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Cellular conditions for intramuscular fat deposition in Japanese Black and Holstein steers

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ABSTRACT

The experiment was conducted to study the development of intramuscular fat in Japanese Black (JB) compared to Holstein (HS) steers and to find breed differences for fat depot development and distribution in the carcass under equal feeding conditions. Additional to slaughter samples, biopsy samples of longissimus muscle (LM) and subcutaneous fat, taken at 10, 14, 18, and 22 months of age, were used for histological and molecular investigations. Japanese Black steers stored about 14% more fat in the LM ($P = 0.001$), resulting in larger marbling flecks ($P < 0.001$). Muscle fibers and intramuscular adipocytes in both breeds responded to the high energy feeding with significant enlargement, which was faster in JB. Histograms of intramuscular adipocytes size showed a shift toward larger cells during growth, but also the abundance of small, developing adipocytes. This development was accompanied by a correlated up-regulation of adipogenic genes until 22 months of age.

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1. Introduction

Visible intramuscular fat (IMF) or marbling is an important meat quality trait which is appreciated by the consumer because of the positive influence on taste, juiciness, and tenderness of meat (Platter, Tatum, Belk, Koontz, Chapman, & Smith, 2005). The development of marbling is not completely independent from other fat depots in the body and fat distribution between different depots in the carcass varies with breed, sex, and nutrition (Pethick, Harper, & Oddy, 2004; Robelin, 1986). In former studies it was shown that IMF develops in different breeds continuously during growth, but with varying intensity, leading to different marbling scores at slaughter (Albrecht, Teuscher, Ender, & Wegner, 2006; Bruns, Pritchard, & Boggs, 2004). A breed with the unique ability to store enormous amounts of fat within the muscle, like Japanese Black (JB), is therefore a useful model to study cellular conditions for IMF deposition. We compared JB with Holstein (HS), another main source of beef for consumers in Japan and Europe with the ability to deposit intramuscular fat. When kept in different production systems in Japan and Europe, animals develop large differences in body composition and IMF content (Gotoh et al., 2009). In the present study, steers of both breeds were kept under the same high energy feeding conditions to support maximum marbling development.

The objective of the study was to quantify breed differences in carcass fat distribution and in the development of intramuscular adipose tissue. Samples were taken by means of shot biopsy to follow changes in cellularity and transcriptional activity of intramuscular and subcutaneous adipocytes during growth. Adipocyte size profile and mRNA abundance of characteristic genes were used as indicators for the maturity state of adipocytes in a tissue. A powerful regulator of the mature phenotype is peroxisome proliferator-activated receptor γ (PPARγ), which induces the expression of many mature adipocyte genes, such as adipocyte fatty acid binding protein (FABP4), fatty acid synthase, and lipoprotein lipase (Boone, Mourot, Grégoire, & Remacle, 2000; Tontonoz, Hu, Graves, Budavari, & Spiegelman, 1994). The suitability of PPARγ or FABP4 mRNA abundance as an indicator for intramuscular fat deposition was tested.

2. Materials and methods

2.1. Animals and sampling

Six JB and 6 HS steers were kept under the same conditions from 10 to 26 months of age. The animals were cared for and slaughtered according to Guidelines for Animal Experiments in the Faculty of Agriculture of Kyushu University and to laws of the Japanese Government (Law No. 105, Notification No. 6). Steers were raised in a pen with group feeding using the standard feeding system for the

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production of marbled beef (Gotoh et al., 2009). They were fed twice daily a high energy diet starting at 10 months of age until slaughter at 26 months of age. From 10 to 18 months of age, the diet consisted of increasing amounts of concentrate (36.8 to 86.4%, formula feed) and respectively decreasing amounts of roughage (jamboree, hay and rice straw). During the last stage from 18 to 26 months of age, the diet consisted of 86.4 to 84.2% of concentrate and 13.6 to 15.8% roughage. All steers had permanent access to water and to mineral salt blocks (Cowstone A, Nippon Zenyaku Kogyo, Japan) that contained minerals, salt, and a diuretic. Consumed feed of the groups was recorded daily.

Steers were weighed monthly and biopsy samples of subcutaneous fat (SCF) and LM were taken from the left side at the 12th to 13th rib region at 10, 14, 18, and 22 months of age. For sampling the established shot biopsy technique (Bellmann, Wegner, Teuscher, Schneider, & Ender, 2004; Schöberlein, 1989; Wegner & Schöberlein, 1984) with a 6 mm canula was used resulting in a total tissue sample of 3 to 5 g. The region, where the biopsy was taken, was prepared in accordance to surgical, aseptic standards of operation. This included clipping of the hair and subsequent wet shaving together with cleaning of the area and aseptic treatment with a disinfectant. Wound treatment included dressing of the wound with an antibiotic spray to avoid infections. The region of sampling was selected to avoid scar tissue of former biopsies. Samples were divided into SCF and 3 pieces of LM. Subcutaneous fat was placed in RNA later (Applied Biosystems, Tokyo, Japan) and frozen at −70 °C. Muscle samples were mounted on cork with Tissue Tek (tissue freezing medium, Sakura Finetechnical, Tokyo, Japan) and snap frozen in liquid nitrogen. Samples were shipped to the Leibniz Institute for Farm Animal Biology (FBN) for further processing, either for histology or for RNA extraction.

Post mortem, the following parameters were assessed: hot carcass weight (HCW), back fat thickness (BFT), rib eye area (longissimus), and weights of subcutaneous fat (SCF), perirenal fat (PER), and visceral fat (VIF) harvested from the right half carcass. Intramuscular fat (IMF) was prepared from large marbling flecks within the LM, to ensure pure adipose tissue without muscle. Samples of different fat depots (intramuscular, intermuscular, SCF, PER, and VIF) and LM (between the 12th and 13th ribs) were taken within 45 min after exsanguination and placed either in Tissue Tek (sample size ~5 × 5 × 5 mm) and snap frozen in liquid nitrogen or in RNA later (1 mL per sample of 100 mg) for two hours and stored at −70 °C. Additionally, a 2 to 3 cm thick muscle slice was removed from the 12th rib area of LM and fixed in 10% neutral buffered formalin (for every 100 g muscle at least 1 L of formalin) for at least 3 weeks. All samples were shipped to the FBN for further processing. Intramuscular fat content was determined by Soxhlet extraction method using petroleum ether as the solvent (Association of Official Agricultural Chemists, 2000).

2.2. Histology and image analysis

Tissue Tek embedded samples were cryosectioned (10 or 20 μm thick, muscle and adipose tissue, respectively) using a Leica CM3050 S (Leica, Bensheim, Germany) cryostat microtome. Sections of PER and VIF were not stained; sections of all other tissues were stained with hematoxylin/eosin (H/E; hematoxylin: Dako, Glostrup, DK; eosin: Chroma Gesellschaft, Münster, Germany) according to Böck (1989) and embedded with Roti-Histokit (Roth, Karlsruhe, Germany). Serial sections of muscle tissue were reacted for actomyosin Ca^{2+} adenosine triphosphatase stability (ATPase) after alkaline pre-incubation (pH 10.4) as described by Wegner et al. (2000) for fiber typing.

Adipocyte size was measured using the interactive measurement module of an image analysis system equipped with a Jenaval microscope (Carl Zeiss, Jena, Germany), an Altra20 CCD camera (OSIS, Münster, Germany), and Cell^D software (OSIS, Münster, Germany). Where available, 200 to 300 adipocytes were randomly selected and measured, after following the contour using the interpolating polygon function. In muscle sections containing less adipocytes, all available cells were measured.

Muscle fiber traits were measured with a special muscle fiber measurement module (MAS, Freiburg, Germany) of the same system. A minimum of 300 muscle fibers per animal in randomly selected muscle fiber primary bundles, usually 4 to 6 fields of one section, were measured and classified as follows. First, a microscopic image of H/E stained muscle fibers was taken and transformed to gray image. A region of interest (ROI) was defined and fiber contours were reconstructed automatically and interactively corrected. In a next step, nuclei were detected by a threshold operation. Nuclei could be deleted or added interactively if required. The reconstructed fiber contours were visible as an overlay when searching the same region in the ATPase stained serial section and acquiring the image. This image was used to define the fiber type for each muscle fiber automatically, again followed by interactive correction, if required. Data of fiber size (from H/E stained section), fiber type (from ATPase stained section), and number of nuclei lying inside a muscle fiber (from H/E stained section) were combined.

For assessment of marbling traits, formalin fixed muscle slices were cut into 2 mm thick slices and stained with oil red O (Chroma Gesellschaft, Münster, Germany), as described in detail by Albrecht, Wegner, and Ender (1996). The stained slices provided a good contrast between fat (red), connective tissue (white), and muscle (gray to pink). Marbling traits were measured with the Cell^D image analysis software. We developed a “marbling”-macro with the following procedure. Pictures of muscle slices including a ruler were taken by a digital camera (Coolpix 8700, Nikon, Düsseldorf, Germany) at a resolution of 3264 × 2448 pixels. After calibration, a region of interest (ROI) was defined, the green channel of the color image was extracted, and contrast enhanced. The muscle area was detected using a threshold operation, holes were closed, and the outline of the muscle slice was defined as the new ROI. This was followed by the next threshold operation for detection of marbling flecks. False detected areas could be deleted interactively. For each marbling fleck, size, position, and shape traits were measured. For each muscle, total area and number of marbling flecks, area percentage, average size of marbling flecks, size of the largest marbling fleck and average distance to the nearest neighbor were assessed as means after measurement of both sides of the muscle slice.

2.3. RNA isolation and RT real-time PCR

Samples stored in RNA later were used for RNA extraction either using the RNeasy Lipid Tissue Mini Kit (QIAGEN, Hilden, Germany) for adipose tissue or TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) for muscle tissue according to manufacturer's instructions. Concentration and quality of the extracted RNA were measured using a NanoDrop ND-1000 Spectrophotometer (Peqlab, Erlangen, Germany). Ratios of absorbance at 260 and 280 nm of all preparations were at least 1.8. Integrity of RNA was checked by denaturing agarose gel electrophoresis and ethidium bromide staining. The iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories, Munich, Germany) was used to synthesize cDNA from 200 ng of total RNA of each sample according to manufacturer's instructions. A negative control, without reverse transcriptase, was processed for each sample to detect possible contaminations of genomic DNA or environmental DNA. The abundance of PPARγ and FABP4 mRNA was quantified by RT real-time PCR (iCycler, Bio-Rad Laboratories, Munich, Germany) according to Löhrke, Viergutz, and Krüger (2005). Briefly, 1-μl aliquots of each RT reaction (1/20 of total) were primed, in each 10 μl PCR, using an iQ-SYBR green supermix (BioRad Laboratories GmbH, Munich, Germany) and gene-specific oligonucleotides (final concentration 0.2 μM). The following specific primers were used: for PPARγ (GenBank accession No: NM_181024; product size: 242 bp) forward: 5′-AAAGCGTCAGGGTTC-CACTAT-3′, reverse: 5′-ATCTCCGCTAACAGCTTCTCC-3′ and for FABP4 (GenBank accession No: NM_174314; product size: 243 bp) forward:

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