactation ($P = 0.10$). Data are means $\pm$ SEM.

Figure 3. Score plots of principal component analysis (PCA; A) and partial least squares-discriminant (PLS-DA; B) of serum acylcarnitine on d -21 (red), 1 (green), 21 (blue), and 70 (cyan) relative to calving. The top 15 metabolites that contributed most significantly to the separation between the 4 time points were identified according to weights in PLS-DA model by using variable importance in projection (VIP; C). The PLS-DA model was further validated by 2,000 permutation tests based on separation distance (D). The histogram shows the group separation distance formed by these data sets randomly reassigned class labels. The red arrow represents the group separation distance of the original classifier. The further away to the right of the distribution formed by randomly permuted data, the more significant the discrimination. The P -value was calculated as the proportion of the times that class separation based on randomly labeled sample is at least as good as the one based on the original data.

observed. Score plots of PCA and PLS-DA of dairy cow muscle ACC are presented in Figure 5A and 5B. The top 15 metabolites that contributed most significantly to the observed separation are shown in Figure 5C.

The top 15 metabolites contributing most significantly (VIP score >1) to the observed separation are shown in Figure 5C. Time course of the selected metabolites identified to have a VIP score >1.0 , and FDR <0.10

Figure 4. Time course of selected metabolites in serum of dairy cows identified to have a variable importance in projection (VIP) score >1.0 and false discovery rate <0.1 : free carnitine (C0; A), acetylcarnitine (C2; B), butyrylcarnitine (C4; C), hydroxybutyrylcarnitine (C4-OH; D), decanoylcarnitine (C10; E), tetradecenoylcarnitine (C14:1; F), octadecenoylcarnitine (C18:1; G), carnitine palmitoyltransferase (CPT) 1 ratio (H), CPT2 ratio (I), and sum of short-chain acylcarnitine (short-chain ACC; C2-C5; J). Data are means $\pm$ SEM. Differences between different time points identified by post-hoc testing (Tukey's HSD) after ANOVA are indicated by different letters (a-c).

are shown in Figure 6. The muscle concentrations of carnitine remained unchanged (data not shown). Muscle short- and medium-chain ACC, including propionylcarnitine (C3:1), C4-OH, hydroxyhexanoylcarnitine (**C6-OH**), and hexenoylcarnitine (C6:1) were elevated from d -21 to 21 and decreased thereafter. Muscle long-chain ACC, including tetradecanoylcarnitine (C14), C14:1, hexadecanoylcarnitine (C16), hydroxyhexadecanoylcarnitine (C16:1-OH), C18:1, hydroxyoctadecanoylcarnitine (C18:1-OH), and sum of the long-chain ACC (**C12-C18**), changed over time ($P \leq 0.002$) and followed a similar pattern; that is, they increased from d -21 to 1, declined to nearly prepartum values by d 21, and then remained unchanged. The CPT1 ratio in muscle was decreased from d -21 to a nadir on d 1 and then increased thereafter. In contrast, the muscle CPT2 ratio increased toward parturition and then decreased thereafter.

mRNA Abundance of Carnitine Acyl Transferases in Skeletal Muscle

No differences were observed between the treatment groups, and thus merged data were analyzed for time-dependent effects. The mRNA abundance of *CPT1B* increased 2.8-fold from d -21 to 1 ($P = 0.02$), followed by a decline to nearly prepartum values by d 70 (Figure 7). The mRNA abundance of *CPT2* remained unchanged over time.

Associations of Acylcarnitine Profiles with Conventional Parameters and mRNA Abundance

As shown in Figure 8, across all time points, correlation analysis revealed a negative correlation between serum FA and CPT1 ratio in muscle and serum ($P < 0.0001$; $\rho = -0.46$ and -0.66 , respectively). Positive correlations were observed between serum FA with most of the muscle and serum long-chain ACC (C14-C18; $P < 0.03$; $\rho > 0.25$), several muscle short- and medium-chain ACC (C3, C4, C6, C7, C10-derived ACC, and C12:1; $P < 0.03$; $\rho > 0.246$), serum C2 ($P < 0.0001$; $\rho = 0.44$), and C4-OH ($P < 0.01$; $\rho = 0.30$), as well as muscle CPT2 ratio ($P < 0.0001$; $\rho = 0.46$). Moreover, FA concentrations were positively related to the *CPT1B* mRNA abundance ($P < 0.0001$; $\rho = 0.64$). The RQUICKI were neither associated with the aforementioned ACC nor mRNA abundance.

DISCUSSION

Skeletal muscle is important for coping with the increasing concentrations of FA at the end of pregnancy

and the onset of lactation. The capacity of oxidizing FA likely changes depending on the supply of FA and the physiological status of the animal. We herein characterized the longitudinal changes in ACC both in circulation and in skeletal muscle in dairy cows during the transition from pregnancy into lactation by means of targeted metabolomics and investigated whether dietary supplementation (from 1 DIM) with CLA altered these compared with control-fat supplemented cows. In laboratory animals, CLA supplementation has been associated with metabolic changes favoring the increase of lipolysis and the reduction of lipoprotein lipase activity, accompanied by the oxidation of FA in the adipose and muscle tissues due to increased CPT-1 activity and action or possibly as a result of inhibiting adipocyte differentiation (Botelho et al., 2005; Churrua et al., 2009; Lehnert et al., 2015). In contrast to our hypothesis, treatment with CLA did not affect the variables targeted herein or other classical variables used to characterize the metabolic effects, such as FA. In consequence, the groups could be pooled thus increasing sample size. The reasons for the lack of a CLA response in the examined variables are not known, but are likely related to the dosage used and the availability of the CLA isomers in the intermediary metabolism as well as the timing of the supplementation, which started only with the first day in milk.

The CLA dosages commonly used in dairy cows are far below those tested in laboratory animals and in humans. However, for the main targeted effect in dairy cows (i.e., milk fat reduction), the relatively low dosages are effective. Likewise, the CLA-treated animals in our study had 12% less milk fat than the control cows; this effect was evident after 28 d of lactation and CLA supplementation (Pappritz et al., 2011a). However, the transfer of CLA into milk was low and largely limited to the *trans*-10,*cis*-12 isoform (0.03 vs. 0.004% in the control group; Pappritz et al., 2011a). In a more detailed approach using cows fitted with ruminal and duodenal cannulas, the actual duodenal availability of *trans*-10,*cis*-12 CLA was determined to be low (i.e., between 5 and 16%; Pappritz et al., 2011b), though this is in accordance with the protection rate of 9 to 34% reported for the calcium salts of the CLA (de Veth et al., 2005). Pappritz et al. (2011b) showed that major portions of the CLA reaching the duodenum are excreted via milk (36–48%) or feces (~50%), and thus only a small proportion of the CLA (i.e., 2–14%) may reach different tissues and cells. Using the same CLA treatment in primiparous cows that were sequentially slaughtered during lactation, von Soosten et al. (2013) reported only low tissue concentrations. For example, in adipose tissues, a maximum of 0.02% of total FA were *trans*-10,*cis*-12 CLA in supplemented cows; the

CLA content in control cows remained below the limit of detection (<0.01% of total FA). In skeletal muscle tissue, CLA were not detectable (von Soosten et al.,

2013). We thus speculated that a substantially higher dose of CLA would be required to affect FA oxidation in muscle or the studied muscle was less sensitive

Figure 5. Score plots of principal component analysis (PCA; A) and partial least squares-discriminant (PLS-DA; B) of dairy cow's muscle acylcarnitine on d -21 (red), 1 (green), 21 (blue), and 70 (cyan) relative to calving. Top 15 metabolites that contributed most significantly to the separation between the 4 time points were identified according to weights in PLS-DA model by using variable importance in projection (VIP; C). The PLS-DA model were further validated by 2,000 permutation tests based on separation distance (D). The histogram shows the group separation distance formed by these data sets randomly reassigned class labels. The red arrow represents the group separation distance of the original classifier. The further away to the right of the distribution formed by randomly permuted data, the more significant the discrimination. The *P*-value is calculated as the proportion of the times that class separation based on randomly labeled sample is at least as good as the one based on the original data.

Figure 6. Time course of selected metabolites in muscle of dairy cows identified to have a variable importance in projection (VIP) score >1.0 and false discovery rate <0.1: propionylcarnitine (C3; A), propenylcarnitine (C3:1; B), hydroxybutyrylcarnitine (C4-OH; C), glutaconylcarnitine (C5:1-DC; D), hydroxyhexanoylcarnitine (C6-OH; E), hexenoylcarnitine (C6:1; F), pimelylcarnitine (C7-DC; G), tetradecanoylcarnitine (C14; H), tetradecanoylcarnitine (C14:1; I), hexadecanoylcarnitine (C16; J), hydroxyhexadecanoylcarnitine (C16-OH; K), hydroxyhexadecanoylcarnitine (C16:1-OH; L), octadecanoylcarnitine (C18:1; M), hydroxyoctadecanoylcarnitine (C18:1-OH; N), sum of the long-chain acylcarnitine (long-chain ACC, C12+; O), carnitine palmitoyltransferase (CPT) 1 ratio (P), and CPT2 ratio (Q). Data are means $\pm$ SEM. Differences between different time points identified by post-hoc testing (Tukey's HSD) after ANOVA are indicated by different letters (a-c).

Figure 7. Time course of muscle mRNA abundance (means $\pm$ SEM) of carnitine acyltransferases (CPT1B and CPT2) in dairy cows during late gestation and early lactation. Differences between different time points identified by post-hoc testing (Tukey's HSD) after ANOVA are indicated by different letters (a–c).

and responsive to CLA. In the current study, muscle samples were biopsied only from the longissimus dorsi muscle, and we also did not determine muscle fiber type composition in the samples. Skeletal muscle, a heterogeneous and highly structured tissue, is composed of a set of fiber types differing in their functional and metabolic profiles (Gunawan et al., 2007), and it is probable that the response of skeletal muscle to the CLA supplement is fiber type dependent, which warrants further investigation.

As expected, we observed greater FA serum concentrations around calving and in the first weeks of lactation, pointing to increased lipolysis around parturition as a response to the massively augmented need for energy to accomplish fetal growth and milk synthesis. Carnitine and its acyl esters (i.e., ACC) are indispensable for the mitochondrial β -oxidation of FA through facilitating the transfer of long-chain FA from the cytoplasm to the mitochondrial matrix across the mitochondrial membranes. Once inside mitochondria, the enzyme *CPT2* reconverts the ACC back into free carnitine and the respective long-chain acyl-CoA, which can then be oxidized for ATP production through β -oxidation and the TCA cycle (Schooneman et al., 2013). Carnitine is mainly synthesized in the liver from the EAA lysine and methionine (Krajcovicová-Kudláčková et al., 2000). Skeletal muscle, harboring the highest concentrations, is unable to synthesize carnitine and thus needs to take carnitine from blood. In accordance with a previous study (Schooneman et al., 2014), we observed carnitine levels being unchanged in muscle but decreasing in se-

rum, likely due to increased carnitine excretion in milk (Shennan et al., 1998) and its uptake by the muscle to maintain the intracellular concentrations. In addition, carnitine maintains the balance between free and esterified CoA and is required for the mitochondrial efflux of excess acyl groups (Sharma and Black, 2009). Thus, changes in individual serum and tissue ACC may imply changes in specific metabolic pathways; therefore, it is commonly used in neonatal screening for metabolic disturbances (Meyburg et al., 2002). The C2, the shortest ACC, derives from acetyl-CoA via the action of carnitine acetyltransferase for transport out of the mitochondria (Flanagan et al., 2010). Acetylcarnitine is the universal degradation product of all metabolic substrates, and is thus the most abundant ACC in the tissues and circulation. In the current study, serum C2 concentrations were elevated around parturition, pointing to an increased FA β -oxidation in mitochondria relative to the TCA cycle flux. In addition, C4-OH, which can be derived from the CoA ester of the ketone body 3-hydroxybutyrate (Soeters et al., 2012; Schooneman et al., 2013), had higher concentrations around parturition in both serum and muscle. The higher C4-OH concentration, which is thought to reflect ketogenesis (Xu et al., 2016), is also consistent with an excess pool of acetyl-CoA around parturition. Amino acid catabolism is a source of odd-chain species such as C3 and C5 (Flanagan et al., 2010). In the current study, muscle concentrations of C3 and C3:1 were slightly higher after than before calving, whereas those in serum did not show time-dependent changes. These data imply that most lipid-derived ACC increased around parturition, which is consistent with the lactation-induced rise in circulating FA.

We hypothesized that lactation-induced alterations in the ACC profiles are caused by incomplete FA oxidation, as long-chain ACC species were elevated around parturition in both serum and muscle (more notable in the latter). Most long-chain ACC species in muscle decreased from d 1 to 21, with little or no changes afterward, suggesting insufficient adaptation of their metabolism in response to the metabolic load of FA around parturition. However, it should be noted that, due to the study design, we were unable to assess potential changes during the first days after calving, a period of rapid and substantial metabolic changes in dairy cows. It is likely that FA oxidation should be in relative excess to oxidation in TCA and respiratory chain to guarantee continuous supply of energy. Peroxisomal β -oxidation, independent of carnitine-mediated transport, is the second pathway through which long-chain FA can be oxidized, and unlike mitochondrial β -oxidation, is not regulated by energy demands of the cell (Osmundsen et al., 1991; Drackley, 1999). For

Figure 8. Significant Spearman rank-order correlation coefficient (ρ) between serum fatty acid concentration with muscle (A) and serum (B) acylcarnitine (ACC) profiles as well as mRNA abundance. The P -values were adjusted for multiple comparisons by calculating the false discovery ratio (FDR). The cutoff condition of correlation analyses was set as $|\rho| > 0.20$ and $FDR < 0.10$.

bovine liver, the relative contribution of peroxisomal β -oxidation to total oxidative capacity has been shown to be $>50\%$, suggesting that this pathway may be a component of the adaptations of FA metabolism in liver during the periparturient period, thus helping the liver cope with the large influx of FA from body fat mobilization (Grum et al., 1994, 1996, 2002). The main function of peroxisomal β -oxidation is the shortening of the FA chains, preparing them to be completely oxidized in the mitochondria (Drackley, 1999). The role of peroxisomes in metabolism of FA in the skeletal muscle of ruminants has not been determined. It is also probable that peroxisomal oxidation plays a role as an overflow pathway to oxidize FA in muscle of dairy cows during extensive FA mobilization that warrants future investigations.

We also evaluated the CPT1 ratio, as a potential marker for CPT1 deficiency (Fingerhut et al., 2001; CPT1 is a rate-limiting enzyme for long-chain FA entry into the mitochondria for β -oxidation. An elevation of this ratio has been described in CPT1 deficiency (Fingerhut et al., 2001). In our study, the ratio significantly decreased with the onset of lactation in both serum and muscle, reflecting increased mitochondrial entrance of long-chain FA. Incomplete FA β -oxidation downstream of CPT1 is associated with elevated levels of plasma ACC (Koves et al., 2008), as acyl-CoA in the

mitochondrial matrix can be converted into ACC for transport out of the mitochondria (Koves et al., 2008; Millington and Stevens 2011; Violante et al., 2013). The CPT2 ratio, calculated as the ratio of $C16:0 + C18:1$ to $C2$, is a potential marker to describe CPT2 deficiency (Gempel et al., 2002). Deficiency of CPT2 is associated with a pronounced elevation of $C16:0$ and $C18:1$ ACC while $C2$ is low, pointing to a significant reduction in long-chain FA oxidation. In the present study, the ratio was increased around parturition, likely indicating deficiency or impaired CPT2 functions. In this situation, long-chain ACC cannot be converted to their corresponding acyl-CoA esters, resulting in accumulation of long-chain ACC in the mitochondrial matrix, which are subsequently transported out of the mitochondria to the blood stream (Flanagan et al., 2010; Schooneman et al., 2013). It has been shown that high-fat overfeeding and an increased lipid exposure to skeletal muscle was associated with an increased expression of genes involved in the FA β -oxidation pathway, including *CPT1* that regulates the entry of acyl-CoA into the mitochondrial matrix (Muoio and Newgard, 2006; Noland et al., 2007; Turner et al., 2007). Interestingly, in the current study, the mRNA abundance of *CPT1B* (muscle isoform) increased 2.8-fold from d -21 to 1, followed by a decline thereafter, whereas that of *CPT2* remained unchanged over time. These data may

suggest a physiological increase in the capacity of long-chain fatty acyl-CoA entry into muscle mitochondria around parturition, but does not seem to coincide with upregulation of downstream metabolic pathways, such as the TCA cycle and respiratory chain. Thus, it is likely that post-CPT1 events, including deficiency or impaired in CPT2 function and depletion of several TCA cycle intermediates, cause an accumulation of ACC in skeletal muscle around parturition.

CONCLUSIONS

The serum and muscle concentrations of the ACC as well as mRNA expression of the carnitine acyltransferases CPT1B and CPT2 changed with time, but they were not affected by the CLA supplement at the dosage used. Muscle carnitine remained unchanged despite a decline in serum concentrations, likely due to increased carnitine excretion with milk and its uptake by the muscle to maintain intracellular concentrations. The elevated concentrations of muscle long-chain ACC species and serum C2 around parturition point to increased FA β -oxidation, which does not seem to coincide with an upregulation of downstream metabolic pathways, such as the TCA cycle and respiratory chain.

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