Effect of increasing body condition on key regulators of fat metabolism in subcutaneous adipose tissue depot and circulation of nonlactating dairy cows

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ABSTRACT

In response to negative energy balance, overconditioned cows mobilize more body fat than thin cows and subsequently are prone to develop metabolic disorders. Changes in adipose tissue (AT) metabolism are barely investigated in overconditioned cows. Therefore, the objective was to investigate the effect of increasing body condition on key regulator proteins of fat metabolism in subcutaneous AT and circulation of dairy cows. Nonlactating, nonpregnant dairy cows ($n = 8$) investigated in the current study served as a model to elucidate the changes in the course of overcondition independent from physiological changes related to gestation, parturition, and lactation. Cows were fed diets with increasing portions of concentrate during the first 6 wk of the experiment until 60% were reached, which was maintained for 9 wk. Biopsy samples from AT of the subcutaneous tailhead region were collected every 8 wk, whereas blood was sampled monthly. Within the experimental period cows had an average BW gain of 243 ± 33.3 kg. Leptin and insulin concentrations were increased until wk 12. Based on serum concentrations of glucose, insulin, and nonesterified fatty acids, the surrogate indices for insulin sensitivity were calculated. High-concentrate feeding led to decreased quantitative insulin sensitivity check index and homeostasis model assessment due to high insulin and glucose concentrations indicating decreased insulin sensitivity. Adiponectin, an adipokine-promoting insulin sensi-

tivity, decreased in subcutaneous AT, but remained unchanged in the circulation. The high-concentrate diet affected key enzymes reflecting AT metabolism such as AMP-activated protein kinase and hormone-sensitive lipase, both represented as the proportion of the phosphorylated protein to total protein, as well as fatty acid synthase. The extent of phosphorylation of AMP-activated protein kinase and the protein expression of fatty acid synthase were inversely regulated throughout the experimental period, whereas the extent of phosphorylation of hormone-sensitive lipase was consistently decreasing by the high-concentrate diet. Overcondition in nonpregnant, nonlactating dairy cows changed the expression of key regulator proteins of AT metabolism and circulation accompanied by impaired insulin sensitivity, which might increase the risk for metabolic disorders.

Key words: fat metabolism, dairy cow, subcutaneous adipose tissue

INTRODUCTION

Many health disorders in dairy cattle are attributed to the process of uncontrolled lipid mobilization in response to excessive negative energy balance in early lactation (Drackley, 1999; Opsomer et al., 1999; Pravettoni et al., 2004; Roche et al., 2009) and are most likely related to reduced antilipolytic action of insulin (Ji et al., 2012; De Koster and Opsomer, 2013). Overconditioned cows with a BCS > 4.0 (Edmonson et al., 1989) at calving showed higher plasma concentrations of NEFA in early lactation until wk 7 postpartum compared with cows with moderate or low BCS (Pires et al., 2013). Hyperlipidemia in turn caused insulin resistance in dairy cows (Pires et al., 2007), which is in accordance to studies linking high BCS to reduced peripheral insulin sensitivity in the lipomobilization state (Holtenius et al., 2003; Hayirli, 2006; Holtenius and Holtenius, 2007). However, whether changes in

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adipose tissue (**AT**) metabolism occur with fattening and possibly account for subsequent insulin resistance in bovine is rarely known.

Beyond the storage and release of metabolites, **AT** exhibits specific enzymatic patterns. In addition, bioactive molecules, so called adipokines, are secreted from **AT**. Both specific enzymes and adipokines can affect peripheral organs (Trayhurn et al., 2006). Leptin, an adipokine, regulates energy intake, storage, and expenditure in ruminants (Chilliard et al., 2005). It positively reflects both **BCS** and nutrient status in cows and heifers (Reist et al., 2003; León et al., 2004) and interacts with insulin depending on the physiological status (Block et al., 2003; Leury et al., 2003). The adipokine adiponectin (**AdipoQ**) improved peripheral insulin sensitivity (Turer and Scherer, 2012), whereas low **AdipoQ** levels were associated with insulin resistance in humans and rodents (Berg et al., 2002). In dairy cows, reduced plasma **AdipoQ** concentrations seemed to be an important control variable for the homeorhetic adaptation to early lactation (Giesy et al., 2012; Singh et al., 2014). In addition, plasma **AdipoQ** was positively correlated with **RQUICKI**, a surrogate marker for insulin sensitivity, whereas a negative association with **BCS** in the periparturient period was recently reported by Singh et al. (2014).

The AMP-activated protein kinase (**AMPK**), a target of **AdipoQ**, controls energy expenditure within the cell by balancing ATP-consuming and ATP-providing pathways (Gauthier et al., 2008; Gaidhu et al., 2009). Both **AdipoQ** and insulin activated **AMPK** in isolated 3T3-L1 adipocytes presumably by increasing the AMP/ATP-ratio (Liu et al., 2010). With regard to the whole organism, **AMPK** activated β -oxidation and reduced glucose and NEFA output into circulation and thus influenced indicators of reduced insulin action in several species (Bijland et al., 2013). In bovine **AT**, lipolysis during early lactation was associated with an increase in phosphorylation of **AMPK** and the ratio of $p\text{AMPK}\alpha 1$ to $\text{AMPK}\alpha 1$ (Locher et al., 2012).

Fatty acid synthase (**FAS**) is one of the key enzymes in de novo synthesis of fatty acids and is positively related to the nutritional status in cattle (Sadri et al., 2011; Khan et al., 2013). Hormone-sensitive lipase (**HSL**) is a key enzyme of adrenergically stimulated lipolysis in adipocytes. This enzyme is activated by protein kinase A (**PKA**) dependent phosphorylation and its activation is controlled by catecholamines and insulin (Holm, 2003; Choi et al., 2010). In dairy cows, an increase in phosphorylation of **HSL** was associated with the onset of lactation and fat mobilization (Elkins and Spurlock, 2009; Locher et al., 2011). The present study aimed to assess the effect of increasing fat accumulation on key regulator proteins of fat metabolism in subcutaneous

AT and circulation of dairy cows. Therefore, we analyzed the enzymes **HSL** and **AMPK** with their respective phosphorylated forms, as well as **FAS**, and **AdipoQ** in subcutaneous **AT** on protein level, whereas **AdipoQ**, leptin, and insulin concentrations were measured in blood. Transcription, translation, and activity of all the aforementioned enzymes and adipokines are largely influenced by pregnancy, parturition, and lactation. Therefore, nonlactating, nonpregnant cows serve as an appropriate model to elucidate the changes of these effectors in the course of overcondition independent from physiological changes related to gestation, parturition, and lactation.

MATERIALS AND METHODS

Animals, Feeding, and Sample Collection

The animal experiment was approved by the Lower Saxony State Office for Consumer Protection and Food Safety, Oldenburg, Germany (File Number 33.9-42502-04-11/0444). The experiment was conducted at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute, Braunschweig, Germany. Nonpregnant, nonlactating, pluriparous German Holstein cows ($n = 8$) were housed in a free-stall barn with free access to straw and water. The cows were gradually adapted to a high-energy ration (corn-grass-silage with increasing the proportion of corn silage), including a successive increase of the proportion of the concentrate feed (within 6 wk from 0% up to 60% of the DM of the daily ration). The ration composition during conditioning and feeding regimen as well as the analyzed composition of the diet are given in Tables 1 and 2 and have been described in detail elsewhere (Dänicke et al., 2014). Blood samples from a jugular vein were collected monthly. Plasma was analyzed for glucose, NEFA, and BHBA concentrations using an automatic analyzer system (Eurolyser CCA180, Eurolab, Hallein, Austria) and were used for calculation of the surrogate indices for insulin sensitivity stated below. Plasma insulin concentration (determined in EDTA plasma) was measured by RIA (IM3210, Immunotech, Beckman Coulter Inc., Brea, CA). Biopsies from **AT** were taken before conditioning (wk 0) and at wk 8 and 15 of the trial. Animals were given 4 mL of procaine (Procaine 2%, Selectavet, Weyarn-Holzolling, Germany) as a lumbosacral epidural anesthesia. After preparation of the surgical field, a 5.0-cm skin incision was made in the region of the tailhead and subcutaneous **AT** from the underlying fat layer was collected. The biopsies from wk 8 and 15 were each made on the contralateral side of the preceding biopsy. To reduce surgically induced blood contamination, the sample was shortly rinsed

Table 1. Ingredients of the ration during conditioning and feeding regimen over 15 wk

Conditioning (wk)	Ingredient of the daily ration (% DM basis)			
	Straw	Corn silage	Grass silage	Concentrate
Before conditioning	100	0	0	0
1	73.1	11	7.3	8.6
2	49.0	19.9	13.2	17.2
3	29.7	26.7	17.8	25.8
4	13.1	31.5	21.0	34.4
5	0	34.2	22.8	43.0
6	0	29.0	19.4	51.6
7 to 15	0	24.0	16.0	60.0

in sterile saline solution. Surrounding connective tissue was removed as far as possible, and biopsies were directly snap frozen in liquid nitrogen and stored at -80°C until further analyses.

Calculation of Surrogate Indices for Insulin Sensitivity

To assess insulin sensitivity, surrogate indices were calculated as follows. The homeostasis model assessment (**HOMA**; Matthews et al., 1985) was given by

$$\text{HOMA} = \text{glucose (mmol/L)} \times \text{insulin } (\mu\text{U/mL}),$$

the quantitative insulin sensitivity check index (**QUICKI**; Katz et al., 2000) was given by

$$\text{QUICKI} = 1 / \{ \log[\text{glucose (mg/dL)}] + \log[\text{insulin } (\mu\text{U/mL})] \},$$

and the revised quantitative insulin sensitivity (**RQUICKI**; Perseghin et al., 2001) was given by

$$\text{RQUICKI} = 1 / \{ \log[\text{glucose (mg/dL)}] + \log[\text{insulin } (\mu\text{U/mL})] + \log[\text{NEFA (mmol/L)}] \}.$$

Serum leptin concentrations were determined by ELISA as described by Sauerwein et al. (2004).

Adiponectin Assay

For preparation of tissue extracts, AT samples were homogenized in 2 volumes of HEPES buffer [10 mM; pH 7.4 with complete protease inhibitor cocktail (one tablet/10 mL buffer, Roche, Mannheim, Germany)] using a homogenizer (Precellys 24, Peqlab Biotechnologies GmbH, Erlangen, Germany). After centrifugation (twice at $14,000 \times g$, 10 min, 4°C), the fat layer was removed and the infranatant was stored at -20°C until analysis. Total protein concentrations were determined by Bradford assay (Roti-Nanoquant K880, Roth, Karlsruhe, Germany).

Serum and tissue extract samples were analyzed for AdipoQ using an in-house developed competitive ELISA as described previously (Mielenz et al., 2013). Assay

Table 2. Components of the concentrate feed and analyzed composition of the feedstuffs (g/kg of DM; data from Dänicke et al. 2014)

Item	Concentrate	Straw	Corn silage	Grass silage
Component				
Soybean meal	268			
Wheat	500			
Corn	208			
Mineral feed ¹	12			
Calcium carbonate	12			
Analyzed composition				
DM	886.8	850.0	354.1	332.5
Crude ash	50.3	57.9	36.3	92.4
CP	221.4	3.7	91.2	128.7
Crude fat	28.1	15.6	30.5	35.0
Crude fiber	30.2	432.9	197.5	274.0
NDF	146.7	833.1	436.4	518.4
ADF	43.5	484.6	226.8	303.0
NE _L (MJ/kg of DM)	8.31	3.8	6.5	5.9

¹Per kilogram of mineral feed: 140 g of Ca; 120 g of Na; 70 g of P; 40 g of Mg; 6,000 mg of Zn; 5,400 mg of Mn; 1,000 g of Cu; 100 mg of I; 40 mg of Se; 5 mg of Co; 1,000,000 IU of vitamin A; 100,000 IU of vitamin D₃; 1,500 mg of vitamin E.

accuracy was confirmed by linearity and parallelism of diluted serum samples. The measuring range of the assay was 0.07 to 1.0 ng/mL and the limit of detection was 0.03 ng/mL. The intra- and interassay coefficients of variation were 7 and 9%, respectively. Correction for residual blood in the tissue extracts was performed by comparing the transferrin content of the tissue extract to the transferrin content of the serum as described recently by Singh et al. (2014). The blood corrected AdipoQ concentrations of the tissue preparations (ng/mL) were normalized and are presented as nanograms of AdipoQ per milligram of total protein.

Western Blot Analyses

For Western blot analyses, homogenates of AT were prepared as reported previously (Locher et al., 2011, 2012). Adipose tissue was ground under liquid nitrogen, and approximately 50 mg of tissue meal was homogenized in 1 mL of prechilled lysis buffer. After centrifugation ($1,000 \times g$, 10 min at 4°C), the fat layer and tissue debris were removed and the infranant was stored at -20°C until analysis. Protein concentrations were measured by Bradford assay (protein quantification kit, Serva Electrophoresis, Heidelberg, Germany). Samples were diluted in loading buffer containing 2% mercaptoethanol and denatured by heating for 5 min at 95°C before loading 15 µg of protein per lane onto a 5% stacking gel and a 8.1% separation gel. Electrophoresis was carried out according to Laemmli (1970).

Detection of AMPK α 1, phosphorylated AMPK α 1 (pAMPK α 1), HSL, and phosphorylated HSL (HSLp563) is described in detail elsewhere (Locher et al., 2011, 2012). After blocking the membranes in 5% fat-free milk/PBS + 0.1% Tween 20 (Roche), at room temperature, they were incubated overnight at 4°C with primary antibodies against AMPK α 1 (Bethyl, Montgomery, TX; 1:1,000), pAMPK α 1 (phosphorylated at Thr 172; 1:1,000), HSL (1:1,000), HSLp563 (1:1,000) purchased from Cell Signaling (Danvers, MA), FAS (1:1,000), and β -actin (1:10,000) from Sigma-Aldrich (St. Louis, MO). Detection of the primary antibodies was performed using secondary anti-rabbit-antibody conjugated with horseradish peroxidase (Cell Signaling, 1:2,000 for pAMPK α 1, HSL, and HSLp563, Sigma-Aldrich, 1:50,000 for AMPK α 1 and FAS) and β -actin antibody using secondary anti-mouse antibody labeled with horseradish peroxidase (Sigma-Aldrich, 1:100,000). Equal protein loading of lanes was assessed by Indian ink staining of total protein at the end of the experiment.

Membranes were incubated with Pierce ECL (Thermo Scientific, Rockford, IL) for detection of pAMPK α 1

and LumiGLO substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for the rest of proteins. Chemiluminescence was measured by an imaging system connected with a digital camera (ChemiDoc, BioRad Laboratories Inc.). Data were processed and analyzed by densitometry using Image Lab 4.0 software (BioRad). The linear range of signal intensity development was assessed by measuring the chemiluminescence at increasing exposition times. Specific band values were normalized to β -actin values as an internal standard. To adjust band values from different membranes, 2 reference samples were blotted on each membrane.

Measurement of Adipocyte Size

Subcutaneous AT was fixed in 4% paraformaldehyde and embedded in paraffin according to the standard procedure described by Romeis (2010). Paraffin-embedded tissue was cut by a microtome (SLEE, Mainz, Germany). Sections (10–14 µm) were deparaffinized in Rotihistol (Roth) and rehydrated through descending grades of isopropanol (Roth). Finally, sections were stained with hematoxylin (Merck) and mounted with Kaiser's glycerol gelatin (Merck). Adipocyte areas (µm²) of 100 adipocytes per section were measured as described by Akter et al. (2011).

Statistical Analyses

Statistical analyses were performed using SPSS (version 21.0, SPSS Inc., Chicago, IL). Data were analyzed for normal distribution using the Kolmogorov-Smirnov test. Homogeneity of variances were tested using the Levene's test. Nonnormal distributed data were log-transformed and back-transformed to the original scale after calculation. Data were analyzed using one way ANOVA for repeated measurements with Bonferroni post hoc test. Mean and SEM are given for BCS and BW, whereas all remaining data are given as median, and first and third quartile. To describe the association between parameters, Spearman correlations were calculated over all data and time points. The statistical significance threshold was set at $P < 0.05$ and $0.05 < P < 0.1$ as a trend.

RESULTS

Variables Describing Body Condition and Insulin Sensitivity in Overconditioned Cows

Cows significantly gained BW from 540 ± 56.8 to 792 ± 81.7 kg ($P < 0.001$) and BCS (5-point scale) increased from 2.31 ± 0.35 to 4.53 ± 0.39 ($P < 0.001$; Figure 1). Circulating concentrations of AdipoQ, leptin, insulin, glucose BHBA, and NEFA as well as

Table 3. Circulating adiponectin (AdipoQ), leptin, and insulin concentrations and surrogate indices for insulin sensitivity of nonpregnant, nonlactating dairy cows ($n = 8$) in the course of experimental induction of overconditioning over 15 wk (median, first and third quartile)

Item ¹	Conditioning (wk)					<i>P</i> -value†
	0	4	8	12	15	
AdipoQ ($\mu\text{g/mL}$)	40.0 (37.0; 46.2)	36.8 (33.4; 41.6)	37.6 (35.4; 39.0)	35.2 (32.6; 36.4)	34.8 (30.6; 38.8)	0.116
Leptin (ng/mL)	4.95 ^a (3.03; 8.68)	8.70 ^b (5.60; 12.1)	12.5 ^c (8.48; 16.1)	16.3 ^d (11.4; 20.3)	14.7 ^{cd} (11.6; 18.8)	<0.001
Insulin (nU/mL)	14.0 ^a (9.40; 22.2)	41.7 ^{ab} (21.3; 58.2)	47.8 ^b (28.4; 86.3)	185 ^c (76.1; 228)	95.5 ^c (74.7; 202)	<0.001
NEFA ² (mmol/L)	0.45 ^a (0.36; 0.52)	0.15 ^b (0.14; 0.19)	0.14 ^b (0.13; 0.20)	0.15 ^b (0.14; 0.18)	0.18 ^b (0.16; 0.20)	<0.001
BHBA ² (mmol/L)	0.44 ^{abc} (0.33; 0.58)	0.28 ^a (0.22; 0.40)	0.34 ^{ac} (0.26; 0.40)	0.62 ^b (0.55; 0.68)	0.39 ^{bc} (0.36; 0.47)	0.015
Glucose ² (mmol/L)	3.06 ^a (2.92; 3.31)	3.75 ^{ab} (3.37; 3.86)	3.98 ^b (3.81; 4.16)	4.38 ^{bc} (4.05; 4.66)	4.72 ^c (4.49; 4.97)	<0.001
RQUICKI	0.39 ^a (0.35; 0.42)	0.39 ^a (0.36; 0.42)	0.38 ^a (0.34; 0.39)	0.31 ^{ab} (0.30; 0.34)	0.32 ^b (0.30; 0.33)	<0.001
QUICKI	0.33 ^a (0.31; 0.36)	0.30 ^{ab} (0.27; 0.32)	0.28 ^b (0.26; 0.30)	0.25 ^c (0.24; 0.27)	0.26 ^c (0.24; 0.27)	<0.001
HOMA	41.7 ^a (31.3; 66.7)	126 ^{ab} (80.8; 213)	198 ^b (119; 321)	741 ^c (299; 1,130)	423 ^c (366; 1,043)	<0.001

^{a-c}Different superscripts indicate significant differences between samplings after Bonferroni post hoc test; $P < 0.05$.

¹RQUICKI = revised quantitative insulin sensitivity check index based on glucose, insulin, and FFA; QUICKI = quantitative insulin sensitivity check index; HOMA = homeostasis model assessment.

²Data from Dänicke et al. (2014).

†Significant P -value of repeated measurement ANOVA.

the calculated surrogate indices for insulin sensitivity (RQUICKI, QUICKI, and HOMA) are shown in Table 3. Surrogate indices were calculated using NEFA and glucose concentrations in plasma. Circulating AdipoQ remained constant throughout the whole experimental period ($P = 0.116$), whereas leptin concentrations increased up to 3-fold until wk 12 of conditioning and remained constant until the end of the experiment ($P <$

0.001). Insulin concentrations rose 11-fold until wk 12 of conditioning and persisted on that level thereafter ($P < 0.001$). Moreover, RQUICKI and QUICKI decreased continuously throughout the whole experimental period, whereas HOMA increased ($P < 0.001$).

Adipocyte Area, Protein Expression of β -Actin, and Tissue AdipoQ Concentration

Within the subcutaneous AT biopsies, adipocyte area tended to increase 1.3-fold ($P = 0.090$) until wk 8 of conditioning and stagnated until the end of the experimental period (Figure 2 A). The protein expression of β -actin was reduced by approximately one-third from wk 0 to 8 ($P = 0.020$) and then remained unchanged until wk 15 (Figure 2 B).

Furthermore, AdipoQ concentrations in subcutaneous AT in wk 15 were about half the amount of wk 0 ($P = 0.03$; Figure 3).

Regulators of Fat Metabolism in Subcutaneous Adipose Tissue

Although total protein expression of AMPK and abundance of its phosphorylated form did not change over time, the proportion of pAMPK α 1 to AMPK α 1 in subcutaneous AT decreased from wk 0 to 8 ($P = 0.010$) and increased ($P = 0.024$) again from wk 8 to 15

Figure 1. Body condition score (●) and BW (kg; ■) from nonpregnant, nonlactating dairy cows were determined every second week of the experiment. Cows received diets with increasing concentrate during the first 6 wk of the experiment until a proportion of 60% was reached, which was maintained until the end of the experiment (mean $\pm$ SEM). $P < 0.05$.

Figure 2. Adipocyte area (μm^2 ; A) and β -actin (arbitrary unit; B) in subcutaneous adipose tissue biopsies from nonpregnant, nonlactating dairy cows at the beginning (wk 0) and at wk 8 and 15 of the experiment. (B) top: representative Western blot of β -actin. Cows received diets with increasing concentrate during the first 6 wk of the experiment until a proportion of 60% was reached, which was maintained until the end of the experiment. Data are presented as medians, first and third quartiles, and minimum/maximum values; ● = extreme value. Different letters (a,b) indicate significant differences between samplings after Bonferroni post hoc test; $P < 0.05$.

(Figure 4 A). Furthermore, the proportion of pHSL to HSL in subcutaneous AT decreased from wk 0 until wk 15 (Figure 4 B). However, absolute amounts of pHSL and HSL remained unchanged.

The protein expression of FAS [absolute (data not shown) and normalized to β -actin, Figure 4C] signifi-

Figure 3. Adiponectin concentrations ($\mu\text{g/mL}$) in subcutaneous adipose tissue biopsies at the beginning (0), and wk 8 and 15 of the experiment. Nonpregnant, nonlactating dairy cows were fed increasing concentrate during the first 6 wk of the experiment until a proportion of 60% was reached which maintained until the end of the experiment. Data are presented as medians, first and third quartiles, and minimum/maximum values. Different letters (a,b) indicate significant differences between samplings after Bonferroni post hoc test; $P < 0.05$.

cantly increased from the start of the high-concentrate diet in wk 0 until wk 8 of the experimental period ($P < 0.001$) and then decreased again between wk 8 and 15 ($P = 0.047$).

Relationship Between Tissue and Blood Variables and Body Condition

The correlations between blood and tissue variables as well as between tissue variables and body condition are shown in Table 4. Circulating AdipoQ concentrations were further negatively correlated with BCS ($r = -0.515$; $P = 0.01$), BW ($r = -0.473$; $P = 0.023$), and adipocyte area ($r = -0.403$; $P = 0.057$). Leptin concentrations were positively related to BCS ($r = 0.784$; $P < 0.001$), BW ($r = 0.885$; $P < 0.001$), and adipocyte area ($r = 0.493$; $P = 0.017$). Very strong correlations were observed between insulin concentrations and BCS ($r = 0.775$; $P < 0.001$), BW ($r = 0.844$; $P < 0.001$), and adipocyte area ($r = 0.510$; $P = 0.013$). In addition, leptin was positively correlated with insulin ($r = 0.779$; $P < 0.001$). No association was observed between β -actin and adipocyte area ($r = -0.117$; $P = 0.596$).

DISCUSSION

Cows being overconditioned before calving usually have more problems to adapt to the needs of lactation

Figure 4. Phosphorylated adenosine monophosphate-activated protein kinase α 1 (pAMPK α 1)/AMPK α 1(A), phosphorylated hormone-sensitive lipase (pHSL)/HSL (B), and fatty acid synthase (FAS)/ β -actin (C) in subcutaneous adipose tissue biopsies before conditioning (wk 0) and at wk 8 and 15 of the experiment. Nonpregnant, nonlactating dairy cows were fed increasing concentrate during the first 6 wk of the experiment until a proportion of 60% was reached, which was maintained until the end of the experiment. (D) Representative images of Western blot analyzes for pAMPK α 1/AMPK α 1, pHSL/HSL, and FAS/ β -actin in subcutaneous adipose tissue at wk 0, 8, and 15 of the experiment. Data are presented as medians, first and third quartiles, and minimum/maximum values; ● = extreme value. Different letters (a–c) indicate significant differences between samplings after Bonferroni post hoc test; $P < 0.05$.

than lean cows, thus showing greater incidence of postpartum metabolic and reproductive diseases (Kim and Suh, 2003; Roche et al., 2009). In the present study, overcondition in nonpregnant, nonlactating cows was experimentally induced within 15 wk. By means of the “fat cow” model investigated herein, key regulators of fat metabolism independently from physiological influences by pregnancy, parturition, and lactation were analyzed to provide further knowledge about the mechanisms of AT metabolism in overconditioned dairy cows.

Body Condition, Adipokines, and Insulin Sensitivity in Overconditioned Cows

As expected, BW and BCS increased in cows throughout the whole trial. Increasing body condition tended to be accompanied by increased adipocyte area in subcutaneous AT. However, after 8 wk of the experiment, adipocyte size reached a plateau, denoting that adipocyte growth stagnated in this particular subcutaneous AT region, although animals continuously gained BW and BCS until the end of the trial. Activated

Table 4. Relationship (correlation coefficients; *P*-values in parentheses below) between tissue variables, blood and body condition variables, as well as surrogate indices of insulin sensitivity of nonpregnant, nonlactating dairy cows (*n* = 8) during the whole period of experimental overconditioning

Item	Tissue variable				Surrogate index of insulin sensitivity		
	AdipoQ	pAMPK α 1/ AMPK α 1	pHSL/HSL	FAS/ β -actin	HOMA	QUICKI	RQUICKI
Blood variable							
Serum AdipoQ	0.604 (0.006)	NS	NS	NS	-0.413 (0.008)	NS	NS
Leptin	-0.661 (0.002)	-0.374 (0.072)	-0.511 (0.011)	0.463 (0.023)	0.769 (0.008)	-0.760 (0.001)	-0.569 (0.001)
Insulin	-0.585 (0.009)	NS	-0.594 (0.002)	NS	0.990 (0.001)	-0.973 (0.001)	-0.886 (0.001)
Body condition variable							
BCS ¹	-0.517 (0.023)	NS	-0.617 (0.001)	NS	0.810 (0.001)	-0.792 (0.001)	-0.630 (0.001)
BW	-0.690 (0.002)	-0.412 (0.051)	-0.642 (0.001)	NS	0.845 (0.001)	-0.849 (0.001)	-0.620 (0.001)
Adipocyte area	-0.602 (0.008)	NS	-0.389 (0.067)	0.394 (0.063)	0.509 (0.013)	-0.560 (0.005)	NS
Tissue variable							
AdipoQ					-0.579 (0.009)	0.605 (0.006)	NS
pHSL/HSL					-0.615 (0.001)	0.626 (0.001)	0.472 (0.001)
pAMPK α 1/AMPK α 1					NS	NS	NS
FAS					NS	NS	NS

¹Spearman correlation was used; significant *P*-value ≤ 0.05 ; trend: $0.05 \leq P \leq 0.1$. Adiponectin = AdipoQ; AMPK = adenosine monophosphate-activated protein kinase; HSL = hormone sensitive lipase; FAS = fatty acid synthase; RQUICKI = revised quantitative insulin sensitivity check index based on glucose, insulin, and FFA; QUICKI = quantitative insulin sensitivity check index; HOMA = homeostasis model assessment.

AMPK acting antilipogenic (as mentioned below) by inactivation of acetyl-CoA carboxylase (**ACC**), might be one of the control mechanisms to arrest or inhibit adipocyte hypertrophy in rodents and humans (Kola et al., 2008). The thickness of the subcutaneous fat layer is reflected by the BCS; therefore, its increase in the present study assumingly was driven by increasing adipocyte numbers with concurrently stagnating adipocyte size in this depot. In humans, adipocyte number is an important determinant for fat mass (Spalding et al., 2008). However, whether the nutrient surplus led to increased adipogenesis (i.e., proliferation, differentiation, or both of preadipocytes into mature adipocytes) was not examined in the present study. In general, growing fat depots upregulate leptin expression (Thomas et al., 2002); therefore, peripheral leptin concentrations are considered to represent the nutritional status in cattle, assessed by BCS and BW (León et al., 2004). In the current study, leptin concentrations first increased until wk 12 up to concentrations that were higher than those usually expected in dairy cattle (Saremi et al., 2014), but then stagnated even though BW and BCS still increased. Apart from body condition, insulin seemed to be an important regulator of leptin in lactating dairy cows: in a hyperinsulemic-euglycemic clamp study in late lactating dairy cows, chronic hyperinsulinemia led to an increase in plasma leptin, whereas the insulin de-

cline due to feed restriction was paralleled by decreased plasma leptin concentrations (Block et al., 2003). We assume that stagnating plasma insulin concentrations observed herein might be the reason for stagnating leptin concentrations after wk 12 of the trial, which is supported by the strong correlation between insulin and leptin plasma concentrations.

Besides leptin, AdipoQ modulates the glucose and fat metabolism in insulin-sensitive tissues such as muscle and liver and sensitizes the whole body for insulin (Yamauchi et al., 2001). Low AdipoQ concentrations can serve as an indicator for decreasing insulin sensitivity in rodents and humans (Kadowaki et al., 2006). In dairy cows, low AdipoQ concentrations around parturition might contribute to decreased insulin sensitivity, to improve glucose supply for milk synthesis (Singh et al., 2014). In the current study, AdipoQ concentrations were comparable with those levels observed in late lactating multiparous dairy cows (Singh et al., 2014). Although AdipoQ released from subcutaneous AT is known to contribute to serum AdipoQ concentrations (Singh et al., 2014), only tissue AdipoQ and not serum AdipoQ was positively correlated with surrogate indices of insulin sensitivity, proposing either a tissue-specific effect or a time-dependent regulatory response of AdipoQ. In hyperinsulinemic conditions, AdipoQ receptors are reduced (Yamauchi et al., 2002), suggesting that

high AdipoQ concentrations observed herein might compensate reduced AdipoQ sensitivity (Mazaki-Tovi et al., 2007).

In ruminants, glucose and propionate are strong insulin secretagogues (Allen et al., 2009). Cows in the present study displayed increasing propionate and glucose concentrations in serum (Dänicke et al., 2014) that probably provoked increased insulin concentrations. The RQUICKI has been assessed in cows in various physiological (Holtenius and Holtenius, 2007; Bossaert et al., 2009) and pathophysiological (Kerstes et al., 2009; Stengärde et al., 2010) conditions in cattle. Decreasing RQUICKI throughout the whole study indicated reduced whole body insulin sensitivity at first glance. Perseghin et al. (2001) intentionally integrated NEFA into RQUICKI calculations to level out diet-induced variations in plasma glucose. With NEFA reflecting AT mobilization during the period of negative energy balance, this parameter perfectly fits to assess the antilipolytic efficiency of insulin in the periparturient dairy cow. However, in the present study rather plasma glucose led to reduced RQUICKI, because NEFA sharply decreased below 0.2 mmol/L with the beginning of concentrate feeding, whereas glucose and insulin increased (Table 3). Therefore, RQUICKI was not an adequate parameter to reflect changes in antilipolytic or prolipogenic effects of insulin in the present study. Nevertheless, changes in QUICKI and HOMA indicate that in the course of permanent intake of a high concentrate diet as conducted herein, insulin sensitivity, in respect of the glucostatic effect of this hormone, seems to decrease in nonpregnant, nonlactating dairy cows. This is in accordance to a recent study, showing that excessive energy intake led to decreased insulin sensitivity on a glucose tolerance test (Leiva et al., 2014).

Regulators of Fat Metabolism in Subcutaneous Adipose Tissue

In the present study, the ratio of pAMPK α 1 to AMPK α 1 on protein level decreased from wk 0 to 8 and then increased again between wk 8 and 15 in subcutaneous AT. In consideration of the regulation of energy balance at both the cellular and the whole-body level, AMPK plays a central role (Rossmeisl et al., 2004; Allen et al., 2009; Bijland et al., 2013). In lactating dairy cows, feed deprivation (60 h of exclusive straw feeding) led to an increase of AMPK phosphorylation in liver (Kuhla et al., 2009). Fat mobilization due to negative energy balance in early lactation was associated with an increase of pAMPK α 1 to AMPK α 1 ratio indicating AMPK activation. Even though cows were fed *ad libitum* at the beginning of the present study, decreasing

NEFA and BHBA concentrations at the beginning of concentrate feeding indicate a moderate lipomobilization. Therefore, decreased AMPK activation seemed to sprout from increasing energy intake. The significant decrease in DMI after wk 12 from 20.3 to 16.7 kg/d at the end of the experiment (Dänicke et al., 2014) might be the reason for reincreasing AMPK activation. However, although DMI decreased, nutrient requirements were still exceeded and plasma NEFA concentrations remained low (Dänicke et al., 2014).

Several adipokines and hormones including leptin and AdipoQ have been identified to activate AMPK in certain tissues (Bijland et al., 2013). Moreover, reduced AMPK activation in the first 8 wk of the study might result from reduced tissue AdipoQ concentrations, because AdipoQ is known to stimulate the phosphorylation and subsequently activate AMPK in adipocytes (Liu et al., 2010). On the other hand, leptin activated AMPK in AT (Gauthier et al., 2011), which may have induced recurring AMPK activation in wk 15 of the study for leptin reached its maximum concentration in wk 12.

The ratio of pHSL/HSL tended to decrease after the first biopsy and was significantly lower at wk 15 compared with the beginning of the study. The negative correlation between pHSL/HSL and all variables describing the body condition reflected the lipogenic/anabolic status of the cows during this fattening period, which was also supported by decreasing NEFA concentrations. Anabolic processes occurring in cows investigated within this trial may be initiated through high plasma insulin concentrations. The antilipolytic effect of insulin is based on the activation of phosphodiesterase, an enzyme which dephosphorylates and subsequently inactivates HSL (Anthonsen et al., 1998) as confirmed in subcutaneous AT from overconditioned cows in the current study.

In the present study, FAS was increased from wk 0 to 8 and then decreased again between wk 8 and 15. Fatty acid synthase is a key enzyme in the lipogenic pathway involved in *de novo* synthesis of fatty acids (Yeaman et al., 1994). The enzyme is highly regulated by nutrients and hormones, including insulin (Carra et al., 2013). Therefore, high insulin concentrations led most likely to higher FAS protein expression after feeding the high-concentrate diet. Furthermore, the activity of FAS was decreased due to starvation in sheep, whereas refeeding restores it (Ingle et al., 1973), which is in accordance with our findings. Intravenous infusion of glucose stimulated FAS mRNA in subcutaneous AT indicating enhanced *de novo* fatty acid synthesis after glucose infusion (Carra et al., 2013). In mid-lactating dairy cows, acetate is the principal precursor for *de novo* lipogenesis (Bergen and Mersmann, 2005); high

plasma glucose concentrations observed in the present study (Dänicke et al., 2014) might have a similar effect. Albeit stagnating, insulin concentrations remain on a high level at the end of the trial, together with decreasing DMI and FAS expression, suggesting that the orexigenic and prolipogenic activity of insulin start to decline. Decreasing FAS expression after wk 8 was associated with stagnating adipocyte size.

CONCLUSIONS

In summary, high energy intake and, subsequently, overconditioning led to alterations in key regulator proteins of fat metabolism in subcutaneous AT and circulation of nonpregnant, nonlactating dairy cows. Although serum AdipoQ concentrations remained constant, tissue AdipoQ were associated with surrogate indices of insulin sensitivity. Reduced peripheral insulin sensitivity referred mainly to the glucostatic effect of insulin. Primarily the initial changes at the beginning of the experimental period were rather of reactive nature, whereas significant changes in peripheral insulin sensitivity, feeding behavior, and lipogenic activity of AT occurred at the end of the trial. However, the precise mechanism of changes in adipokine and lipid mechanism in AT of overconditioned dairy cows remains to be determined.

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