Adipose tissue proteomic analyses to study puberty in Brahman heifers

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ABSTRACT: The adipose tissue has been recognized as an active endocrine organ which can modulate numerous physiological processes such as metabolism, appetite, immunity, and reproduction. The aim of this study was to look for differentially abundant proteins and their biological functions in the abdominal adipose tissue between pre- and postpubertal Brahman heifers. Twelve Brahman heifers were divided into 2 groups and paired on slaughter day. Prepubertal heifers had never ovulated and postpubertal heifers were slaughtered on the luteal phase of their second estrous cycle. After ensuring the occurrence of puberty in postpubertal heifers, abdominal adipose tissue samples were collected. Mass spectrometry proteomic analysis identified 646 proteins and revealed that 171 proteins showed differential abundance in adipose tissue between the pre- and postpuberty groups (adjusted P -value < 0.05). Data are available via ProteomeXchange with identifier PXD009452. Using a list of 51

highly differentially abundant proteins as the target (adjusted P -value < 10^{-5}), we found 14 enriched pathways. The results indicated that gluconeogenesis was enhanced when puberty approached. The metabolism of glucose, lipids, and AA in the adipose tissue mainly participated in oxidation and energy supply for heifers when puberty occurred. Our study also revealed the differentially abundant proteins were enriched for estrogen signaling and PI3K-Akt signaling pathways, which are known integrators of metabolism and reproduction. These results suggest new candidate proteins that may contribute to a better understanding of the signaling mechanisms that relate adipose tissue function to puberty. Protein-protein interaction network analysis identified 4 hub proteins that had the highest degrees of connection: PGK1, ALDH5A1, EEF2, and LDHB. Highly connected proteins are likely to influence the functions of all differentially abundant proteins identified, directly or indirectly.

Key words: *Bos indicus*, Brahman, estrogen signaling, heifer, proteomics, puberty

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INTRODUCTION

Age of puberty is a heritable trait that determines the ability of a heifer to become pregnant in the first breeding season. Age of puberty is also correlated with rebreeding and with the likelihood to remain

in the herd in subsequent years (Schillo et al., 1992; Perry and Cushman, 2013). Puberty impacts on heifer lifetime production. For example, Lesmeister et al. (1973) suggested that beef cows which reached puberty earlier calved earlier throughout their reproductive lives, compared to those that attained puberty later. Consistent with these ideas, early pubertal heifers (first calving as 2 yr olds) generated 0.6 more calves than those calving first as 3 yr olds, over the first 5 yr of productive life (Meaker et al.,

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1980). Therefore, selection for early age at puberty may improve cattle profitability and longevity.

In order to achieve early puberty, heifers need adequate nutritional management. Studies have reported that beef heifers maintained on low energy diets that limit weight gain reached puberty later than those fed high energy diets for greater weight gain (Rathbone et al., 2001; Nogueira, 2004). Insufficient nutrition considerably delays puberty in both *Bos indicus* and *Bos taurus* cattle (Abeygunawardena and Dematawewa, 2004). One of the ways in which nutrition affects the initiation of puberty is by influencing the increase of LH release. Heifers fed an adequate energy diet for growth presented increased LH secretion and attained puberty, while heifers fed a restricted diet failed to present increased LH pulse frequency and did not achieve puberty (Day et al., 1986). Likewise, dietary energy restriction inhibited the prepubertal increase of LH secretion in ovariectomized, ovary intact, and estradiol-treated heifers (Kurz et al., 1990). The role of nutrition and metabolism in pubertal development underpins the hypothesis that the adipose tissue impacts the onset of puberty (Schillo et al., 1992; Kinder et al., 1995; Hiney et al., 1996). This role merits further investigation.

Targeting adipose tissue, which can modulate complex physiological process including metabolism, appetite, BW, inflammation, and reproduction (Kusminski et al., 2016), with mass spectrometry proteomics is expected to enhance our knowledge about the links between nutrition, adipose tissue signaling, the onset of puberty, and the pulsatile LH secretion. Here, we present results from differential abundance analysis using mass spectrometry proteomics on adipose tissue of 6 pre- and 6 postpubertal Brahman heifers. Our aim was to identify differentially abundant proteins and understand their biological functions in relation to the onset of puberty.

METHODS

Animals and Samples

Samples were collected from 12 commercial Brahman heifers of similar age. These heifers were handled and managed as per approval of the Animal Ethics Committee of the University of Queensland, Production and Companion Animal group (certificate number QAAFI/279/12).

Pubertal status was defined using the observation of first corpus luteum with ultrasound (Johnston et al., 2009). Prepubertal heifers were

paired and slaughtered with postpubertal heifers. Prepubertal heifers had never ovulated. Postpubertal heifers were slaughtered on the luteal phase of their second estrous cycle. Experimental set-up took approximately eight months until the last postpubertal heifer to achieve puberty.

Serum progesterone concentration for the prepubertal heifers was 0.4 ± 0.2 ng/mL and for the postpubertal heifers was 2.0 ± 0.7 ng/mL. Associated data collected during tissue harvest of these 2 groups were BW and condition score (CS). Body weights and CS were not different between the groups as previously reported (Fortes et al., 2016; Nguyen et al., 2017).

Protein Sample Preparation, Identification, and Quantification

Abdominal adipose tissues of 6 pre- and 6 postpubertal Brahman heifers were dissected into small fragments. Samples were processed essentially as previously described (Tan et al., 2014). Each fraction was diluted in lysis buffer (6 M guanidine, 10 mM DTT, and 50 mM Tris pH 8) and sonicated at power level 4 for 10 s. After homogenization in a QIAshredder homogenizer (QIAGEN Pty Ltd, Melbourne, VIC, Australia) at 4 °C for 10 min, the samples were vortexed vigorously at 30 °C for 1 h. Next, 25 mM acrylamide was added to samples, followed by another incubation at 30 °C for 1 h. Excess acrylamide was quenched in the samples by the addition of 5 mM DTT, and samples were precipitated with 4 volumes of methanol:acetone (1:1) at –20 °C overnight. After dissolving the precipitate in 50 mM ammonium acetate, the protein concentration was measured using Nanodrop (Thermo Scientific). Approximately 50 µg of protein was diluted to a final volume of 100 µL and digested by addition of 1 µg of trypsin (Sigma) overnight at 37 °C. Samples were kept at –20 °C until mass spectrometry.

Mass Spectrometry and Data Analysis

Peptides were desalted using C18 Ziptips (Millipore), resuspended in 9.25% acetonitrile and 0.1% formic acid and analyzed by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) using a Prominence nanoLC system (Shimadzu) and TripleTof 5600 mass spectrometer with a Nanospray III interface (SCIEX) essentially as described (Bailey et al., 2012). In order to build the fragment ion library for SWATH (sequential window acquisition of all theoretical mass spectra), we performed an information dependent analysis (IDA) on 2 randomly

chosen prepubertal samples and 2 randomly chosen postpubertal samples. Fifteen microliters of each sample was injected. Samples were separated using reversed-phase chromatography on a Shimadzu Prominence nanoLC system. Using a flow rate of 30 μ L/min, samples were desalted on an Agilent C18 trap (0.3 $\times$ 5 mm, 5 μ m) for 3 min, followed by separation on a Vydac Everest C18 (300 Å, 5 μ m, 150 mm $\times$ 150 μ m) column at a flow rate of 1 μ L/min. A gradient of 10% to 60% buffer B over 45 min, where buffer A = 1% acetonitrile/0.1% formic acid and buffer B = 80% acetonitrile/0.1% formic acid were used to separate peptides. Eluted peptides were directly analyzed on a TripleTOF 5600 instrument (ABSciex) using a Nanospray III interface. Gas and voltage settings were adjusted as required. MS TOF scan across 350 to 1800 m/z was performed for 0.5 s followed by information dependent acquisition of up to 20 peptides with intensity greater than 100 counts, across 100 to 1800 m/z (0.05 s per spectra) using collision energy (CE) of 40 ± 15 V. For SWATH analyses, MS scans across 350 to 1800 m/z were performed (0.5 s), followed by high sensitivity data-independent acquisition mode using 26 m/z isolation windows for 0.095 s, across 400 to 1250 m/z . Collision energy values for SWATH samples were automatically assigned by Analyst software based on m/z mass windows.

The IDA files were analyzed with ProteinPilot v5.0.1 (SCIEX). The database used by ProteinPilot to identify peptides and proteins was downloaded from Uniprot (www.uniprot.org) on March 28, 2016, and contained a total of 43,813 entries assigned to *B. taurus*, including 6,870 entries from Swiss-prot and 36,948 entries from TrEMBL. The search in ProteinPilot was done using the 4 IDA files described above with the following parameters: 1) Sample type: identification; 2) Cysteine alkylation: acrylamide; 3) Digestion: Trypsin; 4) Instrument: TripleTOF 5600; 5) Special Factors: None; 6) Species: *B. taurus*; 7) ID focus: biological modifications; 8) Database: *B. taurus* database as described above; 9) Search effort: thorough; 10) the false discovery rate (FDR) analysis: Yes; and 11) User modified parameter files: No. The IDA library prepared was then used for the analysis of the SWATH acquisition runs using PeakView v2.1 (SCIEX). The SWATH acquisition runs were obtained by injecting 2 μ L of sample for all samples (6 pre- and 6 postpubertal samples). The parameters used for analysis in PeakView were as follows: 6 peptides per protein; 6 transitions per peptide; 99% peptide confidence threshold; 1% FDR; exclude modified peptides and shared peptides; 6 min extraction window; and 75 ppm extraction window

width. Statistical analyses were performed using MSstats (v2.6) in R (Choi et al., 2014) as previously described (Zacchi and Schulz, 2016). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaino et al., 2016) partner repository with the dataset identifier PXD009452.

Functional Enrichment Analyses

The list of differentially abundant proteins was analyzed with *B. taurus* protein database in the STRINGv10 database (<http://string-db.org>) for functional enrichment analyses. Adjusted *P*-values for multiple testing were reported. The protein–protein interaction score was downloaded from the STRINGv10 database and then transferred to the Cytoscape software (Shannon et al., 2003) for further visualization and scrutiny of the protein–protein interaction network derived from the adipose tissue proteome data. Network analyses identified proteins that were hubs.

RESULTS

A total of 646 proteins were identified in the adipose tissue of Brahman heifers (FDR < 1%). Using relative quantitative SWATH analysis, 497 proteins were quantified. Among these proteins, 171 proteins had different levels in the comparison between pre- and postpubertal heifers (adjusted *P*-value < 5%) (Supplementary Table S1). Of these differentially abundant proteins, 9 proteins had a fold change |FC| > 1 and adjusted *P*-value < 10^{-5} (Table 1). The proteins galectin 1 (LGALS1), protein kinase cAMP-dependent type II regulatory subunit alpha (PRKAR2A), and cofilin 1 (CFL1) were less abundance at postpuberty, whereas transketolase (TKT), VPS35, retromer complex component (VPS35), extracellular matrix protein 1 (ECM1), vacuolar protein sorting 4 homolog A (VPS4A), clusterin (CLU), and hemoglobin beta (HBB) were more abundance at postpuberty.

Functional enrichment analysis was carried out to provide an overview of gene ontology (GO) categories and pathways of 51 differentially abundant proteins between pre- and postpubertal heifers (adjusted *P*-value < 10^{-5}). These 51 differentially abundant proteins were assigned to 45 enriched GO terms (adjusted *P*-value < 0.05), wherein 15 terms were categorized into biological process, 21 into molecular function, and 18 into cellular component. Oxidation-reduction process, lipid metabolic process, and fatty acid metabolic process were enriched

Table 1. Highly significant differentially abundant proteins in comparison between pre- and postpubertal Brahman heifers in abdominal adipose tissue

Ensembl ID	Uniprot ID	Gene symbol	Fold change	Adjusted <i>P</i> -value
ENSBTAG00000015089	P11116	<i>LGALS1</i>	-1.38	9.4×10^{-06}
ENSBTAG00000014205	P00515	<i>PRKAR2A</i>	-1.11	1.36×10^{-09}
ENSBTAG00000021455	B0JYL8	<i>CFL1</i>	-1.05	3.21×10^{-08}
ENSBTAG00000003758	Q6B855	<i>TKT</i>	1.07	8.1×10^{-07}
ENSBTAG00000002493	Q2HJG5	<i>VPS35</i>	1.08	4.6×10^{-05}
ENSBTAG00000003806	A5PJT7	<i>ECM1</i>	1.08	1.0×10^{-07}
ENSBTAG00000001659	Q2HJB1	<i>VPS4A</i>	1.35	8.5×10^{-08}
ENSBTAG00000005574	P17697	<i>CLU</i>	1.44	7.9×10^{-05}
ENSBTAG00000038748	D4QBB3	<i>HBB</i>	1.64	9.8×10^{-12}

among the biological process GO terms (adjusted *P*-value < 0.05). Proteins involved in these 3 biological process GO terms increased abundance after puberty. Pathway analysis revealed 14 enriched pathways (adjusted *P*-value < 0.05), including glucose and AA metabolism as well as estrogen signaling pathway and PI3K-Akt signaling pathway. These last 2 signaling pathways contribute to the process of puberty as discussed in the following section.

From protein-protein interaction network, hubs of the interaction network that showed a high degree of connectivity were identified. The protein with the highest number of connections (degree = 12) was phosphoglycerate kinase 1 (PGK1). This hub was followed by 3 other proteins that had the same number of connections (degree = 11): aldehyde dehydrogenase 5 family member A1 (ALDH5A1), eukaryotic translation elongation factor 2 (EEF2), and lactate dehydrogenase B (LDHB). Among these hubs, PGK1 and LDHB were annotated to the glycolysis pathway. Figure 1 shows the interaction network among differentially abundant proteins of Brahman heifers.

DISCUSSION

The adipose tissue has been considered as an active endocrine organ with diverse function including immune responses, glucose metabolism, bone metabolism, and reproduction (Kusminski et al., 2016). However, relatively little is known about the association between proteins secreted by the adipose tissues and the metabolic status of pubertal cattle. Adipose proteomic studies were focused on molecular mechanisms underlying fat accumulation or fat deposition for milk production and meat quality (Zhao et al., 2010; Cho et al., 2016; Ceciliani et al., 2018). Our study was the first to screen the differentially abundant proteins of the adipose tissue of Brahman heifers between pre- and postpuberty. This proteomic data set from Brahman heifers adds novel information on abundant proteins in the adipose

Figure 1. Protein-protein interaction network in the abdominal adipose tissue. Each node represents a protein. Protein nodes which are enlarged represent the highly abundance (more or less abundance at postpuberty). Node color range from green to red for low to high connection of a specific protein.

tissue between pre- and postpuberty. Comprehensive analysis of 171 differentially abundant proteins will help to investigate the molecular mechanisms involved in puberty onset of *B. indicus* cattle, a globally important subspecies for tropical systems.

Functional enrichment analyses provided GO and pathway annotation to the reported abundant proteins. Six proteins with increased abundance postpuberty were involved in metabolic process of lipid and fatty acid. These proteins were ALDH1A1, enoyl-CoA hydratase, short chain 1 (ECHS1), Acyl-CoA synthetase medium chain family member 1 (ACSM1), Acyl-CoA synthetase family member 2 (ACSF2), fatty acid synthase (FASN), and ATP synthase, H⁺ transporting, mitochondrial F1 complex, beta polypeptide (ATP5B). It is possible that postpubertal heifers need to mobilize body fat to provide sufficient energy for cycling and in preparation for reproduction. Seven proteins with increased abundance postpuberty including ECHS1, ALDH1A1, LDHB, FASN, succinate-CoA ligase alpha subunit (SUCLG1),

peroxiredoxin 1 (PRDX1), and cytochrome c oxidase subunit 5A (COX5A) were implicated in oxidation-reduction process, suggesting the role of redox pathways in the control of female reproduction. Altering the oxidation-reduction status may influence signaling pathways, transcription factors, and epigenetic mechanisms and impact on oocyte and embryo quality (Agarwal et al., 2008). Moreover, the high representation of catalytic activity and binding activity among differentially abundant proteins suggest that pubertal heifers had strong metabolic activity and enhanced biological reactions. Proteins annotated to these catalytic and binding activities had increased expression levels after puberty, during the luteal phase. Future work using more animals, targeting all phases of the estrous cycle, and with deeper proteome coverage will further our understanding of this process. Experiments relating nutrient restriction during puberty to changes in the proteome may also help in clarifying the biological changes that occur in heifers as a result of puberty.

The wide range of enriched pathways in our study indicated that the function of adipose tissue in relation to puberty is complex. It seems that numerous metabolic pathways working together in the adipose tissue are both influenced by puberty and signal feedback to this process. Some of the pathways mapped by the proteins that increased in abundance after puberty were involved in glycolysis, butanoate metabolism, propanoate metabolism, pyruvate metabolism, and biosynthesis of AA. The increased expression level of these proteins fuelled the upregulation of these pathways associated with energy metabolism. It is possible that the upregulation of these pathways relates to mobilizing sufficient energy for pubertal development and subsequent reproductive capability.

Proteins involved in estrogen signaling pathway included 2 upregulated proteins: phosphoenolpyruvate carboxykinase 2, mitochondrial (PCK2) and heat shock protein 90 alpha family class B member 1 (HSP90AB1) and 3 downregulated proteins: collagen type VI alpha 2 chain (COL6A2), collagen type VI alpha 3 chain (COL6A3), and collagen type I alpha 1 chain (COL1A1). Estrogen signaling is an important factor in gonadostat theory. The positive and negative feedback of gonadal steroids on the secretion of GnRH and gonadotropin that are necessary for ovulation has been known for decades (Docke and Dorner, 1965; Kulin et al., 1969; Clarke and Cummins, 1984; Yasin et al., 1995; Moenter et al., 2009; Mayer et al., 2010). In adipose tissue, estrogens were recognized as regulators of lipid metabolism and glucose metabolism (Kim

et al., 2014). Estrogen signaling is an expected link between the reproductive axis and the adipose tissue.

Proteins involved in the PI3K-Akt signaling pathway included an upregulated protein (heat shock 70 kDa protein 1A [HSPA1A]) and 2 downregulated proteins (G protein subunit alpha o1 [GNAO1] and HSP90AB1). The PI3K-Akt pathway is known as an integrator of reproductive and metabolic functions (Acosta-Martínez, 2011). In fact, this pathway is activated by growth factors and hormones such as estrogen receptor alpha (ER α), insulin receptor (IR), insulin-like growth factor I (IGF1), and leptin receptor (LepR) that are critical for GnRH release, puberty, and sexual behavior (Etgen and Acosta-Martínez, 2003; Morrison et al., 2005; Morton et al., 2005; Gelling et al., 2006; Mayer et al., 2010). Further, estrogen and progesterone are known to modulate the expression or activity of members of PI3K-Akt signaling pathway such as p85 α , IRS-1, and IRS-2 (Acosta-Martínez, 2011). Recently, study in male mice noted that deletion of a catalytic subunit of PI3K (p110 α) in the adipose tissue led to the delay of puberty onset (Nelson et al., 2017). Our results suggest that PI3K-Akt signaling is important in cattle puberty as well.

From the protein-protein interaction network, we identified 4 hub proteins that had the highest degrees of connection: PGK1, ALDH5A1, EEF2, and LDHB. Among these hub proteins, PGK1 and LDHB are involved in glycolysis. In particular, PGK1 is a glycolytic enzyme (Zhao et al., 2016), whereas LDHB is involved in a post-glycolysis process. LDHB encodes an enzyme that can catalyze the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD⁺. The 2 other hub proteins, ALDH5A1 and EEF2, also play a role in metabolic pathways. The protein ALDH5A1 is a member of aldehyde dehydrogenases family that is known to diminish oxidative stress (Vasiliou et al., 2004; Chen et al., 2009; Singh et al., 2013). The link between female infertility and oxidative stress suggested that an increase of ALDH5A1 abundance at puberty in Brahman heifers may reduce the risk of oxidative stress and help animals to attained puberty (Ruder et al., 2009; Gupta et al., 2014). Lastly, the protein EEF2, a member of G-protein super family, has been known as an essential factor for protein translation and synthesis (Kaul et al., 2011; Hekman et al., 2012). These 4 protein hubs should be further investigated in the context of cattle puberty.

Reproductive function, enabled through the processes of puberty, is an energy intense activity.

It cannot be denied that fatty acids, AA, purines, and pyrimidines as well as metabolites are essential compounds required for the final stage of oocyte maturation. The mature bovine cumulus oocyte complex consumes twice as much oxygen, glucose, and pyruvate as the immature cumulus oocyte complex (Sutton et al., 2003a). The maturing cumulus oocyte complex uses glucose for energy production and numerous other cellular processes (Sutton et al., 2003b). Energy for reproductive function is likely mobilized from peripheral tissues, such as abdominal fat. Consistent with this, we have identified candidate proteins that are increased in abundance in postpuberty adipose tissue that are key for fatty acid metabolism, lipid metabolism, and glycolysis/gluconeogenesis. Further investigation of our candidate proteins is necessary to fully understand their roles in the process of puberty in *B. indicus* heifers.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

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