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Comparing mRNA levels of genes encoding leptin, leptin receptor, and lipoprotein lipase between dairy and beef cattle

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Abstract

Body weight and fat mass vary distinctly between German Holstein (dairy cattle) and Charolais (beef cattle). The aim of this study was to determine whether the expression of the obese (Ob) gene and lipoprotein lipase (LPL) gene in fat tissues and expression of the long isoform leptin receptor (Ob-Rb) gene in the hypothalamus were different between these two cattle breeds. Body weight and the area of longissimus muscle cross-section of German Holstein were lower ($P < 0.001$), while body fat content, as well as the omental and perirenal fat mass were higher ($P < 0.001$), compared to Charolais. Plasma insulin and leptin levels between two cattle breeds were determined by radioimmunoassay. Compared to Charolais, plasma insulin concentrations were significantly higher ($P < 0.01$), and plasma leptin levels were tended to be higher ($P < 0.1$) in German Holstein. Ob mRNA levels in subcutaneous and perirenal fat depots, but not in the omental fat depot, were significantly higher ($P < 0.05$) in German Holstein than in Charolais. LPL mRNA expression in the perirenal fat depot of German Holstein was greater in abundance than that of Charolais. No significantly different LPL mRNA levels were found in subcutaneous and omental fat depots, and Ob-Rb mRNA levels in the hypothalamus between these two cattle breeds ($P < 0.05$). Both Ob and LPL expression was greater in perirenal and omental fat depots than in the subcutaneous fat depot ($P < 0.05$). Data indicated that in bovine the Ob and LPL gene expression levels in perirenal fats are an important index that is associated with body fat content, while Ob-Rb in hypothalamus is not.

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1. Introduction

Leptin, the product of the obese gene (Ob), is secreted mainly by white adipose tissue, although low levels have been detected in other tissues [1–3]. Ob mRNA levels are proportional to adipocyte size and increase with increasing levels of body fat [4–6]. Ob expression can be regulated by insulin, glucocorticoids, testosterone, and possibly by other hormones [7,8]. Leptin acts on the central nervous system to regulate body weight and consequently influences fat deposition in animals and humans through the control of appetite and energy expenditure [4,9]. Multiple splice variants of leptin receptor (Ob-R) mRNA encode at least six leptin receptor isoforms [3]. All isoforms share identical extracellular ligand-binding domains, but they differ at their C-terminus. The long isoform leptin receptor (Ob-Rb) is normally expressed at high levels in hypothalamic neurons and at low levels in other cell types, including adipocytes [10] and vascular endothelial cells [1]. Lipoprotein lipase (LPL) is produced in adipose tissue and other tissues, including heart and skeletal muscle. LPL is transferred to the surface of the capillary endothelium, where it catalyzes the rate-limiting step in the hydrolysis of triglycerides from circulating chylomicrons and very low density lipoproteins [11]. LPL controls triacylglycerol partitioning between adipose tissues and muscles, thereby increasing fat storage or providing energy in the form of fatty acids for muscle growth [12]. For this reason, the study of LPL is of particular interest in tissues of meat-producing ruminants.

Different cattle types, like German Holstein as dairy cattle and Charolais as beef cattle, develop differently during postnatal growth. After the period of fattening, the body skeletal muscle and fat contents are quite different between these two breeds. The molecular mechanisms which induce the different levels of fat deposition between German Holstein and Charolais are still unclear. By comparing the expression levels of fat-related genes between these two cattle breeds a greater understanding of the mechanisms involved may be obtained. Therefore, one of the aims of the present study was to examine whether dairy and beef cattle have different expression levels of Ob and LPL mRNA in fat depots and differences in Ob-Rb gene mRNA levels in the hypothalamus. It was also of interest to compare the plasma leptin and insulin levels between German Holstein and Charolais.

2. Material and methods

2.1. Animals

Thirty-six German Holstein and Charolais bulls (18 of each breed) were cared for according to the Animal Protection Committee from the Ministry for Agriculture and Nature Protection, Schwerin, Germany. During the trial two bulls, one German Holstein and one Charolais, were excluded due to illness. All animals were fed the same diet and were reared using a tethering system in individual pens. During the finishing period, animals were fed with hay *ad libitum*, and concentrates (the amount of concentrates was adapted to different growth periods). The composition of concentrates was as follows: 73% barley, 19% soy bean cake, 5.5% beet residue, 2.5% minerals and vitamins. The concentrates contained 16.8% crude protein.

The energy intake of Charolais was 68.8 MJ/kg dry matter/kg daily gain, and the energy intake of German Holstein was 79.5.

2.2. Sample collection

All animals were slaughtered at 18 months of age. Blood samples were collected from the *vena jugularis* at 06:30 h in the Institute's stable, about 1 h prior to transport to the Institute's slaughterhouse. Plasma samples were stored at -70°C after centrifugation of blood at $3000 \times g$ for 20 min at 4°C . After stunning with a shot device animals were killed by bleeding out. The subcutaneous, perirenal, and omental fat, and the hypothalamus were collected within 30 min after slaughter. The subcutaneous fat was taken from a depot overlying the *semitendinosus* muscle. The perirenal fat was sampled from around the kidneys and omental fat was from around the gut. The boundaries used to dissect the hypothalamus were: the rostral edge of the optic chiasm, the dorsal edge of the mamillary bodies, and the width of the optic chiasm. All tissue samples were immediately frozen in liquid nitrogen, and stored at -70°C until RNA extraction.

2.3. Measures of carcass quality

Live weight, along with the perirenal and omental fat pad mass were weighed. *Longissimus* muscle cross-section areas were measured on a 1-cm thick muscle slice, removed from the 12th rib area, using a computerized image analysis system (Quantimet 570, Cambridge Instruments, Leica, Bensheim, Germany). Body fat was chemically determined via the soxhlet extraction method using petroleum ether as the solvent and determined gravimetrically after evaporating the extracting solvent according to standard methods [13]. Carcass fat, (summarized from the different cuts i.e. intra-, and intermuscular fat, and subcutaneous fat), fat in edible organs, and fat in internal fat depots values were individually determined after each respective organ, muscle, or depot were ground and a sample of the ground tissue was analyzed.

2.4. Plasma hormone assay

The determination of insulin was performed by the porcine insulin RIA kit (PI-12K, Linco Research, Inc., St. Charles, MO, USA) which used a purified human insulin as standard, a first antibody raised in guinea pigs, and a goat-anti-guinea pig IgG for the bound/free separation. A standard curve was prepared at concentrations from 2 to 200 $\mu\text{U/L}$. Cross-reactivity with bovine insulin was 90%. All samples were analyzed in duplicates. The sensitivity of the insulin RIA was at 2 $\mu\text{U/mL}$ calculated after measurements by a multi-crystal-gamma counter with a RIA program (LB 2104, Berthold, Bad Wildbad, Germany). Intra- and inter-assay coefficients of variation were 4.3 and 8.2%, respectively.

For leptin measurement, the multi-species leptin RIA kit (XL-85K) from Linco Research was used. The double-antibody assay utilized an anti-human leptin antibody raised in guinea pigs (first antibody) and a human standard hormone. This assay showed a wide cross-reactivity to several species thereby enabling the analysis of plasma leptin in cattle, pigs and horse, as well others. Linearity and parallelism to the standard curve determined for a range of sample volumes between 25 and 100 μL (confirmed the usefulness of the Linco test for our study.

The lowest level of leptin that can be detected by the RIA was 0.9 ng/mL (human equivalent) calculated by the integrated software of the counting instrument. Intra- and inter-assay coefficients of variation were 5.2 and 8.0%, respectively. All steps of RIA methods for plasma insulin and leptin were performed according to the manufacturer's instructions.

2.5. RNA extraction, cDNA synthesis, and polymerase chain reaction

Total RNAs were isolated from tissue samples using the RNeasy mini kit (QIAGEN GmbH, Germany) according to the manufacturer's instructions. RNA yields and purities were assessed by absorbance at 260 and 280 nm in a RNA/DNA Calculator (Pharmacia GeneQuant™). Ratios of absorption (260/280 nm) of all preparations were between 1.8 and 2.0. Aliquots of RNA samples were subjected to electrophoresis to verify their integrity.

The cDNA was synthesized using 1.0 µg of total RNA from each sample. To eliminate residual genomic DNA from the RNA sample, prior to the RT reaction, 1 unit of DNaseI (Roche Diagnostics GmbH, Mannheim, Germany) was added and incubated at 37°C for 30 min followed by heat-inactivation of the enzyme at 75°C for 5 min [14]. RNA samples were denatured at 65°C for 15 min and placed on ice for 5 min before reverse transcription (RT). The final reaction volume, 25 µL, contained 1× reaction buffer, 5 mM MgCl₂, 1 mM of dNTPs, 3.2 µg of random hexamer primer, 50 units of RNase inhibitor, 0.01 mg/mL gelatin and 20 units of AMV reverse transcriptase (1st strand cDNA synthesis kit, Roche Diagnostics GmbH, Mannheim, Germany). The reaction was performed at 25°C for 10 min, 42°C for 60 min, 99°C for 5 min for final enzyme inactivation, and 4°C for 5 min. RT products were either stored at –20°C or used directly for PCR.

PCR was performed in a 50 µL reaction volume containing 2.0 µL tissue specific cDNA (equivalent to 80 ng of starting RNA), 1.5 mM MgCl₂, 2 units of Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany), 0.2 mM of dNTPs and 0.4 µM of each primer. For amplification of the target genes, the following primer pairs were used: Ob gene (GeneBank accession U50365) forward 5'-CATCTCACACACGCAGTCCG and reverse 5'-CTGCCG-CAACATGTCCTGTA, for amplification of a 349-bp fragment of bovine Ob cDNA; leptin receptor (GeneBank accession U62385) forward 5'-GTGCCAGCAACTACAGATG and reverse 5'-AATTTCCCTCAAGTTTCAA, for amplification of a 400-bp fragment of Ob-Rb cDNA [15]; LPL (GeneBank accession M16966) forward 5'-AGTCGCCTTTCTCCTGATGATG and reverse 5'-GAAGGCCTGGTTGGTGTATGTATT, for amplification of a 501-bp fragment of bovine LPL cDNA. To obtain optimal conditions for amplification, in the exponential phase of PCR, the cycle numbers were tested first for each target gene. Plotting of intensity of PCR signals (as expressed by net intensity) against the number of amplification cycles revealed a linear relationship between cycles 24 and 32 for Ob (correlation coefficient $r^2 = 0.975$), between 30 and 38 ($r^2 = 0.972$) for Ob-Rb, and between 20 and 30 ($r^2 = 0.983$) for LPL.

QuantumRNA™ 18S primer and competitor (Ambion, Inc. Austin, Texas) were used as internal controls of amplification. The primer pairs (catalog no. 1716 and 1718) amplify 489 and 324 bp fragments, respectively. The ratio of 18S primer to competitor was 2:8 for Ob and LPL genes, and 1:9 for Ob-Rb. Amplifications were performed in a Biometra Personal Cycler (Biomedizinische Analytik GmbH, Göttingen, Germany). For the Ob gene the following cycle parameters were used: 120 s at 94°C, 28 cycles at 94°C for 40 s, 55°C for 45 s, 72°C for 45 s.

For Ob-Rb, we used 34 cycles at 94°C for 40 s, 55°C for 40 s, 72°C for 45 s. For LPL, we used 24 cycles at 94°C for 40 s, 57°C for 45 s, 72°C for 45 s. Each reaction was followed by 5 min at 72°C and continuous hold at 4°C. After amplification, 10 µL of each PCR product were analyzed by agarose gel electrophoresis (2%). The RT and PCR reactions of each sample were repeated 2–3 times.

To confirm that the amplified fragments were those predicted, the PCR products were isolated from the gel using a QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany) and sequenced.

2.6. Quantitation of PCR products (image analysis)

Gels were stained with ethidium bromide and photographed. Net intensities of individual bands (same area) were measured with the Electrophoresis Documentation and Analysis System 120 (Eastman Kodak Company, Rochester, NY, USA). Ratios of net intensity of target genes to that of the internal control bands (QuantumRNA™ 18S) were calculated before statistical analysis. To minimize the between-assay error, samples from two cattle breeds were always processed in parallel.

2.7. Statistics

The experimental units were bulls of two breeds. For the considered traits, means and standard errors of the means (SEM) were calculated. For the comparison between the breeds, the TTEST procedure of SAS version 8.0 was used. Data from the three fat depots were analyzed using one-way ANOVA.

3. Results

3.1. Body weight, carcass measurement and plasma hormones

German Holstein grew slower than Charolais. An average daily gain of 1023 ± 54 g for German Holstein, and 1188 ± 49 g for Charolais, was obtained during the final three months. After 18 months of fattening, the body weight and *longissimus* muscle cross-section area of German Holstein cattle was lower ($P < 0.001$), as compared to Charolais, but body fat content, as well as the omental and perirenal fat weights were higher ($P < 0.001$) by 42.1, 55.6 and 41.2%, respectively. Therefore, German Holstein cattle are characterized by a higher fat content and lower protein content as compared to Charolais cattle. Compared to Charolais, plasma insulin levels in German Holstein were significantly higher ($P < 0.001$). The plasma leptin concentrations of German Holstein tended to be higher (15%, $P < 0.1$) than that of Charolais (Table 1).

3.2. mRNA levels of candidate genes

Relative mRNA levels of Ob and LPL in the three fat depots were measured by semi-quantitative RT-PCR. Compared with Charolais, relative Ob mRNA levels in subcutaneous and

Table 1

Carcass measurements and plasma hormone levels in bulls of different breeds at 18 months of age (means \pm SEM)

Trait	German Holstein	Charolais	Significance
Number of cattle	17	17	
Live weight (kg)	671.2 \pm 10.2	755.4 \pm 11.0	$P < 0.001$
<i>Longissimus</i> area (cm ²)	82.84 \pm 2.44	127.32 \pm 2.60	$P < 0.001$
Body fat content (%)	26.91 \pm 0.92	18.94 \pm 0.85	$P < 0.001$
Omental fat (kg)	20.93 \pm 0.93	13.45 \pm 0.70	$P < 0.001$
Perirenal fat (kg)	19.72 \pm 0.99	13.97 \pm 0.79	$P < 0.001$
Insulin level (U/mL)	37.15 \pm 3.33	24.76 \pm 2.43	$P < 0.01$
Leptin (ng/mL)	3.82 \pm 0.16	3.33 \pm 0.24	$P < 0.1$

perirenal fat depots were significantly higher ($P < 0.05$) in German Holstein. No significant differences were detected in the omental fat depot between these two cattle breeds (Fig. 1A and D). LPL mRNA levels were also higher ($P < 0.05$) in the perirenal fat depot of German Holstein than that of Charolais, but not in the subcutaneous or omental fat depots (Fig. 1B and E). We also examined whether there was a regional difference in Ob and LPL gene expression among three different fat depots. In both cattle breeds, Ob and LPL mRNA levels were higher in perirenal and omental fat depots than in the subcutaneous fat depot ($P < 0.05$; Fig. 1D and E). The Ob mRNA expression was not different between subcutaneous and omental fat depots in German Holstein. No significantly different Ob-Rb mRNA levels were detected in the hypothalamus between German Holstein and Charolais (Fig. 1C and F).

4. Discussion

Endocrine and molecular studies are useful to understand biochemical pathways that have been altered during the selection process in dairy and beef cattle. The differences in body composition between German Holstein and Charolais in this study are characterized by a higher body fat content and higher perirenal and omental fat mass in German Holstein. It is known that the somatotrophic axis, insulin, and leptin play important roles in regulating the utilization and partition of nutrients [16–18]. Insulin has been shown to stimulate lipogenesis and fatty acid esterification, while inhibiting fatty acid oxidation [19,20]. Therefore, a higher plasma insulin level is likely one of the reasons for higher body fat content in German Holstein.

It has been reported that leptin secretion is highly correlated with body fat mass in mice [21,22], in humans [1,4,5], and ruminants [6,23–25]. Investigations in beef cattle [26] support the concept that there is a positive relationship between circulating leptin and intramuscular fat content in cattle. Therefore, a numerically greater plasma leptin level in German Holstein, in the present study, is consistent with the higher fat deposition in this cattle breed.

Comparison of fat-related gene expression between the two different cattle breeds will be useful in understanding adipogenic mechanisms. It is not yet clear which genetic alterations in German Holstein and Charolais contribute to the differences in body fatness. It was of considerable interest to determine whether the expression of Ob, LPL, or Ob-Rb genes varied

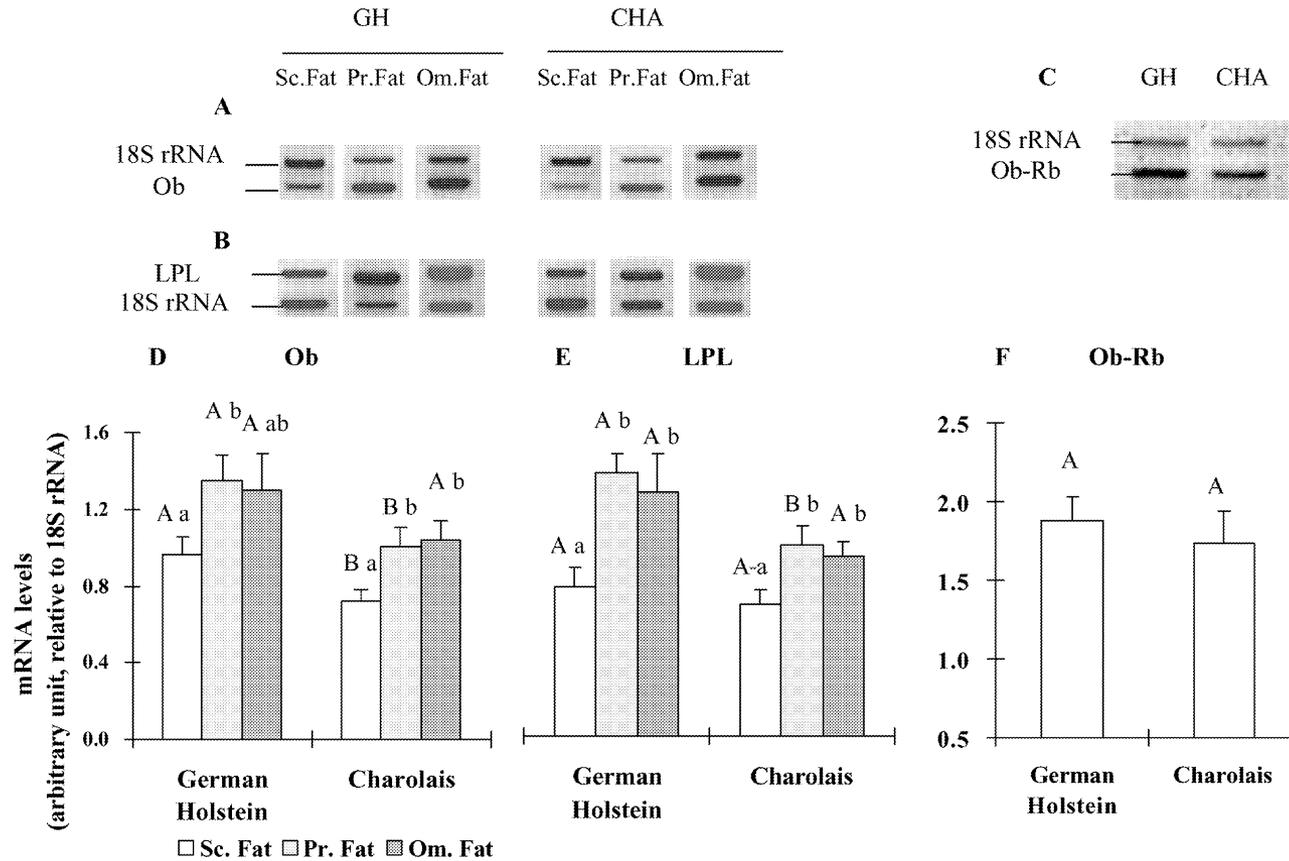


Fig. 1. Expression of mRNA for leptin (Ob), lipoprotein lipase (LPL), and long isoform leptin receptor (Ob-Rb) from German Holstein (GH) and Charolais (CHA) cattle. (A, B) Gel electrophoresis (2%) shows respectively RT-PCR products of Ob, LPL and 18S ribosomal RNA (internal standard) from subcutaneous fat sampled over the *semitendinosus* (Sc. Fat), perirenal fat (Pr. Fat), and omental fat (Om. Fat). (C) Gel electrophoresis (2%) presents RT-PCR products of Ob-Rb gene from hypothalamus in two cattle breeds compared with 18S ribosomal RNA. (D, E, F) Semi-quantitative RT-PCR of the Ob, LPL, and Ob-Rb genes, respectively (duplicate representative tests). RT-PCR products of each tissue in GH ($n = 17$) and CHA ($n = 17$) were normalized with an internal standard. Levels are reported as means \pm SEM. Means with different superscripts are significantly different ($P < 0.05$), lowercase letters (a, b) refer to differences among three fat depots and capital letters (A, B) refer to differences between the breeds.

between dairy and beef cattle. Limited research has been done on Ob mRNA expression in ruminants [27–32]. The expression of Ob mRNA in genetically lean and fat selected lines of sheep was studied by Kumar *et al.* [28]. The difference in body fat contents in lean and fat lines of sheep, adjusted for carcass weight (21.2% versus 29.3%), was nearly the same as in our study between the two cattle breeds (18.9% versus 26.9%). The relative level of Ob expression was higher in the sheep fat line paralleling our results. Housknecht *et al.* [32] demonstrated high concentrations of insulin stimulated bovine Ob mRNA levels *in vitro*. But Kauter *et al.* [33] observed no change in circulating leptin in response to changes in glucose or insulin in sheep, thus suggesting that insulin may not be an important regulator of leptin *in vivo* in ruminants.

In this study the highest level of Ob gene expression was detected in the perirenal fat depot among the three fat depots of both dairy and beef cattle. This is consistent with results reported by Kim *et al.* [31] for cattle. They found the Ob gene to be highly expressed in perirenal fat, moderately in subcutaneous and intermuscular fat, and expressed in lower levels in intramuscular fat. In contrast, Ji *et al.* [27] found similar levels of Ob expression in perirenal, omental, and subcutaneous fat depots evaluated from five finished steers. In rodents, Ob mRNA levels are much higher in the perirenal depot than in subcutaneous depot [34,35]. In humans, Ob has been shown to be expressed at significantly higher levels in subcutaneous than omental fat tissue [5,36]. These data demonstrate that the Ob gene is expressed not only in a fat depot specific manner, but also in a species specific manner. The different expression of Ob gene mRNA may be dependent on different insulin sensitivity between the fat depots [31], or due to differences in the adipocyte size [37].

Research work from Chilliard's group [12,38,39] demonstrated that LPL also plays an important role in ruminant species. In adult sheep at maintenance levels, 55–60% of the total amount of free fatty acids originate from hydrolysis of circulating triacylglycerols by LPL [40]. Hocquette *et al.* [12] demonstrated the levels of LPL transcripts are positively related to LPL activity in bovine tissues, including muscles and adipose tissues. Similar to the result of Ob gene expression, in the present study, numerically greater LPL mRNA levels were detected in all three fat depots from German Holstein compared to Charolais, though significant differences were only found in the perirenal fat depot. LPL gene expression is affected by a number of factors including nutrient level [38] and certain hormones, such as insulin [41]. In the present study, the nutrition level was the same between two cattle breeds. Therefore, in comparison to Charolais higher plasma insulin levels may induce higher LPL gene expression in German Holstein. We also found LPL gene expression to be fat-depot specific. The results are consistent with Hocquette *et al.* [12] who found that LPL mRNA levels were higher in perirenal and omental fat depots than in the subcutaneous fat depot. Depot-specific differences in LPL mRNA levels were related to different metabolism and LPL activity among fat depots which have been previously reported in cattle [42]. These differences also can be correlated with the varied size of adipocytes among these fat depots, as Barber *et al.* [43] reported that expression of the LPL gene in sheep was highly correlated with the size of adipocytes.

To our knowledge, there is currently no published literature describing Ob-Rb gene expression levels in bovine hypothalamus. Numerous studies on other species demonstrated that the hypothalamus is the predominant region for Ob-Rb gene expression, which is considered to be the principal signaling leptin receptor isoform in the brain [44–46]. Leptin binding to

Ob-Rb in the hypothalamus is essential to regulate food intake and energy expenditure [47]. Very recently Cohen *et al.* [48] by selective deletion of leptin receptor in mice proved that the extent of obesity was negatively correlated with the level of leptin receptor in hypothalamus. However, in our experiment the mRNA levels of leptin receptor in bovine hypothalamus were not significantly different between the breeds. One of the reasons may be that ruminants have a different energy metabolism than that of monogastrics: rumen fermentation plays a very important role in the ruminant digestive system. It is thus difficult to understand the function of the leptin system in the energy balance of ruminants. Recent research results from Baskin *et al.* [47] suggest that the expression of Ob-Rb mRNA in the rat hypothalamus is sensitive to nutrition levels. As all animals in our experiment were provided feed according to their live weight development (according to their respective nutritional requirements), this excludes the effect of nutrition level on Ob-Rb mRNA expression in the hypothalamus between German Holstein and Charolais.

In summary, Ob and LPL gene expressions in fat tissues, particularly in perirenal fat, are different between German Holstein and Charolais. The cause for these differences is still unclear. Results also indicate that higher plasma insulin levels and higher LPL gene expression in fat depots are associated with the higher fat content in dairy cattle. Compared to mice, the bovine OB-Rb mRNA level in hypothalamus is not correlated to body fat deposition.

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