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Adipose tissue proteomes of intrauterine growth-restricted piglets artificially reared on a high-protein neonatal formula☆,☆☆

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Abstract

The eventuality that adipose tissues adapt to neonatal nutrition in a way that may program later adiposity or obesity in adulthood is receiving increasing attention in neonatology. This study assessed the immediate effects of a high-protein neonatal formula on proteome profiles of adipose tissues in newborn piglets with intrauterine growth restriction. Piglets (10th percentile) were fed milk replacers formulated to provide an adequate (AP) or a high (HP) protein supply from day 2 to the day prior weaning (day 28, n=5 per group). Adipocytes with small diameters were present in greater proportions in subcutaneous and perirenal adipose tissues from HP piglets compared with AP ones at this age. Two-dimensional gel electrophoresis analysis of adipose tissue depots revealed a total of 32 protein spots being up- or down-regulated (P<.10) for HP piglets compared with AP piglets; 18 of them were unambiguously identified by mass spectrometry. These proteins were notably related to signal transduction (annexin 2), redox status (peroxiredoxin 6, glutathione *S*-transferase omega 1, cyclophilin-A), carbohydrate metabolism (ribose-5-phosphate dehydrogenase, lactate dehydrogenase), amino acid metabolism (glutamate dehydrogenase 1) and cell cytoskeleton dynamics (dynactin and cofilin-1). Proteomic changes occurred mainly in dorsal subcutaneous adipose tissue, with the notable exception of annexin 1 involved in lipid metabolic process having a lower abundance in HP piglets for perirenal adipose tissue only. Together, modulation in those proteins could represent a novel starting point for elucidating catch-up fat growth observed in later life in growing animals having been fed HP formula.
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Keywords: Adipose tissue; Annexins; Low birth weight; Proteomics; Piglet; High-protein formula

1. Introduction

It is now recognized that nutrition imbalance during early life can affect the development of body tissues and confers a greater susceptibility to chronic disease in adulthood [1]. Epidemiological studies have hypothesized that babies born with intrauterine growth retardation (IUGR) may be at a greater risk of developing obesity, type 2 diabetes and hypertension later in life [2,3]. Those babies are often fed neonatal formula enriched in proteins to accelerate their weight gain [4,5]. However, a high protein intake during early life is also suspected to increase the risk of subsequent obesity [6–8]. Therefore, early nutrition may modulate the physiology of various tissues in a way that promotes short-term survival but that leads to a maladapted phenotype in later life.

White adipose tissues are expandable energy reserves and secretory organs that release numerous factors capable of regulating several physiological processes. Visceral adipose tissue is generally more strongly associated with an adverse metabolic risk profile than the subcutaneous adipose tissue in humans [9]. In an IUGR piglet artificially nourished with a high-protein milk replacer, Morise et al. [10] have recently reported a lower relative mass of perirenal adipose tissue at the day of weaning when compared with piglets fed formula with an adequate protein level. The mechanisms underlying short-term adaptations of adipose tissues to early postnatal protein nutrition have been however poorly investigated. To date, lower expression of glucose transporters (GLUT4) have been demonstrated on abdominal adipocyte cell membranes of artificially reared infant rats with high protein intakes [11]. Based on a bottom-up approach using 2-dimensional polyacrylamide gel electrophoresis (2DE) followed by mass spectrometry (MS) protein identification technology, recent studies have shown tissues-specific modulation of various proteins notably involved in intermediary metabolism, protein turnover, immune function or cell growth in response to IUGR in porcine neonates [12,13]. This suggests that 2DE analysis of key tissues may add new information to better understand nutrition-associated problems in animals and humans, such as intrauterine fetal retardation and obesity.

Then, the aim of this study was to identify proteins for which short-term abundance pattern in adipose tissues could have been altered by high protein intake in IUGR suckling piglets. In a

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complementary study, we have demonstrated lower tissue lipid content and depressed activities of lipogenic enzymes in perirenal and subcutaneous adipose tissues of those piglets compared with IUGR piglets fed adequate protein levels until weaning [14]. In the current study, the potential of 2DE-MS to reveal a global picture of the short-term adaptation of adipose tissues was evaluated in piglets subjected to IUGR followed by a high protein intake during sucking.

2. Materials and methods

2.1. Animals and diets

The care and use of piglets were performed in compliance with the guidelines of the French Ministry of Food, Agriculture and Fisheries. The scientific and technical staffs obtained an agreement from the French veterinary services to conduct animal research. The milk replacer powders were supplied by "la laiterie de Montaigu" (France). The current experiment is a subset of a larger study investigating the immediate and lasting effects of early high protein intakes on growth rate and adipose tissue phenotype [14]. Briefly, 10 crossbred full-term IUGR piglets (mean birth body weight of 0.92±0.02 kg, 10th percentile) were obtained from the experimental herd of INRA (Saint-Gilles, France). These piglets were allowed to suckle colostrum from their dams for the first 2 days. Pairs of littermates of the same sex were then randomly assigned to one of the two dietary groups (two entire males and three females in each group). They received milk replacers (Table 1) formulated to provide an adequate (AP, 1.05 g of protein/100 kJ mimicking sow's milk) or a high (HP, 1.5 g of protein/100 kJ) protein supply. After separation from their dam, they were placed individually in incubators (33°C, 60% humidity) and were bottle-fed every 2 h from 7:00 a.m. to 11:00 p.m. and once during the night at 3:00 a.m. from day 2 to day 7 [15]. At day 7, piglets were transferred into stainless steel metabolic cages in a temperature-controlled room (30°C), and they were then fed with an automatic formula feeder up to 28 days of age. The daily formula rations of piglets were calculated in net energy (NE) and were 1305 kJ per kg $BW^{0.75}$ during the whole experimental period. The HP powder was formulated to provide more protein and amino acids per NE than the AP powder, and the amount of proteins offered to the HP piglets was progressively increased to reach 41% more than AP intake from day 8 onwards. At day 28, experimental piglets (average body weight of 4.9 kg±0.2 kg and 5.5 kg±0.4 kg for AP and HP pigs, respectively) were sacrificed 90 min after the last meal at the experimental facilities of INRA (Saint-Gilles, France).

2.2. Sample collection and histological analyses

Adipose tissue at the perirenal location (PAT) was immediately removed and weighed, and a portion was prepared for later analysis. A sample of dorsal subcutaneous adipose tissue (SCAT) was also collected at the last rib level from the left half-carcass within 15 min after death. For histological analysis, samples of adipose tissue were restrained on flat sticks and frozen in isopentane cooled by liquid nitrogen. Other adipose tissue samples were cut into small pieces and frozen in liquid nitrogen. All samples were then stored at −75°C until analyses. Cross-sectional areas of adipocytes were measured from serial cross-sections of frozen PAT or SCAT cut in a cryostat, as previously described [14]. The distribution of adipocyte diameters (μm) into classes was then calculated.

2.3. Adipose tissue proteins solubilization

Frozen adipose tissue samples of approximately 150 mg were crushed with a mortar and a pestle under liquid nitrogen. They were homogenized with a Polytron grinder (Kinematica, Bioblock Scientific, Switzerland) in 1 ml of ice-cold lysis buffer (pH 7.4) containing 10 mmol/L Tris base, 1 mmol/L EDTA, 0.25 mol/L sucrose and compete protease inhibitors (Roche Diagnostics, GmbH, Mannheim, Germany). Homogenates were stirred for 1 h on ice using glass bead agitators (Heidolph, Germany) and were then centrifuged at 10 000*g* at 4°C for 15 min. The resulting supernatants (below the fat cake) contained soluble proteins. The total protein concentration of extracts was assessed by Bradford reagent (BioRad, Hercules, CA, USA) using bovine serum albumin as a standard [16]. The protein extracts were then stored at −75°C until use for later electrophoresis.

Table 1
Composition of AP and HP formula

Item	AP	HP
Protein, g/L	51.4	77.0
Lipid, g/L	82.0	79.0
Carbohydrates, g/L	49	46
Energy, kJ/L	4753	5030
Protein/energy, g/100 kJ	1.05	1.5

2.4. Two-dimensional gel electrophoresis

The 2DE analysis was performed on soluble protein extracts of the two experimental groups, thus allowing the majority of enzymes and some of the low-expressed proteins to be more easily studied. Unless indicated, all the materials used were supplied by GE Healthcare (Saclay, France). For the first dimension, soluble proteins (100 μg) were mixed with Destreack solution and 2% carrier ampholytes to make up the volume to 450 μl. Immobilized pH gradient (IPG) strips (24 cm, 3–10 pH nonlinear) were rehydrated passively with the protein extracts over a period of 16 h. Proteins were then isoelectrically focused on an Ettan IPGphorII system according to the following settings: 1 h at 120 V, 1 h at 200 V, 1 h at 500 V, 6 h at 1000 V, 1 h 30 min at 8000 V and a constant of 8000 V until approximately 48 000 Vh was reached. Subsequently, the IPG strips were equilibrated in two steps of 12 min each at room temperature with gentle agitation. The first equilibration solution contained 50 mmol/L of pH 8.8 Tris-HCl (Sigma) buffer, with 6 mol/L urea, 65 mmol/L dithiothreitol (DTT, Interchim, Montluçon, France), 2% sodium dodecyl sulfate (SDS) (v/w, Interchim) and 30% glycerol (v/v, Interchim). In the second equilibration solution, DTT was replaced by 4.5% iodoacetamide, and 0.03% bromophenol blue (Sigma) was added as a dye. Following equilibration, the strips were gently rinsed in water to remove buffer excess, then applied onto 12.5% SDS–polyacrylamide gel electrophoresis (PAGE) gels and sealed with 0.5% agarose solution. The SDS-PAGE was conducted in a vertical Ettan DALTsix system. A power of 5 W per gel was applied for 45 min, and a power of 17 W per gel was then applied until the bromophenol blue dye front reached the bottom of the gels. Proteins were then visualized by sensitive silver staining [17]. In PAT and SCAT, respectively, the gel with the best resolution and the greatest number of detected spots was used as a master gel for image analysis. Finally, two preparative gels per tissue were made by pooling an equal volume of each extract (200 μg and 400 μg of all protein extracts, respectively) and stained [18] to allow spot picking and further MS identification.

2.5. Image analysis

The gels were immediately scanned with an UMAX ImageScanner (GE Healthcare) at 200 dpi and analyzed by Melanie 7.0 software (GeneBio, Switzerland). A total of 20 analytical 2DE gels were considered with two match sets constructed for the two kinds of adipose tissues. For each match set, two subsequent match sets were built based on the dietary experimental piglet groups (HP and AP) consisting each of five biological replicates. The process of automatic spot matching was done using the master gel as reference both within and between submatch sets. Manual fine editing matching was performed when necessary. The volume of each spot was normalized by dividing its volume by the total volume of all spots in the gel (outliers being removed) to take into account variations due to protein loading and staining. Only spots detected in a minimum of $n-1$ biological replicates from the two submatch sets were considered. The results were expressed according to fold-change value, which represents the expression ratio of the HP group to the AP group. Ratios of abundance of protein spots in HP group relative to AP group are inversed and are preceded by a minus sign for value less than 1.

2.6. Protein identification

Protein spots of interest were manually cut out of the gels using pipette tips. Gel pieces were placed into a 1.5-ml microcentrifuge tube in a solution of 1% acetic acid (Carlo Erba, Val de Reuil, France). Excised spots were processed and digested with trypsin as described previously [19]. Extraction was performed in two successive steps by adding 50% v/v acetonitrile (ACN)/0.1% trifluoric acid (TFA). Digests were dried out and dissolved with 2 mg/ml alpha-cyano-4-hydroxycinnamic acid in 70% ACN/0.1% TFA before spotting onto targets (384 Scout MTP 600 μm AnchorChip, Bruker Daltonik, Bremen, Germany). The peptide fragments produced from each protein spot were used to generate peptide fragment mass data via matrix-assisted laser desorption/ionization–time of flight (TOF)/TOF mass spectrometry analysis (Ultraflex, Bruker Daltonik). The peptide fragment mass data were processed with the FlexAnalysis software (V2.2, Bruker Daltonik). All analyses were performed on the Proteomics Core Facility of Biogenouest (Rennes, France). Autolysis products of trypsin were used for internal calibration. The monoisotopic masses of tryptic peptides were used to query NCBI nonredundant sequences databases of all mammalian proteins using the MASCOT search engine (http://www.matrixscience.com). The mass tolerance was set at 100 ppm for the parent ion and at 0.5 Da for the fragment ions. To avoid incorrect identifications, four matched peptides per protein were required at least, and each matching was carefully checked manually by considering MASCOT probabilistic score and accuracy of the experimental to theoretical isoelectric point and molecular weight.

2.7. Protein categorization

Identified proteins were functionally classified according to their biological process terms provided in Gene Ontology Consortium (http://www.ncbi.nlm.nih.gov/gene) for *Homo sapiens* and considering eventually their parent terms (http://amigo.geneontology.org).

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