Protein deprivation attenuates Hsp expression in fat tissue

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Abstract For ruminants, dietary protein is the first limiting component to the utilization of low-quality forage. Throughout gestation, low-protein intake may result in prenatal programming that causes various metabolic disturbances and physiological modulations to dams and their developing embryos. We studied the effect of long-term low-protein diet (LPD) on physiological, biochemical, and molecular parameters of the energy status in gestating beef cows. LPD resulted in significant reductions in feed intake and heart rate and promoted a negative retained energy status already after 3 weeks. Elevated levels of plasma creatinine and non-esterified fatty acids indicate endogenous degradation of fat and protein as a response to the demands in energy and nitrogen. Increasing levels of β -hydroxybutyrate confirmed the negative energy status obtained by the physiological measurements. At the molecular level, subcutaneous fat, Hsp90, Hsp70, and proteasome subunits decreased significantly after 3 months on LPD, in parallel with an increase of adipocyte fatty acid-binding protein. These results may indicate a decrease in turn-over of proteins, at the cost of induced lipolysis, and suggest that the response to protein deprivation, when examined in an energy-storing tissue, includes downregulation of the constitutive heat shock proteins involved in the protein degradation pathway of energy production and

upregulation of tissue-specific genes such as those involved in energy production from fat degradation.

Keywords Low-protein diet · Hsp70 · Hsp90 · C2 proteasomal subunit · A-FABP · Heart rate · Energy expenditure · Creatinine · BHBA · NEFA · Antioxidant capacity · Subcutaneous fat tissue · Ruminants

Introduction

In ruminants, low-protein intake limits the utilization of low-quality forage as a source of energy (Siebert and Hunter 1982; Koster et al. 1996), due to low supply of nitrogen to the rumen microbes. Under protein-deficient diets, dry matter intake and digestibility in turn fall below requirements for maintenance, leading to detrimental effects on energy balance, performance, milk production and weight gain (Lee et al. 1987; Lanyasunya et al. 2006). Thus, protein-deficient diets in ruminants may in fact be energy-deficient diets, with similar metabolic modulations.

Throughout gestation, a low-protein diet may result in prenatal programming that causes various metabolic disturbances and physiological modulations, such as blunted acute phase response, pancreatic deficiencies, renal dysfunctions and disturbed programming of glutathione metabolism, hypothalamic-pituitary-adrenal axis, and the cardiovascular system. These effects impair the immune system (Langley-Evans et al. 1994; 1997), interfere with the detoxification capacity of glutathione (Langley-Evans et al. 1997), increase blood pressure (Langley-Evans et al. 1996; McMullen et al. 2004), and restrict offspring growth (Snoeck et al. 1990; Dahri et al. 1991).

Recently (Eitam et al. 2009), we have demonstrated that caloric stress promoted attenuation of heat shock protein 70

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(Hsp70) in mammary gland epithelial cells of lactating beef cows. In other experimental systems, induction of Hsp70 was associated with the development of tolerance to caloric stress (Kregel 2002). Hsp70 is a member of the Hsp super family of proteins that are evolutionary conserved polypeptides functioning as molecular chaperones to prevent and repair deleterious damages caused to proteins by a range of environmental and physiological stresses (Georgopoulos and Welch 1993; Parsell and Lindquist 1993; Craig et al. 1994). However, members of the Hsp family are also constitutively expressed in cells. The induced and the constitutively expressed Hsp families help in normal folding of various polypeptides; assist misfolded proteins to attain or regain their native states; help in translocation of proteins to different cellular compartments; and regulate protein degradation (Hartl and Hayer-Hartl 2002; Kovacs et al. 2005).

A pivotal role in protein degradation is attributed to the proteasome. Proteasomes are large multi-subunit proteases that are found in the cytosol and the nucleus of eukaryotic cells. Their ubiquitous presence and high abundance in these compartments reflects their central role in cellular protein turnover (Bochtler et al. 1999). Many subunits of the proteasome contain Hsp. Thus, the potential existence of a coordinated Hsp–proteasome interaction results in a beneficial preservation or upregulation of proteasome activity (Keller et al. 2002). Functioning at both edges of the cellular protein turnover, Hsp and proteasome may be good candidates to monitor changes in protein metabolism in response to protein deprivation.

The aim of the present study was to correlate the overall energy status of gestating beef cows fed LPD with molecular fingerprints of gene products that participate in the metabolism of proteins and lipids (i.e., Hsp, proteasome, A-FABP) in a representative energy-storing tissue, the subcutaneous fat.

Materials and methods

Animals and treatments

Six gestating beef cows (in the third month), from the experimental, Simmental dominated herd of Newe Ya'ar participated in this study. All cows were third parity or greater, with an average age of 6 years. Cows were housed individually to allow measurements of individual feed intake. They were adapted to the individual pens for two weeks prior to the initiation of the experiment. Thereafter, individual control feed intake was measured for 1 week. At the end of this week, cows were adapted for a further 2-week period to the experimental, low-protein diet. Thereafter, individual experimental feed intake was measured for 1 week. Cows were kept on LPD during 3 months, at the

end of which, individual experimental feed intake was measured again for 1 week. The control and LPD were iso-caloric on a metabolizable energy (ME) basis. Their composition is listed in Table 1. Crude protein (CP) was assayed according to AOAC (1990). ME was calculated according to NRC (2001), equations 2-1 and 2-2. While the control diet contained 88% degradable protein, the experimental diet contained 72.5% (calculations according to NRC 1996). Biopsies of subcutaneous fat were taken under local anesthesia (Esrakain; Lidocaine hydrochloride 2%; cat # 020 82 21028 00, Rafa Laboratories LTD, Israel) at the beginning and at the end of the experiment, to follow the expression of Hsp, C2-proteasome subunit, and A-FABP.

All procedures involving animals were approved by the Israeli committee for animal care and experimentation.

Determination of BHBA, NEFA, and creatinine

Plasma β -hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA), and creatinine were measured by COVAS MIRA PLUS (Roche Diagnostics GmbH, Germany), using the following inserts: β -Hydroxybutyrate Reagent Sets (Pointe scientific, inc., USA), NEFA FS (DiaSys Diagnostic systems GmbH, Germany), and CREA (Roche Diagnostics GmbH, Germany).

Energy expenditure measurements

Energy expenditure (EE) was measured by using the heart rate (HR) method as described by (Brosh et al. 1998; 2002; 2004). The method is based on measuring HR throughout several days and then relating it to EE by multiplying HR by the ratio of oxygen consumption to HR, by the O₂ pulse, as measured simultaneously over a short time (10 to

Table 1 Composition of the control and LPD TMRs (grams per kilogram of DM) and calculated ME concentrations

Component	Mix ration	
	Diet	
	Control	Low-protein
Wheat straw	202	313
Wheat hay	233	350
Ensiled poultry liter	332	
Minerals and vitamins ^a	16	16
ME concentration, MJ/kg	7.4	7.7
Crude protein, g/kg	126	56.5

^a Composition per 1 kg of minerals and vitamins premix: calcium carbonate, 450 g; salt, 350 g; dicalcium phosphate, 150 g; Mn, 1.1 g; Zn, 1.1 g; Fe, 1.1 g; Cu, 0.45 g; I, 0.3 g; Co, 7.5 mg; Se, 11 mg; vitamin A, 375,000 IU; vitamin D₃, 75,000 IU; vitamin E, 200 IU

15 min) and by the energy dissipated (heat production) by the animal in consuming a unit of oxygen (kilojoules per liter of O₂). The HR was recorded with a Polar instrument (Polar Electro Oy, Kempele, Finland), model T51H HR transmitter, and a watch model S610 data logger. The devices were attached to the thorax behind the forelegs by means of a specifically designed elastic belt (Pegasus, Eli-ad, Israel). HR was measured continuously for 4 days in each period. In the first period, heart rate measurements were carried out during four consecutive weeks (4 days in each week)—1 week on control and 3 weeks on LPD. The second period of HR measurements was carried out for 3 months on LPD. The data logger was programmed to record HR at 1-min intervals. Oxygen consumption was measured with a face-mask open-circuit respiratory system. The accuracy of the system was checked gravimetrically by injecting nitrogen (N₂ recovery) into the facemask. The measurements on each cow were carried out in a cattle squeeze, between 09:00 and 15:00, immediately after the 4 days HR measurement. During the O₂ pulse measurement, the cow was standing, and the exhaled air was sucked from its face mask and passed through an air filter and a model 50D-15 mass flow meter (McMillan, Georgetown, TX). Changes in exhaled air oxygen concentration in relation to the environmental concentration were measured with a Servomex model 1400 paramagnetic oxygen analyzer (Servomex, Crowborough, East Sussex, UK), and the temperature and relative humidity of the expired air were measured with HygroClip S electronic sensors (Rotronic AG, Basserdorf, Switzerland) to enable calculation of oxygen consumption under standard conditions of temperature, pressure, and zero humidity. These data were recorded and saved simultaneously by the dataTaker model DT 50 (dataTaker, Rowville Melbourne, Victoria, Australia) data acquisition system and logged to a laptop at 5-s intervals; HR was recorded in parallel at the same 5-s intervals. Each cow's O₂-pulse was calculated, and then the EE throughout the day was calculated by multiplying the HR (beats/min) as measured throughout the day by the O₂-pulse (liters per beat), as measured over a short interval, and by the constant value of 20.47 kJ/l of O₂ consumed. The cow's EE for each 5-min interval during the day was adjusted for the metabolic BW (MBW, kilogram of BW^{0.75}) as kilojoules of MBW⁻¹ per day. The use of a constant daily value of O₂ pulse, measured individually in each period, is supported by our previous findings (Brosh et al. 1998; 2002).

SDS-PAGE and Western blot

Whole-cell lysates were boiled in sample application buffer containing 2-mercaptoethanol. Proteins were separated by SDS-polyacrylamide gel (10%) and transferred onto nitrocellulose membranes (Schleicher & Schuell GmbH, Dassel,

Germany). The membranes were probed with monoclonal anti-actin (Sigma, cat # A1978), anti-Hsp70 (recognizing the constitutive and the inducible forms of the protein; Sigma, cat # H5147), anti-Hsp90 (Stressgen, cat # SPA-830), anti-C2 and anti C9 proteasomal subunits (a kind gift from Prof. Chaim Kahana), followed by appropriate secondary antibodies. Proteins were visualized by enhanced chemiluminescence.

A-FABP identification—mass spectrometry analysis

Protein extracts of subcutaneous fat were run on a 10% acrylamide gel and stained with Coomassie blue. The stained protein bands, at a molecular mass of ~15 kDa, were cut from the gel with a clean razor blade, and the proteins were reduced with 10 mmol l⁻¹ DTT and modified with 100 mmol l⁻¹ iodoacetamide in 10 mmol l⁻¹ ammonium bicarbonate. The gel pieces were treated with 50% acetonitrile in 10 mmol l⁻¹ ammonium bicarbonate to remove the stain, followed by drying the gel pieces. The dried gel pieces were rehydrated with 10% acetonitrile in 10 mmol l⁻¹ ammonium bicarbonate containing 0.005 µg µl⁻¹ trypsin and then incubated overnight at 37°C. The resulting peptides were recovered with 60% acetonitrile with 0.1% trifluoroacetate. The tryptic peptides were resolved by reverse-phase high-performance liquid chromatography on 0.1×300-mm fused silica capillaries (J&W, Folsom, CA, USA; 100 µm i.d.) home-filled with porous R2 (Persepective, Framingham, MA, USA).

The peptides were eluted using an 80-min linear gradient of 5–95% acetonitrile with 0.1% acetic acid in water at a flow rate of ~1 µl min⁻¹. The liquid from the column was electrosprayed into an ion-trap mass spectrometer (LCQ; Finnigan, San Jose, CA, USA). Mass spectrometry was performed in the positive ion mode using repetitively full MS scan followed by collision induced dissociation (CID) of the most dominant ion selected from the first MS scan. The mass spectrometry data were compared to simulated proteolysis and CID of the proteins in the NR-NCBI database using the Sequest software (J. Eng and J. Yates, University of Washington and Finnigan, San Jose, CA, USA). The amino terminal of the protein was sequenced on a Peptide Sequencer 494A [Perkin Elmer, (Applied Biosystems), Foster City, CA, USA] according to the manufacturer's instructions.

Antioxidative capacity of the plasma

To test the effect of LPD on the antioxidative capacity of the plasma, 2 ml blood were sampled from the caudal vein of the cows, using evacuated tubes (Greiner bio-one GmbH, Austria) containing EDTA as anticoagulant. The blood was centrifuged at 1,000×g, 4°C, to separate the cells from the

plasma which was immediately frozen in liquid nitrogen until the analysis.

The luminol-enhanced chemiluminescence (CL) assay (Ginsburg et al. 2005) was used to measure the reducing antioxidant potential of plasma. Plasma samples (20 μ l) were added to a reaction mixture containing 10 μ l of luminol (10 μ M), 10 μ l morpholinodisulfonimine (SIN-1, 1 mM), 20 μ l of sodium selenite (2 mM), 10 μ l of bovine serum albumin (100 μ g/ml), and 10 μ l of Co^{2+} (SIN-1 cocktail). This radical-generating cocktail simultaneously generates a flux of peroxide and NO. Luminol enhanced chemiluminescence was measured in a LUMAC/3M Biocounter M2010 connected to a linear recorder, and the resulting light output was recorded as counts per minute (Ginsburg et al. 2005) during 6 min. The antioxidative potential of the plasma is expressed each minute relative to the SIN-1 cocktail.

The antioxidative tendency of plasma was verified by cyclic voltammetry (CV) using a BAS model CV-1B cyclic voltammeter (West Lafayette, IN), equipped with the following electrodes: (1) working electrode (glassy carbon; BAS MF-); (2) Ag:AgCl reference electrode and (3) platinum wire as counter electrode, as described by Dubnov et al. (2000).

Statistical analysis

Repeated measures ANOVA was performed to test for differences in body weight (BW), dry matter (DM) intake, protein content in served feed, refused feed and feces, HR, metabolizable energy intake (MEI), EE, retained energy (RE), antioxidative capacity in response to LPD. Differences in DM intake, refused DM and protein, fecal protein, HR, MEI, EE, and RE between treatments and pregnancy stage, as well as in BHBA were further tested by Bonferroni Mult2012iple Comparison. *T* test for two independent groups was performed to test for differences in HR between the experimental cows and cows of the “pregnancy control” group. We applied one-way ANOVA test for differences in protein content of feeds and in plasma levels of creatinine, NEFA, and BHBA. We used two-tailed Pearson correlation coefficient (*r*) to test the correlation between protein content in refused feed and feces. Statistical analyses were performed using SPSS 14.0 software (SPSS Inc. 2005).

Results

Verification of negative protein balance at the physiological level

Dry matter intake, protein content, and body weight

Three weeks on LPD resulted in a significant decrease in DM intake of gestating beef cows ($F_{2,10}=211.91$, $p<$

0.001). A further decrease was observed after 3 months on LPD ($p<0.05$) (Fig. 1). The protein content in the served diet, refused feed, and feces is presented in Table 2. As planned in the total mixed rations (TMRs), the actual protein content dropped by ca. 50% in the LPD ($F_{2,6}=4163.7$, $p<0.001$). It can also be seen in Table 2 that in response to LPD, the amount of protein in the refused feed decreased significantly ($F_{2,10}=87.83$, $p<0.001$). These differences, however, occurred between control and 3 weeks LPD and control and 3 months LPD ($p<0.05$) but not between 3 weeks and 3 months LPD. A difference in protein content between control and LPD groups was further reflected in the feces. Here, a similar significant decrease was revealed between the control and the LPD groups ($F_{2,8}=107.46$, $p<0.001$), only that in this case fecal protein percentage decreased further ($p<0.05$) from 3 weeks to 3 months LPD. Whereas the protein content in refused feed and feces correlated positively in 3 weeks LPD ($r=0.92$, $p=0.01$, $n=6$), no correlation between them was found in control ($r=0.59$, $p=0.22$, $n=6$) and 3 months LPD ($r=0.54$, $p=0.34$, $n=5$). In spite of a tendency to lose BW in response to LPD (650 ± 59 and 643 ± 57 kg for control and 3 months LPD, respectively), BW did not differ significantly ($F_{2,10}=5.59$, $p=0.06$).

Daily heart rate and energy expenditure

LPD resulted in an immediate decrease in the average daily heart rate of gestating beef cows ($F_{3,15}=13.07$, $p<0.001$). Whereas during the first week, the heart rate (64.1 ± 3.4) tended towards a significant decrease ($p=0.055$), it was significantly lower ($p<0.001$) during the second week on LPD (60.9 ± 3.1).

A maximal effect on heart rate was achieved after 3 months on LPD ($F_{2,10}=22.21$, $p<0.001$). These values (70.1 ± 4.6 , 59.1 ± 4.4 , and 57.6 ± 5.7 beats/min for control, 3 weeks LPD, and 3 months LPD, respectively) differed

Fig. 1 The effect of low protein diet (LPD) on feed intake of gestating beef cows. Results are presented as means $\pm$ SD

Table 2 The actual protein content (grams per kilogram) in TMRs, feed refusals, and feces

	Control	3 weeks LPD	3 months LPD
Diet	12.59±0.09a	5.96±0.08b	5.73±0.13b
Refused feed	13.15±2a	5.32±0.63b	5.64±1.16b
Feces	12.63±1.22a	7.55±0.55b	6.68±0.54c

Results are given as means±SD. Different letters indicate significant differences within rows

significantly between control and 3 weeks LPD and between control and 3 months LPD ($p<0.05$), but no statistical differences in the average daily heart rate were revealed between 3 weeks and 3 months LPD. However, LPD did not affect the circadian pattern of heart rate, which was characterized by two peaks, around 09:00 and 16:00 (Fig. 2).

To eliminate the effect of pregnancy stage, we tested heart rate of four additional cows in the herd, fed control diet, at the same gestating state. This group of cows (at their sixth month pregnancy) is designated “pregnancy control”. As seen in Fig. 2 their average daily heart rate (68.9 ± 4) did not differ significantly from that of the control group ($t_8=0.43$, $p>0.05$) but was significantly higher than the 3 weeks LPD group ($t_8=3.55$, $p=0.007$) and the 3 months LPD group ($t_8=3.41$, $p=0.009$).

The effect of LPD on metabolizable energy intake (MEI) and energy expenditure (EE) is presented in Fig. 3. As seen, MEI decreased significantly upon withdrawal of protein from the diet ($F_{2,10}=222.4$, $p<0.0001$). This fall, in turn, has promoted the adjustment of EE, which tended towards a significant decrease ($F_{2,10}=4.62$, $p<0.057$). By subtracting EE from MEI, we calculated the retained energy (RE). The outcome indicates of significant reduction in RE when comparing control (274 ± 44 kJ×kg BW^{-0.75}×day⁻¹) to 3 weeks LPD (-99 ± 100 kJ×kg BW^{-0.75}×day⁻¹) or 3 months LPD (-116 ± 93 kJ×kg BW^{-0.75}×day⁻¹) ($F_{2,10}=$

Fig. 2 Average daily heart rate of gestating beef cows fed control or low protein diet (LPD; $n=6$). Pregnancy control are cows at the same gestating state as the 3 months LPD cows that were fed control diet ($n=4$). Results are presented as means ± std **Fig. 3** Metabolizable energy intake (MEI) and energy expenditure (EE) of gestating beef cows fed control or low-protein diet (LPD). Results are presented as mean±SD

73.09, $p<0.0001$), with no effect of duration of protein deficiency in the diet on RE.

Verification of negative protein balance at the biochemical level

Plasma levels of creatinine, NEFA, and BHBA

The effect of LPD on plasma creatinine, non-esterified fatty acids (NEFA), and β -hydroxybutyrate (BHBA) is presented in Fig. 4. One-way ANOVA analysis indicate that although creatinine (Fig. 4a) and NEFA (Fig. 4b) levels increased gradually during time on LPD, they did not differ significantly ($F_{2,9}=1.05$, $p=0.39$ and $F_{2,9}=1.68$ $p=0.24$, respectively). Shown in Fig. 4c is a significant difference in BHBA ($F_{2,9}=8.61$, $p=0.008$, respectively). Whereas the Bonferroni multiple comparison test revealed significant increase only between control and 3 months LPD ($p<0.05$), paired t tests indicate significant increase in plasma levels of BHBA also after 3 weeks on LPD ($p=0.02$).

Effects of LPD on Hsp and C2-proteasome expression and antioxidant capacity

In response to 3 months LPD, the expression of Hsp90, Hsp70, and C2-proteasome subunit in the subcutaneous fat tissue was attenuated (Fig. 5). The densitometry of Hsp could not be compared between control and LPD because bands in the LPD lanes were hardly visible. The levels of C2-proteasome subunit, however, decreased significantly ($p<0.05$). In parallel with the attenuation of the above proteins, a marked increase in the levels of A-FABP ($p<0.01$) was observed in the same tissue extracts.

The effect of short- and long-term LPD on the antioxidant capacity of the plasma of the experimental cows is shown in Fig. 6a. The plot presents the reducing

Fig. 4 The effects of low protein diet (LPD) on plasma levels of creatinine (**a**), non-esterified fatty acids (NEFA; **b**) and β -hydroxybutyrate (BHBA; **c**). Results are presented as mean $\pm$ SD

potential of the plasma during a 6-min period. In this assay, lower counts represent higher reducing potential. The reducing potential of the plasma was highest in 3 weeks LPD and lowest in control ($F_{2,6}=6.55, 9.13, 11.08, 11.67, 13.48, 14.87, p=0.031, 0.015, 0.01, 0.009, 0.006, 0.005$, for 1–6 min). CV measurements matched the antioxidant tendency of plasma measured by the CL assay (Fig. 6b). They indicate an increased anodic current, in response to LPD, at ca. 0.3 V, representative of changes contributed by low-molecular weight antioxidants (LMWA) (Fig. 6c).

Fig. 5 Representative Western blot for Hsp70, Hsp90, C2-proteasome subunit, adipocyte fatty acid-binding protein (A-FABP), and actin (as loading control) in subcutaneous fat tissue of gestating beef cows fed 3 months low-protein diet (LPD). C control

Discussion

The deleterious effect of LPD on the energy status of gestating cows

To test the effect of LPD on the expression of the selected proteins in the subcutaneous fat tissue, LPD was designed to be iso-caloric to the control diet and to be lower by ca. 50% in its protein concentration. It was reported that low-protein content may exert detrimental effects on energy balance, performance, and production in ruminants (Lee et al. 1987; Lanyasunya et al. 2006). Hence, the first step was to verify the deleterious effect of LPD on the energy status of gestating beef cows. To do so, we followed the changes in cows' HR, EE, feed intake, and BW.

Average daily HR decreased soon after LPD was applied, reaching values that were lower by ca. 11 and 12 beats/min after 3 weeks and 3 months, respectively. Previously, we reported changes in the average daily HR of heifers and cows, grazing or in confinement, in low or high stocking rates and in response to diets with different ME (Brosh et al. 1998; 2006; Brosh 2007). The current study demonstrates that this measure is applicable also to distinguish between diets with different protein contents, presumably because nitrogen deficiency limits the utilization of the roughage by the rumen microbes (Siebert and Hunter 1982), leading to an indirect effect on the ME of the diet. Despite the changes in the HR values, its pattern remained unaffected in response to LPD.

Brosh et al (2004) reported the effect of feed quality on HR and EE levels. Accordingly, HR and EE values were high during late winter and spring, when the feed quality was high, and declined with the deterioration of herbage quality. A similar phenomenon was reported by Brosh et al

Fig. 6 The antioxidant capacity of plasma of gestating beef cows fed control or low-protein diet (LPD) measured by the chemiluminescence (a) and cyclic voltammogram assays (b, c). Results of chemiluminescence assay (a) are presented as means $\pm$ SD. Please note that in this assay, lower counts represent higher reducing potential. The representative cyclic voltammogram data of cows' plasma under control or 3 months low-protein diet are presented in panels (b) and (c), respectively. Arrows indicate the anodic current contributed by low-molecular weight antioxidants. Note that this current increases in response to LPD (panel c)

(1998) when switching between heifers' diets of low and high ME. The results presented in the current study indicate that in response to LPD, cows reduced their metabolic rate, and that EE measurements are capable of accurately

estimating deterioration of the energy status of gestating beef cows, driven by LPD.

MEI was significantly affected by the protein content in the diet, thus serving as a good indication for the deterioration in the energy status of the experimental cows. However, in spite of the marked significant decrease in feed intake, BW did not change during the entire experiment. Recently (Eitam et al. 2009), we showed that a diet of a reduced ME resulted in significant losses in cows' BW. Being pregnant and in lactation state, the cows in our previous study experienced heavier energetic burden in comparison with the current study in which the cows were not nursing. We assume that protein restriction, although influencing digestibility of feed in ruminants, affects the degradation of endogenous energy resources more moderately than energy restriction (see below).

Biochemical indications for the negative energy status

The fate of protein in the refused feed and feces, in response to LPD, implies that under these conditions, cows tended to utilize more efficiently protein from the diet. For the refused feed, it is better pronounced after 3 weeks on LPD, whereas the fecal analysis for protein demonstrates that protein utilization from the diet becomes more efficient with time on LPD.

A continuous increase in plasma creatinine was observed in the current study. Creatinine is an end-product of creatine metabolism in muscle and is an index of total muscle mass (Eisemann et al. 1989). It was shown in high-producing lactating sows that the increasing levels of serum creatinine tended to match the increasing levels of plasma urea (Richert et al 1996). The authors postulated that it might indicate an increased muscle catabolism to meet the needs of milk production. It is tempting to hypothesize that in the current study, changes in creatinine levels reflect a metabolic response to demands for nitrogen, which is directed to the developing embryo.

In addition to the increase in plasma creatinine, we observed a gradual elevation in NEFA in response to LPD. Elevated levels of NEFA usually indicate body fat mobilization, which in the present study may imply of a response to the demand for available energy.

BHBA is a marker for ketosis, a familiar metabolic disorder in high-producing dairy cattle, which is less common in beef cows. Ketosis is caused by a severe negative energy balance that stems from high production, insufficient energy intake, and excessive body fat mobilization (de Roos et al. 2007). The significant increase of plasma BHBA in response to LPD is the biochemical result of the physiological imbalance of the experimental cows and strengthens the power of O₂P-HR method as a reliable measure of the energy status.

Effects of LPD on cow's energy metabolism in fat tissue—attenuation of basal levels of Hsp and proteasomes

When coming to discuss the effect of LPD on Hsp expression in fat tissue, one should distinguish the roles of Hsp in inducible stress responses and inducible defenses from those of constitutive Hsps, functioning primarily as chaperones under non-stress conditions, to hold non-native polypeptides as soluble folded intermediates in a refolding competent state, thus preventing aggregation (Morimoto and Santoro 1998). In this sense, it is more likely that the diminished Hsp and proteasome responses of the fat tissue reported herein may reflect a local, fat tissue response. In this case, the fat tissue, which is a representative energy-reserve tissue, serves as an autonomous energy-rich entity, the gene expression of which was reprogrammed to be adjusted to the new metabolic state caused by the long term consumption of LPD. While being involved in protein synthesis and protein degradation, Hsp70, Hsp90, and the proteasome machineries promote protein turn-over in the cell, which in the case of LPD may fall to a new state. As shown here and by others, low-protein intake adversely affects also the energy balance in ruminants, and increase the need for energy from endogenous sources. This, in turn, promoted the expression of A-FABP that participates in lipid degradation (Jenkins-Kruchten et al. 2003). In this regard, this finding follows the biochemical indications for lipolysis discussed earlier.

The attenuated Hsp response shown herein is supported by a recent study reporting a diminished chaperoning capacity upon amino acid starvation (Hensen et al. 2011). In a recent study, we showed that while Hsp70 attenuated in mammary gland epithelial cells, in response to caloric stress, FABP3 levels increased (Eitam et al. 2009). We suggested that as Hsp70 assists newly synthesized proteins to attain their native states, regulates protein degradation, and is an important component of cellular networks, this attenuation reflected a decrease in the turn-over of self proteins in favor of milk-components producing factors. These results may be supported now by the current findings, thus broadening our view as for the Hsp response in energy storing and producing tissues upon nutritional deprivation. Specifically, the current results may indicate a shift from protein to fat energy metabolism for a more effective energy production.

Ding and Keller (2001) suggested that proteasome function, which is subjected to inhibition by oxidative stress, may be preserved by Hsp. Considering the above, we propose that the molecular fingerprint of Hsp, proteasome, and A-FABP reflects a local response of the fat tissue to LPD and may be indicative of low-protein intake in beef cows. Further studies, however, are needed in order to confirm this hypothesis.

We postulated a-priori that LPD, due to its effects on nitrogen metabolism or its indirect effect on cows' ME and energy status, would alter their antioxidant capacity. The outcome was a significant increase in the reducing potential of the plasma. Energy restriction is a well-documented method for increasing the lifespan of laboratory animals.

It reduces the development of age-related immune alterations and decreases levels of cellular oxidative stress in several organ systems. The prevailing view as to the mechanism underlying the beneficial effects of diet restriction in retarding the aging process is that because a low calorie intake results in less glucose utilization by cells, there is reduced mitochondrial production and thus less oxidative damage to cells over time (Sohal and Weindruch 1996). In the present study, cows decreased their metabolic rate in response to LPD. Thus, the increased antioxidative potential of the plasma may in fact reflect decreased production of ROS and consequently decreased oxidative burden on the reducing force of the plasma. If so, and as oxidative stress triggers the induction of Hsp, it may support our current finding of attenuated Hsp expression in response to LPD.

The antioxidant capacity of various mice tissues in response to diet restriction was tested by Dubnov et al (2000). The authors reported a tissue-specific response; whereas the antioxidant capacity of heart, kidney, and muscle was enhanced, liver and small intestine deteriorated. They proposed that part of the biological regulation of the antioxidant system could originate from synthesis of endogenous low-molecular weight antioxidants (LMWA), to compensate for lower intake of antioxidants. Based on our CV measurements, the increased anodic current at ca. 0.3 V represents changes contributed by LMWA (Fig. 6c). However, changes in plasma LMWA in response to LPD are yet to be measured.

Summary

The current study provides evidence that dietary protein deprivation results in diminished expression of Hsp and proteasome in a representative energy-storing tissue, the sub-cutaneous fat. Together with previous studies (Eitam et al. 2009), these findings may broaden our view as for the Hsp response in energy storing and producing tissues upon nutritional deprivation—favoring the expression of tissue-specific genes at the cost of Hsp.

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