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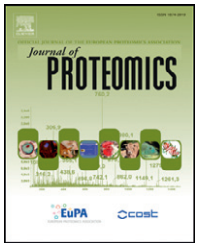

Metabolic proteomics of the liver and mammary gland during lactation☆

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ABSTRACT

The liver and the mammary gland have complementary metabolic roles during lactation. Glucose synthesized by the liver is released into the circulation and is taken up by the mammary gland where major metabolic products of glucose include milk sugar (lactose) and the glycerol backbone of milk fat (triglycerides). Hepatic synthesis of glucose is often accompanied by β-oxidation in that organ to provide energy for glucose synthesis, while mammary gland synthesizes rather than oxidizes fat during lactation. We have therefore compared enzyme abundances between the liver and mammary gland of lactating Friesian cows where metabolic output is well established. Quantitative differences in protein amount were assessed using two-dimensional differential in-gel electrophoresis. As predicted, the abundances of enzymes catalysing gluconeogenesis and β-oxidation were greatest in the liver, and enzyme abundances in mammary tissue were consistent with fat synthesis rather than β-oxidation.
This article is part of a Special Issue entitled: Farm animal proteomics.

1. Introduction

Proteomic analysis of metabolic pathways indicates that changes in enzyme amount often accompany major changes in metabolic output. We have previously commented, however, that although changes in enzyme amount are often interpreted as indicators of metabolic output, in many proteomic studies the proposed relationship between changes in enzyme amount and metabolism is an extrapolation [1]. We have therefore compared the proteomes of the liver and mammary gland in lactating animals to investigate the extent to which differences in protein amount are consistent with the well-documented metabolic output of these organs.

The liver and the mammary gland have complementary metabolic roles during lactation. Glucose synthesized by the liver is released into the circulation and taken up by the mammary gland where major metabolic products of glucose include milk sugar (lactose) and the glycerol backbone of milk fat (triglycerides). In ruminants, short chain fatty acids (acetate, propionate and butyrate) produced by metabolism of carbohydrates in the rumen are used by the host animal as metabolic precursors [2,3].

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Acetate and β-hydroxybutyrate are major sources of energy in ruminants, and are also used for fatty acid synthesis in mammary gland with subsequent incorporation into triglycerides. Propionate is metabolized by gluconeogenesis to glucose in the liver. Under these circumstances it can be predicted that proteomic profiling should detect major differences in metabolic enzymes between the liver and mammary gland, as has been demonstrated using microarray analysis of murine tissue [4]. Most simply, glucose synthetic pathways should predominate in the liver during lactation while fat synthesis should be prominent in the mammary gland. Prevalence of β-oxidation is expected in the liver for production of ATP required for gluconeogenesis in that organ. We have previously shown that many of the cytoplasmic and mitochondrial proteins required for synthesis of lactose and fatty acids can be detected using two-dimensional electrophoresis (2DE) of mammary proteins [1]. We have therefore now examined the extent to which liver and mammary proteomes reflect major pathways of nutrient metabolism.

2. Materials and methods

All methods, except for Western blotting, are as previously described by Beddek et al. [1]. Paired mammary and liver needle biopsies were collected from lactating Friesian cows (age range 2–6 years) during a two-day period and were stored at −80 °C prior to analysis. The animals were 18–112 days post parturition (mean 69 days) and were grazed together at the time of sampling. Ethics approval was from the AgResearch Ruakura Animal Ethics Committee. Protein was extracted by homogenizing 10 mg portions of tissue in 100 μL lysis buffer (30 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS).

Two-dimensional differential in gel electrophoresis (2D-DIGE) [1] with CyDyes (GE Healthcare Ltd, UK) was used to compare the relative abundances of individual proteins. For pH 4–7 focusing, protein samples in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG buffer 4–7, 2% DTT) were loaded onto 7 cm IPG strips (GE Healthcare) by passive hydration overnight prior to focusing in an IPGphor (GE Healthcare) at 20 °C initially at 300 V for 0.5 h, then 1000 V (gradient) for 0. 5 h and 5000 V for 2 h. Cup loading in modified rehydration buffer containing 6–11 IPG buffer was used for pH 6–11 focusing. Focused strips were equilibrated in equilibration buffer (50 mM Tris–HCl, 6 M urea, 30% glycerol, 2% SDS) containing 1% DTT, then in equilibration buffer containing 2.5% iodoacetamide, for 10 min each prior to electrophoresis on 4-12% Bis-Tris NuPAGE gels (Invitrogen, Carlsbad, CA) using MOPS SDS electrophoresis buffer. Electrophoresed gels were stained with CBB G-250 prior to excision of proteins for mass spectrometry. Excised protein spots were processed in an Ettan digester (GE Healthcare) using 4 cycles in 50 mM ammonium bicarbonate–methanol (1:1 v/v) and were digested for 5 h with trypsin (modified sequencing grade; Roche, Mannheim, Germany) in 20 mM ammonium bicarbonate. Peptides were extracted using three 20 min cycles of 0.1% TFA–ACN (1:1 v/v) followed by air drying and resuspension in 2 μL of 10 mg/mL CHCA in 0.2% TFA–ACN (1:1 v/v). The solutions were spotted onto MALDI plates, and allowed to air dry prior to MALDI mass fingerprinting using an Applied Biosystems (Foster City, CA) Voyager DE-Pro mass spectrometer in positive ion mode. Monoisotopic masses were calibrated using internal matrix and trypsin peaks. Peptide mass fingerprints were searched against NCBInr (version 7, November 2007) mammalian taxonomy (572,793 sequences) using Mascot version 2.2.03 and Profound version 2002.03.01. Search parameters included complete modification of cysteine by iodoacetamide and partial modification by methionine oxidation. The peptide tolerance did not exceed 40 ppm, one missed trypsin cleavage was allowed. Match criteria included number of peptides matched, sequence coverage, significance scores and difference in probability between the first and second match. Most matches were to bovine sequences but matches to other mammals were accepted.

Prior to CyDye labelling, the pH of the protein samples solubilized in lysis buffer was adjusted to 8.5 by adding 1.5 M Tris. For each sample, 20 μg of protein was labelled with 80 pmol CyDye for 30 min on ice and in the dark. 2DE was routinely carried out focusing labelled proteins on pH 4–7 and 6–11 Immobiline DryStrips (GE Healthcare). 2D-DIGE gels were scanned using a Fujifilm FLA-5100 (Fuji Photo Film, Japan). Cy2 images were scanned using a 473 nm laser and a BPB1/530DF20 emission filter. Cy3 images were scanned using a 532 nm laser and a PBG/570DF20 emission filter. Cy5 images were scanned using a 635 nm laser and a DBR1/R665 emission filter. Image analysis was performed using DeCyder™ 2D 6.5 software (GE Healthcare). The Biological Variation Analysis (BVA) module was used to match the spot maps to a master gel (typically the gel containing the highest number of spots) and to calculate spot volumes. Statistical analysis was performed using the Student's *t*-test (equal variance two-tailed test, $p<0.01$).

For analysis of inter-individual variation, mammary and liver samples obtained from eight animals were analysed by 2D-DIGE. Individual samples were labelled either with Cy3 or Cy5 and the internal standard was made up of all the samples pooled and labelled with Cy2. Measurement of inter-individual variation in spot abundance was carried out on identified and well-resolved protein spots: 271 in liver and 202 in mammary (Supplementary Table 3). Values for the coefficient of variation of the 473 protein spots ranged from 1 to 16%.

For Western blotting, protein samples extracted in lysis buffer were subjected to one-dimensional electrophoresis on 4–12% Bis-Tris NuPage gels (Invitrogen, USA). Proteins were transferred to Hybond-LFP membrane (GE Healthcare) by wet electro-transfer in 25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol. The membranes were blocked in 8% (w/v) non-fat dried milk in 0.1% Tween-20/TBS. All the subsequent incubation and wash steps were carried out in 0.1% Tween-20/TBS. The primary antibodies were: anti-actin (MAB1501, Merck Millipore, USA), anti-acetyl CoA carboxylase alpha (ACACA) (ab72046, Abcam Ltd, UK), anti-fatty acid synthase (FASN) (ab22759, Abcam), anti-fructose-1,6-bisphosphatase (FBP1) (ab103165, Abcam). Immunoreactive proteins were detected using fluorescence conjugated secondary antibodies: Alexa-Fluor 647 goat anti-rabbit (1:500 for ACACA and FBP1, 1:5000 for FASN), and AlexaFluor 555 goat anti-mouse 1:5000 for actin. Dry membranes were scanned using the Fujifilm FLA-5100.

3. Results

Respectively in liver and mammary tissue lysates, 365 and 220 protein spots were identified using MALDI mass spectrometry of