Effect of increasing body condition on oxidative stress and mitochondrial biogenesis in subcutaneous adipose tissue depot of nonlactating dairy cows

L. Laubenthal,* L. Ruda,† N. Sultana,* J. Winkler,‡ J. Rehage,† U. Meyer,‡ S. Dänicke,‡ H. Sauerwein,* and S. Häussler*¹

*Institute of Animal Science, Physiology and Hygiene Unit, University of Bonn, 53115, Germany

†Clinic for Cattle, University of Veterinary Medicine Hannover, Foundation, 30173, Germany

‡Institute of Animal Nutrition, Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, 38116 Braunschweig, Germany

ABSTRACT

With the onset of lactation, dairy cows with a body condition score >3.5 are sensitive to oxidative stress and metabolic disorders. Adipose tissue (AT) can adapt to varying metabolic demands and energy requirements by the plasticity of its size during lactation. In AT, angiogenesis is necessary to guarantee sufficient oxygen and nutrient supply for adipocytes. Cellular energy metabolism is reflected mainly by mitochondria, which can be quantified by the mitochondrial DNA copy number per cell. In the present study, we aimed to investigate the effect of overconditioning on angiogenesis and mitochondrial biogenesis in AT of nonlactating cows, irrespective of the physiological influences of lactation and pregnancy. Eight nonpregnant, nonlactating cows received a ration of increasing energy density for 15 wk, during which body weight and body condition increased substantially. Subcutaneous AT was biopsied every 8 wk, and blood was sampled monthly. The blood concentrations of indicators of oxidative stress increased continuously throughout the experimental period, possibly damaging mitochondrial DNA. Concomitantly, HIF-1 α , a major marker for hypoxia, increased until wk 8, indicating insufficient angiogenesis in the rapidly expanding AT. Based on the observation that the number of apoptotic cells decreased with increasing hypoxia, the increasing mitochondrial DNA copy numbers might compensate for the hypoxia, reinforcing the production of oxidative stressors. Key transcription factors of mitochondrial biogenesis were largely unaffected. Thus, increased oxidative stress does not impair mitochondrial DNA.

Key words: adipose tissue, dairy cow, mitochondrial biogenesis, oxidative stress

INTRODUCTION

After calving, most cows undergo a phase of negative energy balance, in which the energy demand for milk synthesis is not covered by voluntary feed intake. To meet the increased energy demands, cows mobilize body reserves predominantly from adipose tissue (AT). Over the course of lactation, milk synthesis decreases and energy depots are refilled, leading to a positive energy balance (Drackley et al., 2005). In early lactation, overconditioned cows mobilize more body reserves than thin cows (Treacher et al., 1986) and are more susceptible to metabolic disorders, as well as health and reproduction problems (Gearhart et al., 1990; Goff and Horst, 1997; Roche et al., 2009).

During lactation, AT actively adapts to metabolic needs by mobilizing energy stores (lipolysis) and refilling fat depots (lipogenesis). In obese animals, the blood supply in AT is adapted to dynamic cellular processes via angiogenesis, to provide sufficient nutrients and oxygen for the cells, support fatty acid and glycerol release (Lu et al., 2012; Elias et al., 2013; Lemoine et al., 2013), or both. Vascular endothelial growth factor A (VEGF) is the key regulator of vasculogenesis and angiogenesis (Tam et al., 2009), stimulating the migration, permeability, proliferation, and survival of endothelial cells (Ferrara and Alitalo, 1999; Shibuya, 2001). The angiogenic and mitogenic effects of VEGF are mediated mainly through tyrosine kinase receptor VEGF-R2 (Terman et al., 1991; Shalaby et al., 1995). In AT, VEGF is thought to be involved in energy metabolism (Lu et al., 2012), and its increased expression protects against the negative consequences of diet-induced obesity and metabolic dysfunction (Elias et al., 2013).

Rapid expansion of AT and adipocyte sizes leads to an increase in intercapillary distance, resulting in decreased blood flow and reduced oxygen supply (Cao, 2013). In obese humans and mice, insufficient oxygen supply might cause local hypoxia. In response to hypoxia, AT produces hypoxia-inducible-factor-1 α

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¹Corresponding author: Susanne.Haeussler@uni-bonn.de

(HIF-1 α), a transcription factor that in turn induces angiogenic growth factors (Scannell et al., 1995; Mason et al., 2007; Lemoine et al., 2013). Moreover, upregulation of HIF-1 α can lead to inflammation (Ye et al., 2007) and cell death in AT (Yin et al., 2009).

In cows with a BCS >3.5 before calving and greater BCS loss after calving, metabolic stress is accompanied by increased oxidative stress (Bernabucci et al., 2005). Oxidative stress derives mainly from an imbalance between the production of reactive oxygen species (ROS) by mitochondria and antioxidant defenses that convert ROS to less malign molecules (Sies, 1991; Bernabucci et al., 2005). High concentrations of ROS in periods of increased metabolic demand can damage proteins, lipids, DNA, and the mitochondria themselves (Sawyer and Colucci, 2000; Williams, 2000). Mitochondrial DNA (mtDNA) is not protected by proteins such as histones, so it is more susceptible to damage from oxidative stress than nuclear DNA (Richter et al., 1988). Damaged mtDNA can result in a decline of mtRNA transcription and lead to dysfunction of mitochondrial biogenesis (Wallace, 1999).

Mitochondrial biogenesis describes both the proliferation and differentiation of mitochondria (Izquierdo et al., 1995). One of the main markers of mitochondrial proliferation is the mtDNA copy number per cell (Al-Kafaji and Golbahar, 2013). Genes involved in the transcription, regulation, and maintenance of mtDNA, such as nuclear respiratory factors 1 and 2 (*NRF1* and *NRF2*), mitochondrial transcription factor A (*TFAM*), and peroxisome proliferator-activated receptor- γ co-activator (*PGC-1 α* ; Izquierdo et al., 1995) may change their expression through varying energy supply (Lee et al., 2008).

We hypothesized that overconditioning in cows during positive energy balance leads to local hypoxia in AT due to insufficient angiogenesis. This might change the cellular energy supply and alter the number of mtDNA copies, result in programmed cell death (apoptosis) in AT, or both. Furthermore, oxidative stress might impair the number and function of mitochondria in bovine AT. To describe the local hypoxia and its relation to angiogenesis, we evaluated HIF-1 α and pro-angiogenic factors VEGF-A and VEGF-R2. We determined the mtDNA copy numbers per cell and the abundance of genes involved in the transcription, regulation, and maintenance of mtDNA in subcutaneous AT from overconditioned cows. We also assessed the concentrations of advanced oxidation protein products (AOPP); lipid peroxidation by measuring thiobarbituric acid reactive substances (TBARS); and derivatives of reactive oxygen metabolites (dROM) as indicators of oxidative stress, examining their relationship to mtDNA content and mitochondrial biogenesis.

MATERIALS AND METHODS

Experimental Design and Sample Collection

The animal experiment was performed according to European Community regulations and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Germany. The experimental design has been published previously (Dänicke et al., 2014). In brief, 8 nonpregnant, nonlactating German Holstein cows (age 4–6 y) were kept in an open barn and fed solely with crop straw offered ad libitum for 5 mo. Then, at the start of the observation period, the proportion of straw was gradually decreased and the animals were adapted to a high-energy ration by a weekly increase of the proportions of the corn and grass silage mixture from 0 to 40% of DM and concentrate feed from 0 to 60% of DM over 6 wk. This diet was then maintained for a further 9 wk. Body weight (kg) and BCS (according to the 5-point scale by Edmonson et al., 1989) were monitored every 2 wk.

Blood samples from the jugular vein were collected monthly, and subcutaneous AT biopsies were taken from the tailhead region at the beginning of the experiment (0 wk), and at 8 and 15 wk, as described previously (Locher et al., 2015). Tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C to isolate DNA and RNA for quantitative PCR (qPCR) or were fixed in 4% paraformaldehyde (Roth, Karlsruhe, Germany) for histological evaluation.

Variables Indicative of Oxidative Stress

Oxidative stress was determined in serum by dROM test using *N,N*-diethyl-para-phenyldiamine (DEPPD) as a chromogenic substrate (Alberti et al., 2000) with the modifications of Regenhard et al. (2014). The results were expressed as H_2O_2 equivalents.

In plasma, AOPP were determined by modified spectrophotometric methods (Witko-Sarsat et al., 1998; Celi et al., 2011). Different dilutions (6.25 to 100 μM) of Chloramin-T (Sigma-Aldrich, Darmstadt, Germany) in PBS (pH 7.3) were used to generate standard curves, and PBS without Chloramin-T served as a blank. Samples and standards were incubated with 40 μL of pure acetic acid (Roth) for 5 min at room temperature, and 20 μL of potassium iodide (Sigma-Aldrich) was added to the standards. The absorption was measured spectrometrically at 340 nm (Genesys 10 UV, Thermo Fisher Scientific, Darmstadt, Germany), and AOPP concentrations were expressed in relation to albumin concentrations ($\mu\text{mol/g}$), which were determined by an automatic analyzer (Eurolyser CCA180; Eurolab/

Eurolyser Diagnostica GmbH, Salzburg, Austria) as reported by Dänicke et al. (2014).

The formation of lipid peroxides was measured in serum using a biochemical assay for TBARS (Quant-Chrom DTBA-100, BioAssay Systems, Hayward, CA) according to the manufacturer's protocol. In brief, 100 μ L of serum was mixed with 200 μ L of trichloroacetic acid (10%), incubated for 15 min on ice, and centrifuged at $18,000 \times g$ for 5 min at 4°C. Different dilutions of malondialdehyde in H₂O (0.25–4.5 μ M) served as standard curves. For the color reaction, 200 μ L of thiobarbituric acid (TBA) reagent was added to the samples and standards and heated at 100°C for 60 min, and TBARS were determined photometrically (excitation 560 nm; emission 585 nm; FluoroMax; Spex, HORIBA Scientific, Unterhaching, Germany).

Histological Evaluations

Immunohistochemistry on paraffin-embedded AT sections (12 μ m) was performed according to protocols developed earlier (Häussler et al., 2013). Immunostaining of HIF-1 α was based on a polyclonal rabbit antiserum against human HIF-1 α (1:200; GTX 127309; Genetex, Irvine, CA). To detect VEGF-R2, a polyclonal rabbit anti-VEGF-R2 antibody (1:100; bs-0565R; Bioss Inc., Woburn, MA) was used. Specific primary antibodies were incubated overnight at 4°C. Then, the sections were incubated with horseradish peroxidase-labeled goat-anti-rabbit IgG (1:200; 30 min at room temperature; Southern Biotech, Birmingham, AL). Immunostaining was achieved with 3-amino-9-ethylcarbazol (Toronto Research Chemicals Inc., North York, ON, Canada), and counterstaining was performed by Mayer's Haemalaun (Merck Millipore, Billerica, MA). Bovine placenta (VEGF-R2) and kidney (HIF-1 α) served as negative and positive controls. For negative controls, the primary antibodies were replaced by PBS.

Apoptosis was determined using a modified terminal deoxynucleotide transferase-mediated dUTP nick-end-labeling (TUNEL) assay (Gavrieli et al., 1992), as described previously (Häussler et al., 2013). Bovine lymph node samples from slaughterhouse animals served as negative and positive controls.

The sections were evaluated at 200-fold magnification by light microscope (Leica DMR; Leica Microsystems, Wetzlar, Germany) equipped with a JVC digital color camera KY-F75U (Hachioji Plant of Victor Company, Tokyo, Japan). For each section, 10 randomly selected fields (350 $\times$ 450 μ m) were captured, and the number of positive-stained cells was counted, as well as the total number of cells. Results are presented as the mean percentage of positive cells per total cell number in the evaluated fields. Adipocyte areas (μ m²) were

determined in 100 randomly selected adipocytes per histological section, as described recently (Akter et al., 2011).

Gene Expression Assays

Extraction of total RNA and cDNA synthesis was conducted as described by Saremi et al. (2012). The qPCR analysis was carried out using an Mx3000P cyclor (Stratagene, LA Jolla, CA). Each run included an inter-run calibrator, a negative template control for qPCR, a negative template control, and a no-reverse-transcriptase control of cDNA. Quantification of samples was performed against a cDNA standard curve with serial dilutions. The results for the genes of interest (*HIF-1 α* , *VEGF-R2*, *VEGF-A*, *NRF1*, *NRF2*, *TFAM*, *PGC-1 α*) were normalized based on the geometric mean of amplified reference genes *MARVELD1* (marvel domain containing 1), *EIF3K* (eukaryotic translation initiation factor 3), and *LRP10* (lipoprotein receptor-related protein 10). Primer sequences and accession numbers are given in Table 1.

DNA Isolation and Multiplex qPCR

The mtDNA copy number per cell was assessed by multiplex qPCR (Cawthon, 2009) according to the protocol described by Laubenthal et al. (2016). In brief, total genomic DNA from subcutaneous AT biopsies was extracted using a commercially available DNA isolation kit (PowerPlant Pro DNA Isolation Kit; MO BIO Laboratories, Carlsbad, CA), according to the manufacturer's protocol (Gordon-Bradley et al., 2014). To determine the relative quantity of mtDNA products, total DNA was mixed with 2 sets of primers: one amplified 12S rRNA, a sequence specific in the mitochondrial genome, and the other was specific for bovine β -globin, a housekeeping gene that acted as a nuclear control with a known copy number of 2 per cell (Brown et al., 2012). Primer sequences of β -globin were adopted from Brown et al. (2012). Relative mtDNA copy numbers were calculated according to Nicklas et al. (2004).

Statistical Analyses

Statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL). Data for all variables were tested for normal distribution using the Kolmogorov-Smirnov test and for homogeneity of variances by the Levene's test. Not normally distributed variables and mRNA values were log-transformed for statistical analyses and back-transformed to the original scale after calculation. Data were analyzed using linear mixed models with sampling date as a fixed effect and

Table 1. Primer sequences used to quantify target and reference genes¹

Item	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Accession number
Gene			
<i>VEGF-A</i>	CATGGATGTCTACCAGCGC	TGTTGAACTCCTCAGTGGGC	NM_174216.1
<i>VEGF-R2</i>	CCAGACCATGCTTGACTGCT	TGCCATCCTGTTGAGCGTTA	NM_001110000.1
<i>HIF-1α</i>	TTGGCAGCAATGACACAGAGA	TCTTGGTTGAGTGCAGGGTC	NM_174339
<i>PGC-1α</i>	GAAGGCAATTGAAGAGCGC	TCGACCTGCGCAAAGTGTAT	NM_177945.3
<i>TFAM</i>	ATGCTTACAGGGCAGACTG	AGCTTTACCTGTGATGTGCCA	NM_001034016.2
<i>NRF1</i>	CCCAAACCTGAGCACATGG	GTAAAGTATGTCGAATCGTC	NM_001098002.2
<i>NRF2</i>	TTCCAGCATCAGTGCAGTCT	CTGGCCATTGTTTCCTGTTT	NM_001075437.2
<i>MARVELD1</i>	GGCCAGCTGTAAGATCATCACA	TCTGATCACAGACAGAGCACCAT	NM_001101262
<i>EIF3K</i>	CCAGGCCCACCAAGAAGAA	TTATACCTTCCAGGAGGTCCATGT	NM_001034489
<i>LRP10</i>	CCAGAGGATGAGGACGATG	ATAGGGTTGCTGTCCCTGTG	BC149232
mtDNA copy number			
12S rRNA	CGCGGTCATACGATTAACCC	AACCCTATTTGGTATGGTGCTT	NM_U01920.1
β-globin	CGGCGGGCGGGCGGCGGGCTGG	GCCGCGCCGCGCGCCCGTCCCGC	
	GCGGAAGGCCCATGGCAAGAAG	CGCTCACTCAGCGCAGCAAAGG	

¹12S rRNA = mitochondrially encoded 12S ribosomal RNA; *EIF3K* = eucariotic translation initiation factor 3; *HIF-1α* = hypoxia-inducible-factor 1α; *LRP10* = lipoprotein receptor-related protein 10; *MARVEL1* = Marvel domain containing 1; *NRF1* = nuclear respiratory factor 1; *NRF2* = nuclear respiratory factor 2; *PGC-1α* = peroxisome proliferator-activated receptor gamma coactivator 1α; *TFAM* = mitochondrial transcriptional factor A; *VEGF-A* = vascular endothelial growth factor A; *VEGF-R2* = vascular endothelial growth factor receptor-2.

cow as a random effect, as well as the Bonferroni post hoc test. Values are expressed as mean ± SEM or as median, first, and third quartile. Correlations (ρ) were assessed by Spearman analysis. Results with a P -value ≤ 0.05 were considered to be significantly different, and $0.05 < P \leq 0.1$ considered a trend.

RESULTS

Variables Describing Body Condition, Oxidative Stress, and Mitochondria in Nonlactating, Overconditioned Dairy Cows

The cows gained BW and BCS throughout the experiment, from 540 ± 57 kg to 792 ± 82 kg and 2.31 ± 0.35 to 4.53 ± 0.39 , respectively (both $P < 0.001$; see Supplemental Figure S1; <https://doi.org/10.3168/jds.2016-12356>).

The concentrations of dROM, AOPP, and TBARS in plasma described oxidative stress. From wk 0 to 15, plasma concentrations of dROM and TBARS increased 2.5- and 2.2-fold ($P \leq 0.007$), respectively (Figures 1A and C), whereas AOPP concentrations tended to decrease after the first biopsy ($P = 0.106$; Figure 1B).

We examined the abundance of mtDNA copies per cell using multiplex qPCR. From wk 0 to wk 8, mtDNA copies per cell increased 4.7-fold ($P < 0.001$) and remained constant from wk 8 to 15 (Figure 2).

Variables Describing Angiogenesis and Mitochondrial Biogenesis in Subcutaneous AT

We detected immunohistochemical staining of VEGF-R2 in the cytoplasm of cells in subcutaneous AT

(Figure 3A). Although the statistical tests indicated an increase in the proportion of VEGF-R2-positive cells throughout the experiment (1.2-fold, $P = 0.028$), the data suggest that this increase occurred only between wk 8 and 15 (1.4-fold, $P = 0.001$; Figure 4A). The number of HIF-1α-positive cells was also determined by immunohistochemistry in subcutaneous AT (Figure 3B) and increased 3.3-fold ($P = 0.003$) from wk 0 to 15 (Figure 4B). From wk 0 to 8, HIF-1α protein levels increased 2.6-fold ($P = 0.045$) and stagnated thereafter.

The apoptotic cell rate decreased 2.5-fold from wk 0 to 8 ($P = 0.026$) and showed no further change for the duration of the experiment (Figure 5).

The mRNA abundances of pro-angiogenic factors (*VEGF-A*, *VEGF-R2*, and *HIF-1α*) and genes related to mitochondrial biogenesis (*NRF1*, *NRF2*, *PGC-1α*, and *TFAM*) in subcutaneous AT are shown in Table 2. Expression of *VEGF-A* and *VEGF-R2* tended to decrease from the beginning of the high-concentrate diet until the end of the experiment, whereas *HIF-1α* mRNA decreased 1.7-fold from wk 0 to 8 ($P = 0.037$). The mRNA abundances of *NRF1*, *NRF2*, and *TFAM* remained stable throughout the experiment, whereas *PGC-1α* abundances tended to increase 2.4-fold ($P = 0.087$) from wk 0 to 15.

Relationship Between Angiogenic Variables, Oxidative Stress, and Mitochondrial Biogenesis in Subcutaneous AT

The coefficients of correlation between protein and mRNA expression of angiogenic variables (*VEGF-A*, *VEGF-R2*, *HIF-1α*), mtDNA copy numbers, and mitochondrial biogenesis genes (*TFAM*, *PGC-1α*) with indicators for oxidative stress (TBARS, dROM), body

Figure 1. Time-dependent changes in (A) derivatives of reactive oxygen metabolites (dROM: $\mu\text{g of H}_2\text{O}_2/\text{mL}$), (B) advanced oxidation protein products (AOPP)/albumin ($\mu\text{mol/g}$), and (C) thiobarbituric acid reactive substances (TBARS; nmol/mL) levels in serum from nonpregnant, nonlactating dairy cows at wk 0, 8, and 15 of the experiment. Cows were fed a diet with increasing proportions of concentrate (reaching 60% of DM within 6 wk) that was then maintained for a further 9 wk. Data are presented as medians, first and third quartiles, and individual values. Asterisks indicate significant differences between samplings after the Bonferroni post hoc test: * $P < 0.05$, ** $P < 0.005$.

composition, and blood variables are shown in Table 3. Neither *NRF1*, *NRF2*, nor AOPP was associated with these parameters.

Furthermore, mtDNA copy numbers were positively related to HIF-1 α protein ($\rho = 0.658$; $P = 0.001$) and negatively related to the number of apoptotic cells ($\rho = -0.488$; $P = 0.039$); *HIF-1 α* mRNA was associated with *VEGF-R2* mRNA ($\rho = 0.542$; $P = 0.02$).

We observed very strong correlations between TBARS and BW ($\rho = 0.755$; $P < 0.001$) and BCS ($\rho = 0.877$; $P < 0.001$). Moreover, TBARS concentrations were moderately related to adipocyte areas ($\rho = 0.496$; $P = 0.016$), which have been quantified previously (Locher et al., 2015), and insulin concentrations ($\rho = 0.587$; $P = 0.003$). In addition, dROM concentrations were positively correlated with BW ($\rho = 0.585$; $P = 0.003$), BCS ($\rho = 0.537$; $P = 0.007$), and adipocyte areas ($\rho = 0.488$; $P = 0.018$) and tended to be correlated with insulin concentrations ($\rho = 0.370$; $P = 0.075$).

DISCUSSION

The present study aimed at investigating the effects of excessive fat accumulation on oxidative stress and

Figure 2. Mitochondrial DNA (mtDNA) copy number/cell in subcutaneous adipose tissue biopsies at wk 0, 8, and 15 of the experiment. Nonpregnant, nonlactating dairy cows were fed a diet with increasing proportions of concentrate (reaching 60% of DM within 6 wk) that was then maintained for a further 9 wk. Data are presented as medians, first and third quartiles, and individual values. Asterisks indicate significant differences between samplings after the Bonferroni post hoc test: *** $P \leq 0.001$.

Figure 3. Examples of (A) vascular endothelial growth factor receptor 2 (VEGF-R2) and (B) hypoxia-inducible-factor 1 α (HIF-1 α) immunoreactivity in histological sections from subcutaneous adipose tissue. Positive cells appear as weak red staining in the cytoplasm of subcutaneous adipose tissue cells (marked with arrows). Bovine placenta was used as positive and negative controls (C and D). Original magnification: 200 $\times$. Scale bars represent 100 μ m.

key regulators of mitochondrial biogenesis in the subcutaneous AT of cows. During negative energy balance, overconditioned cows mobilize more body reserves than lean cows and are more susceptible to health problems and metabolic disorders (Bernabucci et al., 2005; Roche et al., 2009). Obesity in humans is often related to the development of high levels of oxidative stress (Higdon and Frei, 2003), as well as to dysfunctions in AT angiogenesis (Kabon et al., 2004; Gealekman et al., 2011) and mitochondrial biogenesis (Yin et al., 2014). We investigated whether these incidents occurred in overconditioned cows during positive energy balance, independent of the physiological changes related to parturition and lactation.

Oxidative Stress in Overconditioned Dairy Cows

In the present study, increasing body condition was accompanied by elevated dROM and TBARS concentrations, indicating enhanced oxidative stress (Bernabucci et al., 2005). It appeared that increasing dROM concentrations corresponded to diet changes at the beginning of the experiment, and increasing TBARS concentrations corresponded to changes in energy balance.

Furthermore, unchanged plasma AOPP concentrations led us to assume that excessive protein oxidation products were not generated. Compared with thin cows, oxidative stress was more pronounced in overconditioned dry cows with greater BCS loss at calving, (Bernabucci et al., 2005). In the present study, insulin sensitivity tended to decrease with a high-energy diet (Locher et al., 2015). Moreover, increasing insulin concentrations were associated with TBARS and tended to be associated with dROM concentrations.

Effect of Oxidative Stress on Mitochondrial Biogenesis in Subcutaneous AT

Excessive accumulation of ROS in adipocytes can impair mitochondrial function (Kusminski and Scherer, 2012) and may further result in insulin insensitivity, as detected in human adipocytes (Wang et al., 2013). We tested whether mtDNA content and mitochondrial biogenesis were affected in response to excessive energy intake and increased oxidative stress in subcutaneous AT. Metabolic processes such as glucose and lipid metabolism increase energy demands during lipogenesis in AT and further stimulate local mtDNA content. In the

present study, indicators of lipid metabolism (i.e., cholesterol and triglycerides) were increased throughout the experimental period (Dänicke et al., 2014), probably to provide substrates for lipogenesis. In addition,

increasing mtDNA copy numbers might be an adaptive response to compensate for mtDNA damage caused by increased ROS (Lee et al., 2000). The positive relationship between mtDNA copy number and oxidative stress variables indicate that besides their importance for cellular energy metabolism, mitochondria are a major source of ROS production (Sawyer and Colucci, 2000). In contrast, increasing ROS may cause more oxidative damage to mitochondria and other cell organelles (Al-Kafaji and Golbahar, 2013), impairing cellular energy metabolism and resulting in cell senescence or apoptosis (Chen et al., 1998; Passos and von Zglinicki, 2005). However, the decreasing rate of apoptosis in the present study supports a compensatory mechanism for oxidative damage in adipocytes via increasing mtDNA copy numbers.

Recently, we measured the number of mtDNA copies in subcutaneous AT from early (50.9 ± 3.14 mtDNA copies per cell) and late (80.8 ± 7.08 mtDNA copies per cell) lactating high-yielding dairy cows (Laubenthal et al., 2016). Although animals at the beginning of the present study showed lower adipocyte sizes, the number of mtDNA copies per cell was 4-fold higher in nonlactating, overconditioned animals than in late-lactating dairy cows. The reason for this finding is not

Figure 4. Proportion of positive cells (%) for (A) vascular endothelial growth factor receptor 2 (VEGF-R2) and (B) hypoxia-inducible-factor 1 α (HIF-1 α) in subcutaneous adipose tissue at wk 0, 8, and 15 of conditioning. Nonpregnant, nonlactating dairy cows were fed a diet with increasing proportions of concentrate (reaching 60% of DM within 6 wk) that was then maintained for a further 9 wk. Data are presented as medians, first and third quartiles, and individual values. Asterisks indicate significant differences between samplings after the Bonferroni post hoc test: * $P < 0.05$, ** $P < 0.005$.

Figure 5. Proportion of apoptotic cells (%) in subcutaneous adipose tissue from nonpregnant, nonlactating dairy cows at wk 0, 8, and 15 of the experiment. Cows were fed a diet with increasing proportions of concentrate (reaching 60% of DM within 6 wk) that was then maintained for a further 9 wk. Data are presented as medians, first and third quartiles, and individual values. Asterisks indicate significant differences between samplings after the Bonferroni post hoc test: * $P < 0.05$.

Table 2. Relative mRNA abundance (mean $\pm$ SEM) of angiogenic and mitochondrial biogenesis genes in subcutaneous adipose tissue at wk 0, 8, and 15 of conditioning^{1,2}

Gene	Conditioning (wk)		
	0	8	15
Angiogenic			
<i>VEGF-A</i>	1.51 $\pm$ 0.31	1.11 $\pm$ 0.15	0.80 $\pm$ 0.09
<i>VEGF-R2</i>	1.30 $\pm$ 0.19	1.09 $\pm$ 0.12	0.78 $\pm$ 0.09
<i>HIF-1α</i>	1.38 $\pm$ 0.21 ^a	0.79 $\pm$ 0.09 ^b	1.05 $\pm$ 0.12 ^{ab}
Mitochondrial biogenesis			
<i>NRF1</i>	1.11 $\pm$ 0.18	0.81 $\pm$ 0.16	1.06 $\pm$ 0.11
<i>NRF2</i>	0.81 $\pm$ 0.16	0.91 $\pm$ 0.18	1.24 $\pm$ 0.12
<i>PGC-1α</i>	0.61 $\pm$ 0.19	0.95 $\pm$ 0.25	1.46 $\pm$ 0.40
<i>TFAM</i>	0.96 $\pm$ 0.20	1.01 $\pm$ 0.17	1.37 $\pm$ 0.14

^{a,b}Different superscript letters between weeks of conditioning indicate significant differences ($P \leq 0.05$).

¹Nonpregnant, nonlactating cows were fed a diet with increasing proportions of concentrate (reaching 60% of DM within 6 wk) that was then maintained for a further 9 wk.

²*HIF-1 α* = hypoxia-inducible-factor 1- α ; *NRF1* = nuclear respiratory factor 1; *NRF2* = nuclear respiratory factor 2; *PGC-1 α* = peroxisome proliferator-activated receptor gamma coactivator 1 α ; *TFAM* = mitochondrial transcriptional factor A; *VEGF-A* = vascular endothelial growth factor A; *VEGF-R2* = vascular endothelial growth factor receptor-2.

clear. However, we found parallels when we compared cows in the present study (fed 100% straw for several months before the start of the experiment) to calorie-restriction models: the mtDNA content in liver from calorie-restricted rats was higher than in ad-libitum-fed rats (Picca et al., 2013). In addition, the efficiency of mitochondrial function seems to be enhanced after caloric restriction, which also prevents decreasing mtDNA content (Picca et al., 2013).

The mRNA abundance of key transcription factors of mitochondrial biogenesis (i.e., *PGC-1 α* , *NRF1*, *NRF2*, and *TFAM*), which might control the amount and func-

tion of mtDNA in AT mitochondria (Villarroya et al., 2009), were related to increased ROS production. Although *PGC-1 α* is known to induce *NRF1*, *NRF2*, and *TFAM* (Puigserver et al., 1998), the mRNA abundance of these transcription factors remained unchanged, and *PGC-1 α* tended to increase with body condition. Gene expression of transcription factors for mitochondrial biogenesis might change after prolonged enhanced oxidative stress levels, as suggested for rats with chronic cholestasis (Arduini et al., 2011).

In the present study, mtDNA copy number was positively associated with BCS and BW, and negatively

Table 3. Relationships (Spearman correlations) between angiogenic and mitochondrial biogenesis variables and indicators for oxidative stress, body condition, and blood variables in nonpregnant, nonlactating dairy cows throughout the experiment¹

Item	Angiogenesis					Mitochondrial biogenesis		
	Protein expression		Gene expression			Gene expression		
	VEGF-R2	HIF-1 α	<i>VEGF-R2</i>	<i>HIF-1α</i>	<i>VEGF-A</i>	<i>mtDNA</i>	<i>TFAM</i>	<i>PGC-1</i>
Oxidative stress indices								
dROM	NS	0.380†	−0.713*	−0.486*	NS	0.550*	NS	NS
TBARS	0.463*	0.446*	−0.550*	NS	−0.430†	0.447*	0.406†	0.461†
Body condition variables								
BCS ²	0.516*	0.453*	−0.516*	NS	−0.588*	0.503*	NS	0.409†
BW ²	0.512*	0.516*	−0.517*	NS	−0.672*	0.596*	NS	0.443†
Adipocyte area ³	NS	0.455*	NS	NS	−0.478*	0.388†	NS	0.439†
Blood variables								
Leptin ³	0.362†	0.548*	−0.498*	NS	−0.575*	0.707*	0.434†	0.492*
Insulin ²	NS	0.482*	NS	NS	NS	0.616*	NS	NS
Nonesterified fatty acids ²	NS	−0.541*	NS	NS	0.450†	−0.381†	NS	NS

¹dROM = derivatives of reactive oxygen metabolites; *HIF-1 α* = hypoxia-inducible-factor 1 α ; *mtDNA* = mitochondrial DNA; NS = not significant; *PGC-1* = peroxisome proliferator-activated receptor gamma coactivator 1; TBARS = thiobarbituric acid reactive substances; *TFAM* = mitochondrial transcriptional factor A; *VEGF-A* = vascular endothelial growth factor A; *VEGF-R2* = vascular endothelial growth factor receptor-2

²Dänicke et al. (2014).

³Locher et al. (2015).

* $P \leq 0.05$; † $0.05 < P \leq 0.10$.

related to fatty acid concentrations. Furthermore, the positive association between mtDNA copies and circulating leptin, an adipokine related to BCS and adipocyte sizes in cattle (Ehrhardt et al., 2000; Delavaud et al., 2002), indicates a role for mtDNA content in the lipogenesis of bovine AT, as proposed for humans (Kaaman et al., 2007).

Large adipocytes require more mitochondria to meet the increased ATP demand of larger cells (Yin et al., 2014). However, in obese humans (body mass index >36.9 kg/m²) no further increase in mtDNA copy number with larger adipocytes was observed (Yin et al., 2014). The number of mtDNA copies from cows in the present study was positively correlated with adipocyte size, reported recently by Locher et al. (2015); both increased 1.3-fold until wk 8 and stagnated thereafter. Stagnating mtDNA copy numbers may limit energy supply in adipocytes.

Effect of Overconditioning and Its Cellular Consequences for Angiogenic Variables in Subcutaneous AT

Mitochondrial biogenesis was related to tissue oxygenation in the brains of neonatal rats (Lee et al., 2008). In general, hypoxia plays an important role in the context of obesity and obesity-related diseases, and, we hypothesized that AT from overconditioned cows might undergo hypoxia. In AT, angiogenesis is adapted to hypertrophic adipocytes to ensure sufficient oxygen and nutrient supply (Lemoine et al., 2013). Enlarged adipocytes are prone to hypoxia and respond by activation of HIF-1 α (Trayhurn et al., 2008). In the present study, increased HIF-1 α -positive cells were positively correlated with adipocyte size, as well as with BW and BCS from wk 0 until wk 8. Due to the rapid enlargement of adipocyte size, the capillary density probably failed to meet the hypertrophy and resulted in insufficient nutrients and oxygen supply, as has been found in mice (Pang et al., 2008) and humans (Karpe et al., 2002; Pasarica et al., 2009). Due to hypoxia, adipocytes might undergo apoptosis or necrosis (Yin et al., 2009). However, despite increased levels of HIF-1 α , the number of apoptotic cells decreased until wk 8. Increasing mtDNA copy number might act as a feedback mechanism for counterbalancing the energy deficit in the cells (Carabelli et al., 2011).

Given that the number of HIF-1 α -positive cells stagnated from wk 8 until the end of the experiment, the increase of VEGF-R2-positive cells from wk 8 to 15 might be a response to the hypoxic condition in subcutaneous AT. To initiate remodeling of blood vessels, HIF-1 α enhances the expression of angiogenic growth

factors, such as *VEGF* and its receptors in human skeletal muscle (Görlach et al., 2001).

The positive association between the number of mtDNA copies per cell and HIF-1 α protein expression in the present study might point to compensation for the hypoxic condition through increased mtDNA, as previously postulated for rats with hypoxia in liver (Carabelli et al., 2011) and in brain (Lee et al., 2008). Although HIF-1 α has been considered an important regulator of mitochondrial biogenesis in skeletal muscle (Mason et al., 2007), we observed no association between mRNA abundance of mitochondrial genes and *HIF-1 α* mRNA in AT in the present study.

Whether the observed changes in the present study could be explained by positive energy balance or by the tremendous dietary changes at the beginning of the experiment remains unknown. However, due to rapid fat accumulation, blood concentrations of markers of oxidative stress increased in overconditioned, nonpregnant, nonlactating cows. Increasing numbers of mtDNA copies might improve the energy supply in expanding AT as a compensatory mechanism for oxidative stress. In contrast, increasing mitochondria generate more ROS, leading to more mtDNA damage. Local hypoxia accompanied adipocyte growth and might be counterbalanced by angiogenic remodeling of blood vessels. Further studies will be necessary to elucidate vascularization and angiogenesis in bovine adipose tissue.

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