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Hepatic patatin-like phospholipase domain-containing protein 3 sequence, single nucleotide polymorphism presence, protein confirmation, and responsiveness to energy balance in dairy cows

Christine C. McCann,* Molly E. Viner,* Shawn S. Donkin,† and H. M. White‡[1]
*Department of Animal Science, University of Connecticut, Storrs 06269
†Department of Animal Sciences, Purdue University, West Lafayette, IN 47907
‡Department of Dairy Science, University of Wisconsin, Madison 53706

ABSTRACT

Patatin-like phospholipase domain-containing protein 3 (PNPLA3), commonly known as adiponutrin, is part of a novel subfamily of triglyceride lipase enzymes with potential effects on triglyceride metabolism in adipose and hepatic tissues. The predicted bovine *PNPLA3* sequence has been identified, but expression of the gene had not been examined. The objectives of this study were to confirm the predicted bovine *PNPLA3* gene sequence, determine expression of the bovine *PNPLA3* gene in response to whole-animal energy balance, identify single nucleotide polymorphisms present in dairy cows, and verify the presence of the protein in the liver. Using liver biopsy samples collected from cows at +28 d relative to calving (DRTC), RNA was isolated and used to generate a cDNA template for amplification of the entire predicted coding sequence of PNPLA3 via PCR. To determine if energy balance alters the expression of PNPLA3, RNA was isolated and mRNA expression quantified in liver samples from mid-lactation cows after a 5-d ad libitum period (n = 5) and after a subsequent 5-d 50% feed restriction period (n = 5), and in samples collected from cows at −14, +1, +14, and +28 DRTC (n = 16). The presence of PNPLA3 protein was detected by Western blot in liver protein samples collected at +28 DRTC. Expression of hepatic PNPLA3 was decreased after a period of feed restriction (8.14 vs. 1.08 ± 2.17 arbitrary units, ad libitum vs. fasted). Expression of PNPLA3 mRNA was decreased at +1 and +14 DRTC compared with −14 DRTC (23.35, 7.28, 10.17, and 14.5 ± 4.9 arbitrary units, −14, +1, +14, and +28 DRTC, respectively). The presence of PNPLA3 protein was detected as a 55-kDa band in hepatic protein isolations from liver tissue collected at +28 DRTC. These data confirm the presence and sequence of the bovine hepatic *PNPLA3* gene and single nucleotide polymorphisms. Furthermore, these data indicate responsiveness of bovine hepatic PNPLA3 to energy balance.

Key words: phospholipase domain-containing protein 3 (PNPLA3), adiponutrin, lipase, transition cow

INTRODUCTION

Patatin-like phospholipase domain-containing protein 3 (**PNPLA3**), also known as adiponutrin, is a 441-AA, membrane-associated protein, found in both adipose tissue and the liver (Moldes et al., 2006). Although the metabolic role of PNPLA3 is not fully understood, evidence of regulation supports a role of PNPLA3 in lipid storage and deposition in adipose tissue and the lipolysis of triglycerides in hepatocytes (Browning et al., 2010; Chen et al., 2010). The expression of *PNPLA3* mRNA in adipose and hepatic tissue is suppressed during fasting in rodents and humans, and is increased by subsequent refeeding with a high-protein or high-carbohydrate diet (Hoekstra et al., 2010; Huang et al., 2010; Oliver et al., 2012). Responsiveness of adipose PNPLA3 to the fed and fasted states in rodents is mediated by insulin (Johansson et al., 2006; Kershaw et al., 2006). Additionally, PNPLA3 has been demonstrated to be transcriptionally and posttranslationally regulated by FA in rodent and human hepatocytes (Huang et al., 2010).

The importance of PNPLA3 in hepatic lipid metabolism has been highlighted by recent correlations between presence of an inactivating *PNPLA3* SNP (I148M; rs738409) and central adiposity and nonalcoholic fatty liver disease (**NAFLD**; Rotman et al., 2010; Speliotes et al., 2010; Corbin et al., 2013). Presence of the SNP increases liver lipid content as well as the onset and progression of NAFLD in both children and adults (Browning et al., 2010; Speliotes et al., 2010; Corbin et al., 2013). Correlations between protein inactivation by SNP presence and disease onset and progression has led to use of this SNP as a marker for NAFLD predisposition (Corbin et al., 2013).

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[1]Corresponding author: hwhite4@wisc.edu

The putative bovine *PNPLA3* gene [National Center for Biotechnology Information (**NCBI**) Entrez gene ID: 786474] was identified by the Bovine Genome Project and is 80% homologous to the human *PNPLA3* gene (Zimin et al., 2009). The extensive homology between bovine and human *PNPLA3*, and the role of PNPLA3 in lipid metabolism of other species, suggests that PNPLA3 may play a role in lipid metabolism in dairy cattle. Expression of *PNPLA3* was undetectable in bovine subcutaneous adipose tissue (Ji et al., 2012) but noted to change in hepatic tissue across the peripartum period (Khan et al., 2014). Expression of bovine hepatic *PNPLA3* mRNA at parturition and protein abundance in bovine, have not been previously reported. Microarray analysis of liver tissue from feed restricted beef steers indicated that *PNPLA3* was detected and increased on the first day of feed realimentation, but was not further examined (Connor et al., 2010). We hypothesize that PNPLA3 is present in dairy cattle and that expression will be decreased during negative energy balance as a result of feed restriction or calving. The objectives of this study were to confirm the predicted bovine *PNPLA3* gene sequence, determine expression of the bovine *PNPLA3* gene in response to whole-animal energy balance, identify SNP present in dairy cows, and verify the hepatic protein presence.

MATERIALS AND METHODS

Animal Handling and Sample Collection

Feed Restriction and Transition Cow Experiments. Liver biopsy samples from feed-restricted and transition cows were collected during experiments conducted at Purdue University (West Lafayette, IN). Full details regarding experimental design, methods, animal performance, and plasma NEFA have been reported previously (Carvalho et al., 2011; White et al., 2011a). Liver biopsy samples collected at −14, +1, +14, +28 d relative to calving (**DRTC**) in control-fed transition cows, and in mid-lactation cows after 5 d of ad libitum access to feed or 50% feed restriction were collected for isolation of RNA. Animal use and animal-handling protocols were approved by the Purdue University Animal Care and Use Committee.

Postcalving Cow Experiment. Seventeen multiparous Holstein and Jersey cows in early lactation from the University of Connecticut (Storrs) dairy herd were housed in group freestalls at the Kellogg Dairy Center and allowed ad libitum access to diets formulated to meet or exceed the NRC (2001) guidelines for a 650-kg early-lactation cow. Cows were milked 3 times daily at approximately 0500, 1300, and 2100 h, and individual milk yield was recorded electronically at each milking. Liver biopsy samples were collected at +28 DRTC via percutaneous biopsy, frozen in liquid nitrogen, and stored at −80°C for subsequent sequence, SNP, and protein analysis. Animal-use and -handling protocols were approved by the University of Connecticut Animal Care and Use Committee.

Gene Expression Analysis

Expression of *PNPLA3* mRNA was examined in samples collected during the feed-restriction (n = 13) and transition-cow (n = 16) experiments. Total RNA was isolated using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) and quantified by absorbance at 260 nm using an ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). The ratio of absorbance at 260 and 280 nm was between 1.95 and 2.00 for all samples. The integrity of the samples was also determined using a ratio of 28S to 18S rRNA after separation through a denaturing agarose gel. A 50-μg RNA pool was formed by combining equal quantities of total RNA from all cows and for all sampling times for the transition-cow study and was treated with DNase I and further purified using an RNeasy Mini Kit (Qiagen Inc., Thousand Oaks, CA). Cleaned samples (2 μg) were reverse transcribed using an Omniscript reverse-transcription kit (Qiagen Inc.), oligomeric deoxythymine (oligo-dT; Qiagen Inc.), and random decamers (Ambion Inc., Foster City, CA).

Abundance of *PNPLA3* mRNA was determined by real-time quantitative PCR and normalized to 18S as described previously (White et al., 2011a, 2012) in samples collected from feed-restricted and transition cows. Primers were as follows: PNPLA3: AGACATGCCCGGCGACATCCA (forward) and CAGCAGGTACACTGGGAC CTCTGA (reverse); 18S (*Bos taurus* gene number 493779): ACCCATTCGAACGTCTG CCCTATT (forward) and TCCTTGGATTGTGGTAGCCGTTTCT (reverse). Reactions were as follows: 1 cycle at 95°C for 10 min; 40 cycles at 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s; and 1 cycle at 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. Only reaction efficiencies that were between 90 and 110% based on standard curve of pooled samples were used for further analysis. All samples, standards, and controls were analyzed in triplicate, and mean values normalized to 18S mRNA abundance within each sample. The use of 18S for data normalization was verified by the lack of an effect of treatment or DRTC on the threshold cycle or quantity, and differences in threshold cycle values of less than 1 between treatment groups, indicating a lack of bias by the housekeeping gene. Although methods of normalization and selection of reference genes vary, 18S is consistently expressed in hepatic tissue during peri-