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Proteomic analysis of bovine omental, subcutaneous and intramuscular preadipocytes during *in vitro* adipogenic differentiation

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ABSTRACT

Given the substantial rise in obesity, depot-specific fat accumulation and its associated diseases like diabetes, it is important to understand the molecular basis of depot-specific adipocyte differentiation. Many studies have successfully exploited the adipocyte differentiation, but most of them were not related to depot-specificity, particularly using freshly isolated primary preadipocytes. Using 2-dimensional polyacrylamide gel electrophoresis coupled with sequencing mass spectrometry, we searched and compared the proteins differentially expressed in undifferentiated and differentiated preadipocytes from bovine omental, subcutaneous and intramuscular adipose depots. Our proteome mapping strategy to identify differentially expressed intracellular proteins during adipogenic conversion revealed 65 different proteins that were found to be common for the three depots. Further, we validated the differential expression for a subset of proteins by immunoblotting analyses. The results demonstrated that many structural proteins were down-regulated during differentiation of preadipocytes from all the depots. Most up-regulated proteins like Ubiquinol–cytochrome-c reductase complex core protein I (UQCRC1), ATP synthase D chain, Superoxide dismutase (SOD), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Sulfotransferase 1A1 (SULT1A1), Carnitine O-palmitoyltransferase 2 (CPT2) and Heat-shock protein beta 1 (HSPB1) across the three depots were found to be associated with lipid metabolism and metabolic activity. Further, all the up-regulated proteins were found to have higher protein expression in omental than subcutaneous or intramuscular depots.

1. Introduction

Adipocytes play an important role in regulating energy balance, both as a reservoir, storing and releasing fuel and as endocrine cells, secreting factors that regulate whole-body energy metabolism (Gregoire et al., 1998). Adipose tissue is restricted to certain areas in the body and its growth is a result of preadipocyte hyperplasia (increase in number) and hypertrophy (increase in size). Adipose tissue can be found in areas composed of loose connective tissues, such as the subcutaneous layer between muscle and dermis. Fat depots can also be found around inner organs and also in the muscle tissue, known as visceral and intramuscular fat, respectively. Interestingly, preadipocytes in different fat depots seem to have distinct adipogenic potential and the metabolic activity differs between mature adipocytes of various depot origins (Rosen et al., 2000). It has been demonstrated that visceral abdominal (omental) fat is related to an increased risk of atherosclerosis, diabetes, hypertension and dyslipidemia in comparison to the other depots (Despres, 2006; Despres and Lemieux, 2006) and therefore, studies related to the understanding of this cell type and its development are gaining much importance.

Preadipocyte differentiation is the transformation from a fibroblast-like cell to a lipid-filled cell. Although *in vivo* studies elucidating the molecular mechanisms that take place during preadipocyte differentiation are still limited, cellular *in vitro* models, such as 3T3-L1 preadipocyte cell line, or freshly isolated primary preadipocytes have been instrumental in studying this process (Fajas, 2003). The process of preadipocyte differentiation is complex and is initiated by exposure of the preadipocytes to many adipogenic stimuli such as hormones like insulin, glucocorticoids and IGF-1, paracrine and autocrine effectors, including free fatty acids and cyclic AMP (Kirkland et al., 2002; Unger, 2005). These stimuli activate pathways that modulate the expression and activity of a set of adipogenic transcription factors that in turn, lead to the expression of downstream differentiation-specific genes (Kirkland and Dobson, 1997; Perera et al., 2006).

Abbreviations: UQCRC1, Ubiquinol–cytochrome-c reductase complex core protein I; SOD, Superoxide dismutase; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; SULT1A1, Sulfotransferase 1A1; CPT2, Carnitine O-palmitoyltransferase 2; HSPB1, Heat-shock protein beta 1; ALDH1A1, Aldehyde dehydrogenase; PHB, Prohibitin; IGF-1, Insulin-like growth factor 1; CEBPA, CCAAT/enhancer binding protein α; PPARγ, Peroxisome proliferator-activated receptor gamma; SREBF1, Sterol Regulatory Element Binding factor 1; AKT1, AKT2, Serine/threonine kinase 1 and 2; KRB, Krebs Ringer Bicarbonate; HBSS, Hank's Balanced Salt Solution; CBB, Coomassie Brilliant Blue; CHCA, α-Cyano-4-hydroxycinnanic acid.

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Limited research with bovine stromal vascular cells (Sato et al., 1996; Gregoire et al., 1998) and a clonally derived preadipocyte cell line from Japanese Black cattle (Aso et al., 1995) have shown that these cells are capable of differentiation in response to the adipogenic stimuli. Research report from Yamada et al. (2007) showed significant differences during adipogenesis of preadipocytes from different depots. Further, they showed that an important adipogenic transcriptional factor CCAAT/enhancer binding protein α (CEBPA) was induced more in subcutaneous and intramuscular than in omental depots. In another important study by Huff et al. (2004), peroxisome proliferator-activated receptor gamma (PPARγ), a key transcriptional factor for adipogenesis was found to be more expressed in intramuscular depots of Holstein cattle. Also gene expression studies involving different depots during differentiation showed increased expression of important adipogenic-specific genes across depots (Kirkland et al., 1996). However, limited information is available on the proteomic analysis of preadipocyte differentiation from different depots in bovine. Also, the identification of differentially expressed proteins between preadipocytes from different depots during differentiation might be helpful in understanding the functions of certain proteins in fat metabolism, especially from the pharmacologically important 'omental depot'. Hence, the main objective of this study was to analyze and compare the differential protein expression from undifferentiated and differentiated preadipocytes with respect to the depots.

2. Materials and methods

2.1. Animals

Five heads of Hanwoo (Korean cattle, *Bos taurus*) steers were fed and managed in the feeding barn at Livestock Research Institute under the high quality beef production program (1997) and slaughtered when they were 24 months old. All experimental procedures and care of animals were conducted in accordance with the guidelines of the Animal Care and Use Committee (IACUC) of the National Institute of Animal Science in Korea.

2.2. Cell culture and differentiation

Immediately, after stunning and exsanguination, the muscle and fat portions between the 6th to 7th ribs were removed, and the subcutaneous and intramuscular fat depots were sampled from this rib section aseptically. The omental adipose tissue was taken within the lesser curvature of the abomasum. All these tissue samples were kept in sterile saline (0.154 M NaCl, 37 °C) for recovery of stromal vascular cells (Cianzio et al., 1982). The stromal vascular fraction of adipose tissue was prepared as described by Cryer et al. (1987). Tissue was sliced and cells were released by collagenase digestion in Krebs Ringer Bicarbonate (KRB) buffer (1.22 mM $CaCl_2$) for 1 h. The digested tissue was filtered through a nylon mesh screen to separate the cells from undigested tissue fragments and debris. The filtrate was centrifuged at 600 *g* for 5 min at room temperature. The pellet was washed twice by centrifugation (600 *g*, 5 min) with Hank's Balanced Salt Solution (HBSS) and resuspended in medium containing M199 supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) and seeded in 10 mM Petri-dish at a density of approximately 2500 cells/cm^2. The cells were incubated at 37 °C in 5% CO_2. Culture medium was changed every second day allowing the cells to proliferate until confluence (about 10 days). For differentiation, the cells were exposed to differentiation–induction media: DMEM-F12 medium plus penicillin (100 U/mL) and streptomycin (100 μg/mL) supplemented with combinations of triiodothyronine (T3, 2 nM), bovine insulin (5 ng/mL), dexamethasone (10 nM), 3-isobutyl-1-methylxanthine (0.1 μM), lipids (10 μL/mL), L-ascorbic acid (50 μM), biotin (10 nM), and pantothetic acid (100 μM). Differentiation media were replaced with fresh media twice during the differentiation period. On the 8th day of incubation in the differentiation media, the cells were taken for Oil red O staining.

2.3. Oil red O staining

Oil red O staining was performed to monitor progression of adipocyte differentiation as described previously (Ramirez-Zacarias et al., 1992). Briefly, preadipocytes of day 8 (after adding differentiating medium) from omental, subcutaneous and intramuscular depots were washed with PBS and then fixed using 3.7% formaldehyde for 2 min. Oil red O (0.5%) was prepared in isopropanol, mixed with water at 3:2 ratio and filtered through a 0.45 μm filter. The fixed cells were incubated with Oil red O reagent for 1 h at room temperature and then washed with water. The stained fat droplets in the cells were visualized by light microscopy and photographed.

2.4. Protein preparation for 2-dimensional electrophoresis

Proteins from stromal vascular cells of three depots on days 0 and 8 of differentiation were extracted in 0.5 mL of lysis buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 100 mM dithiothreitol (DTT), protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany)). Following 15 min centrifugation at 16,000 *g*, the supernatant was collected and precipitated with either one or three volumes of acetone at −20 °C for 2 h. The pellets were collected by centrifugation and then completely dried using a speed-vac. Dried samples were re-dissolved in the 2-dimensional electrophoresis sample buffer (7 M urea, 2 M thiourea, 2% CHAPS, 100 mM DTT, 0.5% pH 3–10 NL IPG [Immobilized pH gradient]) for isoelectric focusing (IEF)). The concentration of total protein in the sample was determined by Bradford's protein assay method.

2.5. 2-Dimensional electrophoresis and image analysis

Protein (500 μg) was loaded onto the immobiline dry strips pH 3–10 NL (GE Healthcare). The rehydrated strips were focused on IEF system (AP Biotech, Sweden) for ~80 kVh at a maximum of 8000 V in a rapid ramping mode with maximum current per strip of 50 μA. Equilibration of the immobilized pH gradient strips was performed in two steps: reduction followed by alkylation (Ahmed and Bergsten, 2005). The second dimension was run on 12.5% polyacrylamide sodium dodecyl sulphate gels (26×20 cm) with a constant voltage of 100 V for 30 min and 250 V for 6 h using the EttanDALT II system (Amersham Bioscience, Piscataway, NJ, USA). The proteins were visualized using a Coomassie Brilliant Blue (CBB) G-250 staining method. A total of 5 gels were run for protein sample from each depot.

The CBB-stained gels were scanned using a GS-800 scanner (Bio-Rad) at an optical resolution of 300 dpi. Spot detection, quantification and matching were performed using Image Master ver 7.0 (GE healthcare). A match set consisting of three images, each from one depot was created. To correct for variability due to CBB staining and reflect the quantitative variations of protein spot, the individual spot volumes were normalized by dividing their optical density (OD) values by the total OD values of all the spots present in the gel. The significance of the difference in protein expression between undifferentiated and differentiated preadipocytes from three depots was estimated by Student's t-test, $p<0.05$ by using Image Master (ver 7.0) software.

2.6. Protein identification

The CBB-stained protein spots were excised from gels using a punch and placed in 500 μL Eppendorf tubes. The proteins were digested in-gel with trypsin as described by Hellmann et al. (1995). Briefly, each spot was destained with 50 μL 50% acetonitrile (ACN) in 50 mM NH_4HCO_3, incubated at 37 °C for 30 min and repeated once. Then the gels were reduced and alkylated. The gel pieces were digested overnight with