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Expression of metabolic sensing receptors in adipose tissues of periparturient dairy cows with differing extent of negative energy balance

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We recently showed that the mRNA expression of genes encoding for specific nutrient sensing receptors, namely the free fatty acid receptors (FFAR) 1, 2, 3, and the hydroxycarboxylic acid receptor (HCAR) 2, undergo characteristic changes during the transition from late pregnancy to lactation in certain adipose tissues (AT) of dairy cows. We hypothesised that divergent energy intake achieved by feeding diets with either high or low portions of concentrate (60% v. *30% concentrate on a dry matter basis) will alter the mRNA expression of* FFAR 1, 2, 3, *as well as* HCAR2 *in subcutaneous (SCAT) and retroperitoneal AT (RPAT) of dairy cows in the first 3 weeks* postpartum *(*p.p.*). For this purpose, 20 multiparous German Holstein cows were allocated to either the high concentrate ration (HC,* n *= 10) or the low concentrate ration (LC,* n *= 10) from day 1 to 21* p.p. *Serum samples and biopsies of SCAT (tail head) and RPAT (above the peritoneum) were obtained at day −21, 1 and 21 relative to parturition. The mRNA abundances were measured by quantitative PCR. The concentrations of short-chain fatty acid (SCFA) in serum were measured by gas chromatography-flame ionisation detector. The* FFAR1 *and* FFAR2 *mRNA abundance in RPAT was higher at day −21 compared to day 1. At day 21* p.p. *the* FFAR2 *mRNA abundance was 2.5-fold higher in RPAT of the LC animals compared to the HC cows. The* FFAR3 *mRNA abundance tended to lower values in SCAT of the LC group at day 21. The* HCAR2 *mRNA abundance was neither affected by time nor by feeding in both AT. On day 21* p.p. *the HC group had 1.7-fold greater serum concentrations of propionic acid and lower concentrations of acetic acid (trend: 1.2-fold lower) compared with the LC group. Positive correlations between the mRNA abundance of* HCAR2 *and peroxisome proliferator-activated receptor* γ*-2 (*PPARG2*) indicate a link between* HCAR2 *and* PPARG2 *in both AT. We observed an inverse regulation of* FFAR2 *and* FFAR3 *expression over time and both receptors also showed an inverse mRNA abundance as induced by different portions of concentrate. Thus, indicating divergent nutrient sensing of both receptors in AT during the transition period. We propose that the different manifestation of negative EB in both groups at day 21 after parturition affect at least* FFAR2 *expression in RPAT.*

Keywords: transition period, bovine adipose tissue, metabolic sensing

Implications

At the onset of lactation, the feed intake does not increase to the same extent as the energy requirements for milk synthesis resulting in a negative energy balance in high-yielding dairy cows. To cover the energy requirements of increasing milk synthesis, the mobilisation of energy from adipose tissue is crucial during the peripartal period. We studied the effects of two different concentrate portions in the feeding ration on the transcriptional regulation of four different nutrient sensing receptors, involved in adipose tissue metabolism. We used adipose tissue from a subcutaneous and a visceral localisation. The findings of our study may help to understand, in parts, how the energy density of the ration may affect the metabolism of adipose tissue due to the expression of the investigated receptors.

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Introduction

One of the most challenging times for high-yielding dairy cows is the transition from late pregnancy to early lactation. The energy requirements in early lactation cannot be entirely met by voluntary feed intake and the animals therefore enter a state of negative energy balance (EB) during which lipolysis increases to provide non-esterified fatty acids (NEFA) from adipose tissue (AT) as energy substrates for other organs or precursors for milk fat synthesis (Drackley, 1999). In a previous study we observed differential expression of particular G protein-coupled receptors (GPCR) mRNAs, which are involved in energy and metabolic sensing, due to lactation-induced changes in EB (Friedrichs *et al.*, 2014). These GPCRs belong to the family of free fatty acid receptors (FFAR) 1, 2, and 3 (also known as GPCR40, GPCR43 and GPCR41, respectively). They enable free fatty acids (FFA), as their ligands, to act as signaling molecules (Stoddart *et al.*, 2008). The FFAR1 is a target for saturated and unsaturated medium and long chain fatty acids (LCFA) (Brown *et al.*, 2005). The FFAR1 is most abundant in insulin-producing pancreatic β-cells, but it is also expressed in other tissues; however, its physiological role in adipose is not clear. In contrast to FFAR1, the physiological functions of FFAR2 and FFAR3 have been identified and comprise an inhibition of lipolysis in adipocytes through activation of FFAR2 (Hong *et al.*, 2005) and increased leptin secretion by activation of FFAR3 (Xiong *et al.*, 2004). The receptors FFAR2 and FFAR3 are activated by short-chain fatty acids (SCFA). In cattle the affinity of FFAR2 and FFAR3 for FFA is different to human or murine receptors with preference for FFA with a longer carbon backbone: FFAR2 displaying affinity $C6 > C5 > C4 = C7 > C3 = C8 > C2 = C9$ and the bovine FFAR3 displays no affinity for C1 (Hudson *et al.*, 2012). Besides serving as major substrates for energy production in ruminants, SCFA have various other regulatory effects (Bergman, 1990). They may increase blood insulin and glucagon concentrations in ruminants (Harmon, 1992), regulate gene expression *in vitro* (Li *et al.*, 2007) and exert immune-modulatory effects; for example FFAR2 is involved in granule release from bovine neutrophils induced by propionate (Carretta *et al.*, 2013). It is well documented that the energy density of the diet influences the microbial SCFA production in the rumen, for example a high energy content or high concentrate proportion in the diet increases the production of propionate (Rabelo *et al.*, 2003).

The hydroxycarboxylic acid receptor (HCAR) 2 (previously termed GPCR109A) is mainly expressed in adipocytes and can be activated by niacin and β-hydroxybutyrate (BHBA) as an endogenous ligand. Due to its function as metabolic sensor suppressing lipolysis during starvation, HCAR2 is an important target for a group of antilipolytic drugs (Offermanns *et al.*, 2011). We recently reported that the HCAR2 ligand niacin stimulates the expression of *HCAR2* in differentiated bovine preadipocytes *in vitro* (Kopp *et al.*, 2014). Comparable to other mammalian species, the retroperitoneal AT (RPAT) from dairy cattle seems to have a higher lipolytic activity than subcutaneous AT (SCAT) due to the higher expression of hormone-sensitive lipase and the greater lipolytic response to adrenergic stimulation in RPAT (Locher *et al.*, 2011; Kenéz *et al.*, 2013). In dairy cattle two isoforms of the transcription factor peroxisome proliferator-activated receptor γ (PPARG), namely PPARG and its isoform PPARG-2 were described (Sundvold *et al.*, 1997). Both members of the nuclear-receptor family bind various fatty acids and their activation is associated with the improvement of insulin sensitivity by increasing glucose and fatty acid uptake. The isoform PPARG2 is the leading isoform in adipose tissue and is involved in the regulation of adipocyte differentiation (Hammarstedt *et al.*, 2005; Tyagi *et al.*, 2011). The mRNA abundance of *PPARG* in mice and of both receptors in SCAT of dairy cows were shown to be positively linked with *HCAR2* (Wanders *et al.*, 2012; Friedrichs *et al.*, 2014).

Therefore, we hypothesised that differing portions of concentrate in and niacin supplementation to the diet of dairy cattle in the first 3 weeks *postpartum* (*p.p.*) will alter the expression of metabolic sensing receptors in two adipose depots. In this study we describe the mRNA expression of genes encoding *FFAR1*, *FFAR2*, *FFAR3* and *HCAR2* in a SCAT and RPAT of cows in the transition period as influenced by time and the diet fed *p.p.*

Material and methods

Animals, feeding and sample collections

This study was conducted at the experimental station of the Friedrich Loeffler Institute, Federal Research Institute for Animal Health, Braunschweig, Germany. All animal experiments were conducted according to the European Community regulations concerning the protection of experimental animals and were approved by the Lower Saxony state office for consumer protection and food safety (LAVES, Oldenburg, Germany). The experimental design has been described in detail elsewhere (Locher *et al.*, 2011). Briefly, 20 multiparous pregnant German Holstein cows, dried off 8 weeks before calving, were fed according to the recommendations of the German Society of Nutrition Physiology (GfE, 2001). On day 1 *p.p.* one half of the cows ($n = 10$) was allocated to either the high-concentrate (HC) group receiving a diet with a 60 : 40 concentrate-to-roughage ratio (on a dry matter (DM) basis) or the low-concentrate (LC) group receiving a diet with a 30 : 70 concentrate-to-roughage ratio. The HC diet comprised 24% and 16% and the LC diet 42% and 28% corn and grass silage, respectively. One half of each dietary group also received as part of the pelletized concentrate 24 g powdered niacin per day which contained at least 99.5% nicotinic acid (Lonza Ltd, Basel, Switzerland). All diets were fed individually as a total mixed ration. The detailed composition of the diet as well as nutrient, fiber, and energy content of the different feed ingredients fed *p.p.* is provided in Supplementary Table S1 and elsewhere (Locher *et al.*, 2011). Milk yield was recorded daily by a milk meter (Lemmer-Fullwood GmbH, Lohmar, Germany) and milk composition was analysed twice a week by a milk analyser

Table 1 *Sequences of the primer and real-time PCR conditions used for the quantification of the target genes in the adipose tissue of dairy cows*

Gene[1]	Forward Primer Sequence (5′-3′) Reverse Primer Sequence (5′-3′)	Acc. no.[2]	bp	Con. (nM)[3]	Mean Cq[4]	Annealing (s|°C)[5]	Elongation (s)[6]	Efficiency (%)
FFAR1	AATTCCACCAGCTCCTTGGGCAT GGCCGCCTTTAGCTTCCGTCT	NM_001309646	213	800	35.7	60|60	60	100.2
FFAR2	CGCTCCTTAATTTCCTGCTG CAAAGGACCTGCGTACGACT	NM_001163784	174	800	34.6	60|60	60	109.6
FFAR3	ACCTGATGGCCCTGGTG GGACGTGAGATAGATGGTGG	NM_001145233	215	200	35.0	40|60	30	104.0
HCAR2	GGACAGCGGGCATCATCTC CCAGCGGAAGGCATCACAG	XM_010823378	140	200	28.9	30|61	30	100.5
PPARG2	ATTGGTGCGTTCCCAAGTTT GGCCAGTTCCGTTCAAAGAA	Y12420	57	400	26.2	60|60	60	108.0

bp = base pairs.
[1]FFAR1 = free fatty acid receptor 1 (Friedrichs *et al.*, 2014); FFAR2 = free fatty acid receptor 2 (Hosseini *et al.*, 2012); FFAR3 = free fatty acid receptor 3 (Friedrichs *et al.*, 2014); HCAR2 = hydroxycarboxylic acid receptor 2 (Lemor *et al.*, 2009); PPARG2 = peroxisome proliferator-activated receptor gamma 2 (Saremi *et al.*, 2014).
[2]Acc. No. = NCBI Accession Number.
[3]Concentrations for each primer.
[4]Mean quantification cycle from SCAT and RPAT.
[5]Initial denaturation for 10 min at 90°C; denaturation for 30 s at 95°C.
[6]Extension at 72°C.

based on Fourier transform infrared spectroscopy (Milkoscan FT 6000; Foss Electric, Hillerød, Denmark). The individual DM intake was recorded by a computerised feeding system (Insentec BV, Marknesse, the Netherlands). Data for milk yield, milk composition and DM intake were pooled for each week of lactation. Energy balance was calculated as follows: EB = NE_L intake – energy in milk – NE_M (GfE, 2001). On day −21, 1, and 21 relative to parturition, blood and AT samples were obtained. Blood samples were drawn from the jugular vein in the morning and centrifuged at 2000 × **g** for 10 min to separate serum and plasma. The AT samples were obtained by biopsy as described previously (Locher *et al.*, 2011), whereby the SCAT samples were taken from the tail head region and the RPAT samples were taken directly above the peritoneum each time alternating from the left and right flank. The AT samples were snap-frozen in liquid nitrogen. The serum, plasma and AT samples were stored at −80°C until further processed.

Measurement of SCFA in serum

The concentrations of serum SCFA were determined according to the method described by Kristensen (2000). Briefly, 450 μl plasma was treated an internal standard solution (50 μl 2-ethyl butyrate, 75.45 μmol/10 ml), 700 μl 2-chloroethanol and 700 μl acetonitrile. Samples were centrifuged (15 min, 4°C, 3000 × **g**) and 1600 μl of the supernatant was combined with 20 μl 0.5 N NaOH and 1600 μl heptane. After mixing for 30 s, the aqueous phase (1300 μl) was removed and treated with 10 μl 37% HCl, 100 μl pyridine and 50 μl 2-chloroethyl chloroformate. After 5 min at RT, the reaction mixture was extracted with 2500 μl H_2O and 100 μl chloroform and the organic phase was separated after centrifugation (3 min, RT, 3000 × **g**). The organic phase was dried with Na_2SO_4 (15 mg) for 30 min and 1 μl injected into a gas chromatograph equipped with a flame ionisation detector (Series 17A; Shimadzu Corp., Kyoto, Japan). Separation was achieved on a free fatty acid phase column (length 25 m, internal diameter 0.25 mm, particle diameter 0.25 μm) (Kristensen, 2000).

Relative quantification of mRNA

After homogenisation of the AT samples with the Precellys®24 system (peQLab Biotechnology, Erlangen, Germany), total RNA was extracted from each sample using Trizol (Invitrogen, Karlsruhe, Germany) with a subsequent DNase (Qiagen, Hilden, Germany) treatment in solution and purified using spin columns (RNeasy® Mini Kit, Qiagen, Hilden, Germany). From 1 μg total RNA a reverse transcription with RevertAid™ (Fermentas, St. Leon-Rot, Germany) in a Multicycler PTC 200 (MJ Research, Watertown, MA) was performed resulting in an 80 μl cDNA reaction volume. The real-time PCR mixes, with a total volume of 10 μl consisting of 2 μl cDNA (diluted 1 : 4) as template, 1 μl primer mix, 2 μl water and 5 μl SYBR Green JumpStart Taq Readymix (Sigma-Aldrich, Nümbrecht, Germany), were performed in an Mx3000P (Agilent, Santa Clara, CA, USA) in three replicates. The sequences of the primers used and the conditions used in qPCR are provided in Table 1. All PCR products were confirmed by sequencing. Relative quantification of the target genes using efficiency corrected data was performed with standard curves diluted from cDNA except in case of *FFAR3*, for which a dilution series based on the purified amplicon was used. The qPCR efficiency of the target genes are also provided separately in Table 1 and those of the reference genes were in the range from 98.3% to 103.1%.

Reference gene stability and data analysis

To determine the most stably expressed genes for subsequent data normalisation, a set of seven genes was tested with qBASE^{plus} 2.0 (Biogazelle, Ghent, Belgium) separately

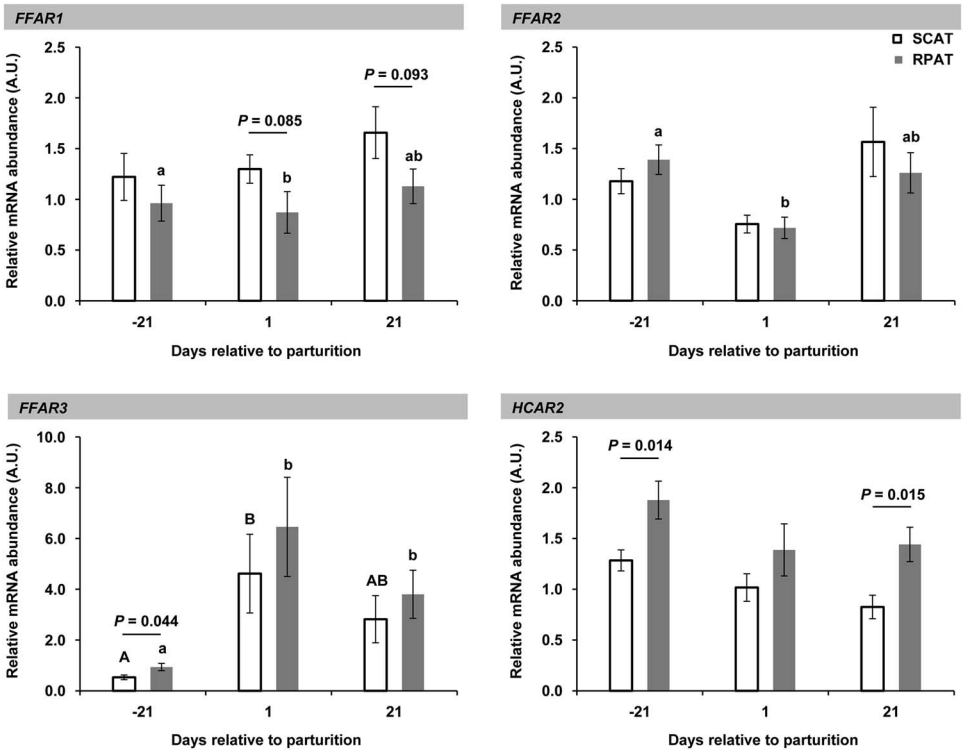

Figure 1 Relative mRNA abundance of the free fatty acid receptor 1, 2, and 3 and hydroxycarboxylic acid receptor 2 at day −21, 1 and 21 relative to parturition in subcutaneous (SCAT, white bars) and retroperitoneal adipose tissue (RPAT, grey bars) of dairy cows. Pooled data from the cows fed either low or high portions of concentrate low are shown (means ± SEM) to compare the mRNA abundances between both AT. A line indicates a difference between the tissues with the corresponding *P*-value written above. Different small letters and different capital letters indicate significant differences in between the sampling dates in SCAT and in RPAT ($P < 0.05$), respectively. For normalisation enabling a comparison of both tissues, the ratio of the mRNA abundance of the gene of interest and the geometric mean of the mRNA abundance of low density lipoprotein receptor-related protein 10, RNA Polymerase II and emerin was used.

for each tissue and for both tissues combined. Based on the stability of their expression as final reference genes, low density lipoprotein receptor-related protein 10, RNA Polymerase II and emerin were used for RPAT and comparison of both tissues; for SCAT, marvel domain containing 1 was additionally used for normalisation. Data are presented as ratio of the mRNA abundance of the gene of interest and the geometric mean of the corresponding reference genes. The characteristics of the primers and their real-time PCR conditions used for the reference genes are described elsewhere (Saremi *et al.*, 2012).

Statistical analyses

The statistical analyses were performed with the software package SPSS (version 21.0, SPSS Inc., Chicago, IL, USA). All data are presented as arithmetic means ± SEM, significance was set at $P < 0.05$ and a trend was noted when $0.05 < P < 0.10$. Data were tested for normal distribution using the Kolmogorov–Smirnov test and homogeneity of variances was tested using the Levene's test. Accordingly, the general linear model or Mann–Whitney U test was used for comparing the HC *v.* the LC group or the groups with and without niacin supplementation. To test for differences between the sampling dates, the ANOVA or the Wilcoxon signed-rank test followed by Bonferroni correction was used. Parametric testing was performed for *FFAR1* mRNA abundance in RPAT and *HCAR2* mRNA abundance in both tissues. For correlation analyses, the Spearman's rank correlation coefficient (two-tailed) was calculated.

Results

Temporal effects

As shown in Figure 1, the mRNA abundance of *FFAR1* and *FFAR2* in SCAT remained unchanged during the transition period. In contrast, an effect of time with greater abundance of *FFAR1* and *FFAR2* mRNA on day −21 in comparison to day 1 relative to calving ($P < 0.01$) was observed in RPAT. The mRNA abundance of *FFAR3* was lower at day −21 compared to day 1 relative to calving in both tissues (SCAT: $P < 0.05$; RPAT: $P < 0.05$); in RPAT day −21 was also lower compared to day 21 *p.p.* ($P < 0.05$). The *HCAR2* mRNA was neither affected by time nor by treatment.

At day 1 after parturition propionic acid, n-butyric acid, n-valeric acid, and n-caproic acid were detectable albeit at low levels in <50% of the samples. For these SCFA we compared only day −21 with day 21 relative to calving (Figure 2). Except for n-butyric acid and n-valeric acid, all SCFA and total SCFA concentrations were lower on day −21 than on day 21; acetic acid ($P < 0.01$) and total SCFA

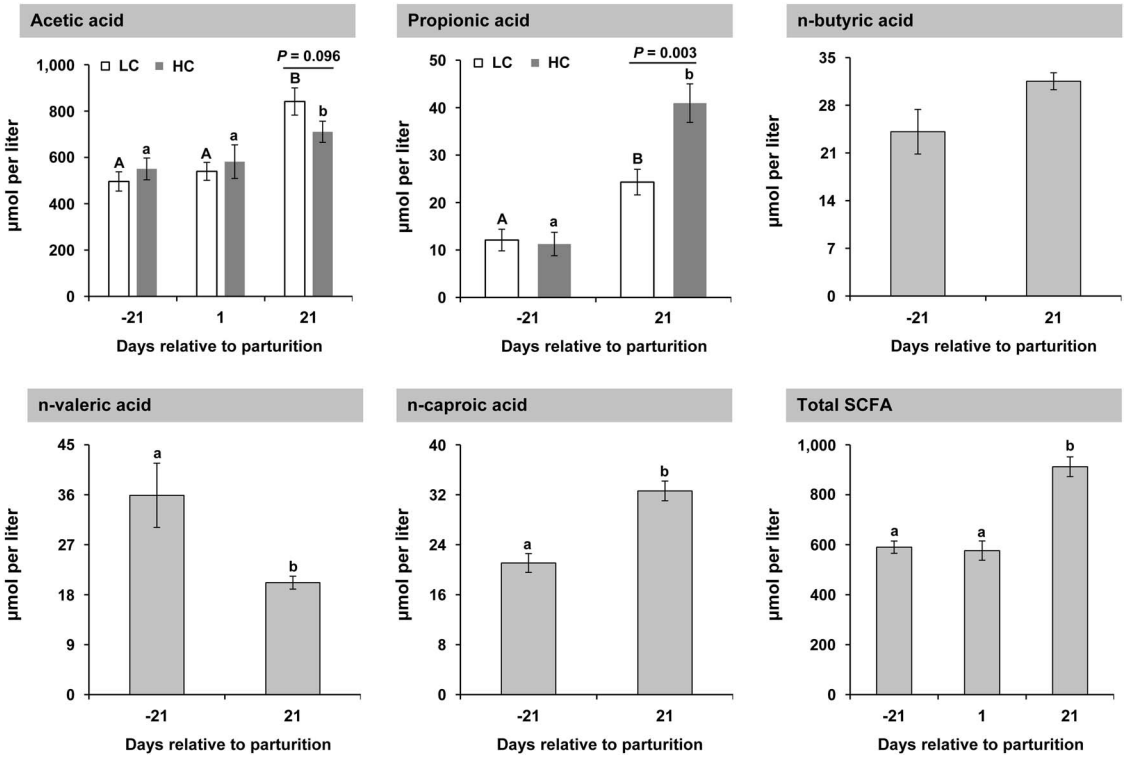

Figure 2 Specific short-chain fatty acids (SCFA) and total SCFA in serum of dairy cows on day −21 and 21 relative to calving, for acetic acid and total SCFA additionally at day 1 relative to calving. Except for acetic and propionic acid, pooled data from cows fed either low (LC; fed a diet with 30 : 70 concentrate-to-roughage ratio; white bars) or high portions of concentrate (HC; fed a diet with 60 : 40 concentrate-to-roughage ratio; dark grey bars) are shown (means ± SEM; light grey bars), because no effect of different portions of concentrate in the diet were observed. Different letters indicate significant differences between the sampling dates ($P < 0.05$), whereas in the first two graphs capital letters indicate differences in the LC group and small letter indicate differences in the HC group. The effects of different portions of concentrate in the diet are defined using a line with the corresponding *P*-value written above.

($P < 0.01$) in serum were also higher at day 21 compared with day 1 after parturition. The concentrations of n-butyric acid did not change with time and n-valeric acid was higher at the beginning compared to the end of the transition period ($P < 0.05$). Acetic acid formed the highest portion of total SCFA.

Dietary effects

In this study we found no effect of supplementing niacin on any of the variables investigated. For this reason, data of the subgroups (with or without niacin) were each pooled within the HC and the LC group for all further analyses.

With the exception of *FFAR2* in RPAT, we detected no differences between the HC and the LC group (Table 2); in RPAT the mRNA abundance of *FFAR2* was greater in the LC animals than in the HC cows at day 21 relative to calving ($P < 0.01$). The abundance of *FFAR3* mRNA tended to lower values in SCAT of LC *v.* HC animals ($P < 0.1$).

On day 21 after calving the LC group, compared to the HC group, had 1.7-fold lower serum concentrations of propionic acid ($P < 0.01$) and 1.2-fold higher concentrations of acetic acid serum as a trend ($P < 0.01$; Figure 2). In Table 3 the performance data and the concentrations of particular and total SCFA in serum from day 21 *p.p.* are listed separately for the LC and the HC group.

Location effects

When comparing both tissues, we observed a trend for a lower *FFAR1* mRNA abundance in RPAT compared to SCAT at day 1 ($P < 0.1$) and 21 ($P < 0.1$) after parturition. The expression of *FFAR2* was not different between SCAT and RPAT at the different sampling dates. In RPAT, *FFAR3* mRNA abundance was greater compared to *FFAR3* mRNA abundance in SCAT at day −21 relative to calving ($P < 0.05$). Also *HCAR2* mRNA abundance was higher in RPAT compared to SCAT at day −21 ($P < 0.05$) and also at day 21 ($P < 0.05$) relative to calving.

Results of the correlation studies

As summarised in Table 4 (*r* values reported in the table), in SCAT the mRNA abundance of *FFAR1* and *FFAR2* was positively correlated ($P < 0.01$), but not in RPAT. In both AT the mRNA abundance of *HCAR2* was positively correlated with *FFAR3* (SCAT: $P < 0.01$; RPAT: $P < 0.01$) and peroxisome proliferator-activated receptor γ-2 (*PPARG2*; SCAT: $P < 0.01$; RPAT: $P < 0.01$). The *HCAR2* mRNA abundance in SCAT was related to the one in RPAT ($P < 0.01$); *HCAR2* and *PPARG2* were interrelated among RPAT and SCAT ($P < 0.05$). The independent analysis of both feeding groups revealed partly higher correlation coefficients which was associated at least partly with the observation that the correlation between the

Table 2 *Relative tissue mRNA abundance of free fatty acid receptor 1, 2, and 3 and hydroxycarboxylic acid receptor 2 in subcutaneous and retroperitoneal adipose tissue of dairy cows fed either high or low portions of concentrate*

			Relative tissue mRNA abundance[3]		
Gene[1]	Day	F[2]	SCAT	RPAT	SEM[4]
FFAR1	−21	–	1.18	1.17	0.20
	1	–	1.11	1.06	0.17
	21	HC	0.96	1.53	0.25
		LC	1.93	1.20	0.31
FFAR2	−21	–	1.12	1.53	0.13
	1	–	0.77	0.72	0.10
	21	HC	2.11	0.74*	0.34
		LC	0.98	1.85*	0.26
FFAR3	−21	–	0.64	0.73	0.12
	1	–	4.28	4.91	1.74
	21	HC	6.03†	3.16	1.25
		LC	1.20†	3.09	1.18
HCAR2	−21	–	1.43	1.59	0.15
	1	–	1.25	1.17	0.20
	21	HC	1.47	1.34	0.20
		LC	1.40	1.09	0.19

*Values within a column marked with a star differ significantly at $P < 0.05$ between the HC and LC group at day 21 after parturition.
†Values within a column marked with a cross differ at $P < 0.10$ (trend) between the HC and LC group at day 21 after parturition.
[1]FFAR1 = free fatty acid receptor 1; FFAR2 = free fatty acid receptor 2; FFAR3 = free fatty acid receptor 3; HCAR2 = hydroxycarboxylic acid receptor 2.
[2]F = feeding; HC = high concentrate group fed a diet with 60 : 40 concentrate-to-roughage ratio; LC = low concentrate group fed a diet with 30 : 70 concentrate-to-roughage ratio. Both diets were fed from day 1 to 21 relative to calving.
[3]SCAT = s.c. adipose tissue; RPAT = retroperitoneal adipose tissue. Given are means presented as ratios of the mRNA abundance of the gene of interest and the geometric mean of the corresponding reference genes. For SCAT, lipoprotein receptor-related protein 10, RNA Polymerase II, emerin and marvel domain containing 1 were used for normalisation. For RPAT, lipoprotein receptor-related protein 10, RNA Polymerase II and emerin were used for normalisation. The values for day 21 are shown separately concerning to feeding on a high concentrate *v.* on a low concentrate diet.
[4]SEM = standard error of the mean. The pooled standard error of the mean relative mRNA abundance in SCAT and RPAT is given.

receptor mRNAs exist only within the LC group. This was relevant for FFAR3 compared to FFAR1 ($r = -0.516$; $P < 0.05$) as well as compared to FFAR2 ($r = -0.598$; $P < 0.01$), HCAR1 and FFAR1 ($r = -0.654$; $P < 0.01$) within SCAT. In RPAT HCAR2 correlated with FFAR2 only within the LC group ($r = 0.494$; $P < 0.01$) but with FFAR3 only within the HC group ($r = 0.606$; $P < 0.01$).

We observed a positive correlation between *FFAR1* mRNA abundance in SCAT and n-caproic acid concentration in serum ($r = 0.322$; $P < 0.05$) and a negative correlation between *FFAR3* mRNA abundance in SCAT and n-butyric concentration in serum ($r = -0.369$; $P < 0.05$). The *FFAR2* mRNA abundance in SCAT was correlated with the glucose concentrations in serum ($r = 0.346$; $P < 0.01$). The target mRNA measured in RPAT showed no correlation with the measured SCFA at all. The *FFAR2* mRNA abundance in RPAT and the BHBA concentration in the circulation were correlated ($r = 0.307$; $P < 0.05$). In RPAT the *FFAR3* mRNA abundance was negatively correlated with the triglyceride concentrations in serum ($r = -0.406$; $P < 0.01$) and positively with the ones of NEFA in serum ($r = 0.287$; $P < 0.05$), respectively. We also observed a negative correlation between *FFAR3* mRNA abundance in RPAT and EB ($r = -0.585$; $P < 0.05$).

Discussion

Changes in FFAR1 expression

Less information is available about the influence of dietary components or metabolites on *FFAR1* expression (Kebede *et al.*, 2012). In the present study in dairy cows, the concentrate portion in the diet had no effect on the *FFAR1* mRNA expression in both AT depots. A recent study in mice showed that the digestibility of fibre affects *FFAR1* expression in AT, that is, soluble fibre compared to insoluble fibre intake was accompanied with an increased SCFA production in the colon and was associated with increasing *FFAR1* mRNA abundance in the epididymal AT (Isken *et al.*, 2010). However, this link could not be confirmed with our results for dairy cows. The reasons for the downregulation of this receptor from late pregnancy to early lactation observed herein and its physiological consequences in RPAT remain unknown as does the function of this receptor in adipocytes. Generally, the *FFAR1* gene expression in AT is very low compared to pancreas and brain in humans (Itoh *et al.*, 2003), and lower in AT compared to liver in cattle (Friedrichs *et al.*, 2014). Therefore, the importance of the regulation of FFAR1 mRNA or the corresponding protein should be verified in future using adequate models.

Table 3 *Performance data and serum concentrations of metabolites and short chain fatty acids of dairy cows fed either high or low portions of concentrate*

Item	HC[1]	LC[2]	SEM[3]	*P*-value
DMI (kg/day)[4]	18.93[a]	16.32[b]	0.58	<0.01
NE_L (MJ/day)[4]	141.7[a]	113.7[b]	4.14	<0.01
Milk yield (kg/day)[4]	37.0	31.4	1.82	<0.10
Milk fat (%)[4]	4.3[a]	5.0[b]	0.19	<0.05
Milk protein (%)[4]	3.4[a]	3.1[b]	0.07	<0.05
ECM (kg/day)	40.6	37.9	2.39	0.44
EB (MJ/day)[4]	−15.3[a]	−33.7[b]	5.85	<0.05
BHBA (mmol/l)[4]	0.47[a]	0.76[b]	0.08	<0.05
Triglyceride (μmol/l)	0.13[a]	0.22[b]	0.03	<0.05
NEFA (μmol/l)	674.5	848.8	170.79	0.29
Acetic acid (μmol/l)	710.5	841.2	52.15	<0.10
Propionic acid (μmol/l)	40.9[a]	24.3[b]	3.38	<0.01
n-Butyric acid (μmol/l)	32.2	31.0	1.78	0.66
n-Valeric acid (μmol/l)	21.9	18.4	1.54	0.14
n-Caproic acid (μmol/l)	32.5	32.7	2.28	0.95
Total SCFA (μmol/l)	867.2	957.5	54.63	0.26

[a,b]Values within a row with different superscript letters differ significantly at $P < 0.05$ between the HC and LC group at day 21 after parturition or in the third week of lactation.
[1]HC = high concentrate group fed a diet with 60 : 40 concentrate-to-roughage ratio.
[2]LC = low concentrate group fed a diet with 30 : 70 concentrate-to-roughage ratio. Both diets were fed from day 1 to 21 relative to calving. Given are means. Performance data are given as means for the third week of lactation; all variables assessed in serum were obtained on day 21 *postpartum*.
[3]SEM = standard error of the mean. The pooled standard error of the mean values from the HC group and LC group is given.
[4]Data has been published previously by Locher *et al.* (2011).

Table 4 *Coefficients of correlation (Spearman) between relative mRNA abundance of receptors involved in nutrient sensing and peroxisome proliferator-activated receptor γ-2 in subcutaneous and retroperitoneal adipose (SCAT and RPAT, respectively) tissue of dairy cows*

		SCAT				RPAT
Tissue	Gene[1]	*FFAR1*	*FFAR2*	*FFAR3*	*HCAR2*	*HCAR2*
SCAT	*FFAR1*	–	0.40**	ns	−0.29*	ns
	FFAR2		–	−0.38*	−0.31*	ns
	FFAR3			–	0.50**	ns
	HCAR2				–	0.44**
	PPARG2	−0.32*	ns	ns	0.54**	0.30*
RPAT	*FFAR1*	ns	ns	ns	ns	ns
	FFAR2	ns	ns	ns	ns	ns
	FFAR3	ns	ns	ns	ns	0.46**
	HCAR2	ns	ns	ns	0.44**	–
	PPARG2	ns	ns	ns	ns	0.45**

**$P < 0.01$; *$P < 0.05$.
[1]FFAR1 = free fatty acid receptor 1; FFAR2 = free fatty acid receptor 2; FFAR3 = free fatty acid receptor 3; HCAR2 = hydroxycarboxylic acid receptor 2, PPARG2 = peroxisome proliferator-activated receptor γ-2.

Changes in FFAR2 and FFAR3 expression related to SCFA content in blood

In differentiated murine adipocytes, acetic and propionic acid were demonstrated to stimulate the gene expression of *FFAR2 in vitro* and both fatty acids also affected adipogenesis and adipocyte differentiation (Hong *et al.*, 2005). In the current study, the LC diet expectedly resulted in decreased concentrations of propionic acid in serum and in a trend for increased acetic acid concentrations. Concomitantly the mRNA abundance of *FFAR2* was increased in RPAT. Even though acetic acid is the most abundant SCFA in the circulation, the pEC_{50} potency values for the bovine FFAR2 and FFAR3 reported by Hudson *et al.* (2012) are in the millimolar range and let us assume that the circulating concentrations of acetic acid recorded in the cows of the present study were not able to activate either receptor. Based on their characterised affinities for SCFA (Hudson *et al.*, 2012) we speculate that the plasma concentration of butyric acid may stimulate both receptors.

It remains open as to whether the observed changes of the abundance of *FFAR2* mRNA in RPAT were triggered by the altered circulating SCFA concentrations in the animals or not. An *in vitro* study on bovine adipose tissue explants showed no effect of propionic acid on *FFAR2* and *FFAR3* expression (Hosseini *et al.*, 2012) and a recent study on human cell culture explants from omental AT showed no effect of neither acetic acid nor propionic acid on *FFAR2* expression during differentiation (Dewulf *et al.*, 2013). The previously reported link between peroxisome proliferator-activated receptor γ (PPARG) and FFAR2 in mice (Dewulf *et al.*, 2013) could not be confirmed by our study. Thus, the observed weak correlation between the mRNA abundance of *PPARG2*, the most prominent PPARG isoform in AT (Tyagi *et al.*, 2011) and *FFAR2* in cattle, point to species specificity. As reported previously (Locher *et al.*, 2011), animals from the LC group underwent a more negative EB in the third week of lactation, showed higher BHBA concentrations and had numerically higher NEFA concentrations (although there was no significant dietary effect), at day 21 after parturition, indicating a more extensive fat mobilisation in LC animals. Expressional up-regulation and activation of *FFAR2* in these animals seems to counteract extensive lipolysis in RPAT due to its role in inhibition of lipolysis in adipocytes (Hong *et al.*, 2005) supported by the negative correlation between *FFAR2* mRNA abundance and EB. However, the herein observed decline in *FFAR2* mRNA abundance in RPAT from late pregnancy to the onset of lactation might be associated with the declining EB during this time period to reduce the inhibitory effects of FFAR2 on lipolysis and thus to enable the necessary *p.p.* catabolism. In previous works, we also observed no differences of *FFAR2* mRNA abundance in SCAT comparing day −21, 1 and 21 relative to calving, but differential expression between the different adipose depots (Friedrichs *et al.*, 2014). The expression of *FFAR3* mRNA was inversely related to *FFAR2* mRNA with higher *FFAR3* mRNA abundance in late pregnancy compared to the onset of lactation in RPAT. Another indication for the inverse expressional regulation of both receptors is the detected trend with lower values for *FFAR3* mRNA abundance in the SCAT of animals from the LC group and the negative correlation between *FFAR2* and *FFAR3* mRNA abundance in SCAT. The negative correlation between *FFAR2* and *FFAR3* mRNA abundance in SCAT occurred solely in the LC group which might indicate a greater importance of these receptors in the animals that underwent a more severe negative EB.

Based on the higher responsiveness of RPAT *v.* SCAT towards lipolytic stimuli in cattle (Locher *et al.*, 2011; Kenéz *et al.*, 2013) and the herein observed changes with time and energy intake, the differential expression of *FFAR2* in RPAT suggests that energy sensing might be more important in RPAT than SCAT. In addition, the circulating concentrations of propionic acid achieved by the feeding regime using either high or low portions of concentrate were related to the mRNA abundance of *FFAR2* in RPAT, but not in SCAT, also indicating that metabolic sensing through FFAR2 to adapt lipolysis might be more important in RPAT than SCAT. We also provide evidence for an inverse differential expression of both receptors in RPAT of dairy cattle in the periparturient period. With the results for the correlation between *FFAR1* and *FFAR2* mRNA abundance we can confirm the previously suggested concordant regulation of these receptors expression in SCAT (Friedrichs *et al.*, 2014).

Changes in HCAR2 expression

The circulating concentrations of BHBA, the endogenous ligand of HCAR2, were different between HC and LC animals, but the mRNA abundance of *HCAR2* in both AT was not affected by the diet. In an *in vitro* study on bovine SCAT and RPAT explants the lack of BHBA-induced change on *HCAR2* expression has already been described, attributed the lack to the short duration of the treatment (Hosseini *et al.*, 2012). Based on our previous and present results we speculate that BHBA is not regulating the transcription of *HCAR2* in bovine AT. However, in our study the *HCAR2* expression did not differ between the animals fed a diet supplemented with niacin compared to the animals fed a diet without niacin. A possible explanation for the missing effect could be the ruminal degradation of the niacin, as the supplement used herein was not rumen-protected.

In another study we found a decrease of the receptor mRNA abundance from late pregnancy (day −21) to mid- (day 105) and late-lactation (day 252 relative to calving), but also not in the time interval considered in the current study (Lemor *et al.*, 2009; Friedrichs *et al.*, 2014). We thus assume that the expressional regulation of *HCAR2* and the feedback of BHBA on lipolysis via this receptor are only relevant if BHBA concentrations are in a range observed during ketosis. Effects of high BHBA concentrations on lipolysis were shown by Kenéz *et al.* (2014) *in vitro* but not evident in the current study at least at sampling on day 21 *p.p.* Alternatively or in addition it might be that up-regulation is more relevant in stages of positive EB to limit lipolysis. Irrespective of time, in our study the mRNA abundance of HCAR2 was higher in RPAT than in SCAT, whereas a recent report describes greater HCAR2 protein abundance in SCAT than RPAT (Kenéz *et al.*, 2014). However, a study in rumen-fistulated Holstein steers showed no difference in HCAR2 protein as well as mRNA abundance between SCAT and RPAT, respectively (Titgemeyer *et al.*, 2011).

A correlation between *HCAR2* and *PPARG2* in SCAT has been reported for cattle previously (Friedrichs *et al.*, 2014) and this association can be extended to bovine RPAT based on our present results. Similarly, *HCAR2* was shown to be positively associated with *PPARG* in mice (Wanders *et al.*, 2012). Feeding a high-fat diet reduced *HCAR2* expression in murine epididymal adipose tissue and correlated with a decline in *PPARG* expression (Wanders *et al.*, 2010). In both AT investigated the mRNA abundance of *PPARG2* was not affected by the concentrate portion fed to the animals; considering the potential regulatory impact of PPARG on *HCAR2* expression, this might explain the unaltered *HCAR2* mRNA abundance. Thus we assume that *HCAR2* is regulated through PPARG rather than by its endogenous ligand BHBA.

This assumption is in line with the study of Kopp *et al.* (2014) in which the mRNA abundance of *HCAR2* was not down-regulated in bovine adipocytes *in vitro* by uncoupling of G-protein signalling using pertussis toxin.

Conclusions

Energy balance after parturition affects the mRNA abundance of *FFAR* an *HCAR2* in RPAT and SCAT differently. Based on our results divergent nutrient sensing by FFAR2 and FFAR3 in AT of dairy cows is indicated. The adjustment of lipolysis by *HCAR2* mRNA abundance may take place only in case of subclinical or clinical BHBA concentrations during the transition period, alternatively in case of positive EB to limit lipolysis.

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Supplementary Material

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