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Impact of high-fat diet on the proteome of mouse liver

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Abstract

Chronic overnutrition, for instance, high-fat diet (HFD) feeding, is a major cause of rapidly growing incidence of metabolic syndromes. However, the mechanisms underlying HFD-induced adverse effects on human health are not clearly understood. HFD-fed C57BL6/J mouse has been a popular model employed to investigate the mechanisms. Yet, there is no systematic and comprehensive study of the impact of HFD on the protein profiles of the animal. Here, we present a proteome-wide study of the consequences of long-term HFD feeding. Utilizing a powerful technology, stable isotope labeling of mammals, we detected and quantitatively compared 965 proteins extracted from livers of chow-diet-fed and HFD-fed mice. Among which, 122 proteins were significantly modulated by HFD. Fifty-four percent of those 122 proteins are involved in metabolic processes and the majority participate in lipid metabolism. HFD up-regulates proteins that play important roles in fatty acid uptake and subsequent oxidation and are linked to the transcription factors PPARα and PGC-1α. HFD suppresses lipid biosynthesis-related proteins that play major roles in *de novo* lipogenesis and are linked to SREBP-1 and PPARγ. These data suggest that HFD-fed mice tend to develop enhanced fat utilization and suppressed lipid biosynthesis, understandably a self-protective mechanism to counteract to excessive fat loading, which causes liver steatosis. Enhanced fatty acid oxidation increases reactive oxygen species and inhibits glucose oxidation, which are associated with hyperglycemia and insulin resistance. This proteomics study provides molecular understanding of HFD-induced pathology and identifies potential targets for development of therapeutics for metabolic syndromes.
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1. Introduction

Diet and nutrients in the diet have significant impact on physiological well being and pathological development. For instance, human studies have shown that high-fat diets (HFDs) can cause over weight or obesity, with varied degree due to different genetic background [1–7]. HFD is one of those nutritional conditions that accounts for the largest incidence of metabolic syndromes and these metabolic syndromes include obesity and related metabolic disorders such as nonalcoholic fatty liver disease (NAFLD), diabetes, cardiovascular diseases and so on [8]. Yet, the molecular mechanisms underlying HFD-induced adverse effects on human health are unclear.

To investigate those mechanisms, several animal models have been developed. One of such is HFD-fed (60% of calories provided from fat) C57BL6/J mouse. These mice have been employed particularly often as a model for the development of metabolic syndromes [8–10], as they have high feed efficiency [11] and are sensitive to HFD [11–13]. This model has been used to characterize phenotypes of obesity, to identify genes or proteins that play important roles in the development of metabolic disorders and to evaluate pharmacological interventions. There is a vast reservoir of data in the literature on this animal model [12,14–24]. However, thus far, there has been no systemic and comprehensive study on the impact of this HFD itself on the proteome of the animal. This is partially due to limitations in the dynamic range of detection methods such as two-dimensional differential in gel electrophoresis (2D-DIGE) utilized previously.

To overcome this limitation, we have utilized stable isotope labeling of mammalian (SILAM) technology for accurate relative quantification. SILAM technology uses ^{15}N metabolically labeled mice to assess proteome-wide changes caused by genetic or environmental manipulations.

Liver regulates the integrative maintenance of whole body glucose and lipid metabolism through the dynamic control of lipogenesis, lipolysis, gluconeogenesis and glycolysis. It is estimated that, at any given time point, more than 10,000 biochemical reactions occur in liver [25]. Those reactions are carried out by proteins involved in basic carbohydrate, fat and protein metabolism, storage of vitamins and minerals and many regulatory processes that control blood lipid, sugar and hormone levels. More importantly, obesity-related metabolic disorders manifest in liver, causing liver steatosis, NAFLD, eventually leading to inflammatory steatohepatitis [26]. Hence, proteome in liver

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is a better representative of the proteome in the whole body, as opposed to other tissues.

In this current study, employing powerful SILAM technology, we investigate and reveal the global impacts of HFD on protein profiles in the livers of the mouse model and their implications in metabolic processes.

2. Materials and methods

2.1. Animal experiments

Wild-type C57BL6/J mice at age 5–6 weeks were purchased from the Jackson Laboratory. All experimental procedures done with mice were approved by the Institutional Care and Use Committee at Albert Einstein College of Medicine in accordance with the “Guide for the Care and Use of Laboratory Animals” published by the National Institute of Health. ^{15}N Metabolically labeled mice were prepared as previously described [27]. Briefly, C57BL6/J mice were fed with ^{15}N-labeled specially fortified chow diet (CD, providing calories 24%, 12% and 63% from protein, fat and carbohydrate, respectively) (Research Diets, Inc., New Brunswick, NJ) using ^{15}N-labeled spirulina (Cambridge Isotope Laboratories, Tewksbury, MA) starting immediately after weaning (21 days of age) for a total period of 12 weeks. Metabolic labeling efficiency was monitored noninvasively by the analysis of urine sediment while the labeling process was ongoing. Greater than 95% atomic enrichment of liver proteins was achieved. HFD (providing calories 20%, 60% and 20% from protein, fat and carbohydrate) was purchased from Research Diets (New Brunswick, NJ). Mice were fed with food and water *ad libitum*. Their body weight was monitored weekly. Intraperitoneal glucose tolerance test (IGTT) was performed on conscious mice after 12-h fasting and intraperitoneally injected with glucose (20% glucose in saline, 2 g/kg total body weight). Two weeks after IGTT, mice were fasted for about 12 h and sacrificed. Liver tissues were collected, weighed and examined by Oil Red O staining.

2.2. Oil Red O staining

Liver tissues were fixed in 10% neutral buffered formalin (Fisher Scientific) for at least 48 h, followed by rapid freezing in Tissue Tek OCT (Sakura Finetek) for cryosectioning. Cryosections of 5 μm were stained by routine Oil Red O methods (Poly Scientific R&D Corp.) and lightly counterstained in Mayer's hematoxylin solution.

2.3. Preparation of samples for proteomic analysis

Liver tissue from four HFD-fed mice was mixed with equal weight of liver tissue from ^{15}N metabolically labeled CD-fed mice. Tissues were homogenized and lysed in RIPA buffer with protease inhibitor cocktail for protein extraction. Fifty micrograms of extracted proteins was separated with SDS-PAGE. Complete lanes were excised using disposable grid cutters (The Gel Company, San Francisco, CA) to produce 40 bands (1 mm×10 mm) per lane, and band slices were placed in 96-well plates and in-gel digested using an Ettan Digester (GEHealthcare, Piscataway, NJ). To optimize analysis, considering time efficiency and the resolution of SDS-PAGE, digested peptides from two bands between 20 and 200 kDa molecular markers or three bands above or below that range were pooled before analysis. Replicate experiment with another set of mice (two groups (CD and HFD), 4 mice/group) was performed following the same experimental scheme (Supplementary Fig. 1).

2.4. Liquid chromatography (LC)–tandem mass spectrometry (MS/MS) analysis

The Agilent 1100 Series Nano HPLC interfaced to a QStar XL mass spectrometer (AB SCIEX, Ontario, Canada) was used for analysis. Samples were loaded onto a ZORBAX 300SB-C18 trap column (5 μm, 300 Å, 0.3 mm) at a flow rate of 8 μl/min with 2% CH_3CN/0.1% trifluoroacetic acid and delivered to an Acclaim 300 (C18, 3 μm, 300 Å, 75 μm i.d.×15 cm; Dionex Coorporation, CA) nanocolumn by a switching mechanism. Peptides were eluted from the nanocolumn at a flow rate of 250 nl/min with 2% CH_3CN/0.1% formic acid (solvent A) and 90% CH_3CN/0.1% formic acid (solvent B). The gradients used were as follows: 0–30 min, 5% B (desalting); 30–80 min, 5–25% B; 80–95 min, 25–90% B; 95–110 min, 90% B; 110–120 min, 90–5% B; and 120–130 min, 5% B.

A nanospray voltage in the range 2000–2400 V was optimized daily. All nano-LC-MS/MS data were acquired in data-dependent acquisition mode in Analyst QS 1.1 (AB SCIEX, Ontario, Canada). Time-of-flight mass spectrometry survey scans with an m/z range of 300–1600 for 1 s, followed by a product ion scan with an m/z range of 50–1600 for 2 s each. Collision energy was automatically controlled by the IDA CE Parameters script.

2.5. Protein identification

Once obtained, peak lists were generated from MS/MS spectra using AB SCIEX MS Data Converter version 1.2 and searched against the IPI mouse database (version 3.73) concatenated with a reverse decoy using Mascot (version 2.3; Matrix Science). Fixed modification of cysteines to *S*-carbamidomethyl derivatives and variable oxidation (M) were defined for the database search. One missed cleavage was allowed with trypsin, and mass tolerance was set to 100 ppm for precursor ions and 0.2 Da for fragment ions. Searched results were exported as Mascot DAT files and grouped to protein matches using ProteoIQ (version 2.6.03; Nusep). Protein hits were filtered to include only those that were identified with at least two peptides with less than 1% false discovery rate. DAT files generated from the Mascot searches were exported to ProteoIQ version 2.3.06 for protein grouping and quantitation. For quantitation, the raw LC-MS/MS files converted into mzXML with Trans Proteomic Pipeline version 4.4. Precursor intensities extracted from mzXML using area under the curve were matched to identify peptides for protein quantitation. Peak intensity ratios were calculated after the baseline extraction and peak smoothing. The product of the square of the correlation coefficients (R^2) was measured between the theoretical and experimental isotopic distributions for the quantitative precursors. Presence of an interfering precursor that overlaps with the distribution of interest was detected and removed from quantitation using the coefficient. Systematic bias was corrected with normalization factors. The normalization factors were calculated such that the total intensity for all peptides in each replicate was equal. The normalization factors were then applied to the ion intensities for each peptide. Identified proteins were relatively quantified using ^{15}N metabolically labeled proteins. Identified proteins were annotated with Gene Ontology (GO) terms using the Panther Classification system [28]. The protein list with relative quantitation and GO annotation is reported in Supplementary Table 1.

2.6. Western blot

Protein samples (extracted from the same liver tissues that were used for proteomic analysis) were fractioned on SDS-PAGE and transferred to polyvinylidene difluoride membrane overnight. The membrane was incubated in 5% milk in TBST at room temperature for 1 h and subsequently incubated with anti-Acaa1 (Sigma Cat# HPA007244, dilution 1:500), anti-Acss2 (abcam, Cat# ab133664, 1:1000), anti-Acaca1 polyclonal antibody (Cell Signaling, Cat# 3676, 1:1000) or anti-Fasn (R&D Cat# AF5927, 1:200) in 5% milk (0.5–1 μg/ml) at 4°C overnight. The membrane was washed three times with TBST, 10 min each time, before incubation with secondary antibody (goat anti-rabbit IgG HRP-linked antibody) in wash buffer with 0.5% milk (1:2000). Secondary antibody was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) followed by autoradiography. The protein bands were quantified using ImageJ.

2.7. Statistical and network analysis

Significantly regulated proteins were determined based on *P* values ($P<.005$) acquired from distribution-free, permutation-based hypothesis testing [29]. Intensity ratios of light and heavy labeled peptides were used for the testing of 10,000 iterations. Out of 9774 from the identified proteins, 8666 peptides were used for the testing. Adjusted *P* values by Benjamini–Yekutieli are presented in Supplementary Table 1 as well for a reference.

Ingenuity Pathway Analysis 8.5 (IPA) (Ingenuity Systems, Redwood City, CA) was used to associate significantly regulated proteins into pathway networks. To analyze the interactions among HFD dysregulated proteins found in this study, we uploaded 29 significantly differentially regulated proteins associated with lipid metabolism to STRING database (version 10) [30] to search for their protein–protein interactions. Four functional association algorithms were tested on those 29 proteins. Interactions with confidence scores above 0.7 were considered high confidence. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were generated using DAVID bioinformatic resources 6.7 [31,32] and shown in Supplementary Table 1.

3. Results

3.1. HFD-induced obesity and liver steatosis

To establish HFD-induced obesity, we fed C57BL/6J mice, which are sensitive to HFD [9–11], with CD or HFD. Sixteen weeks of feeding with HFD produced obese mice that had body weight 60% higher than those with CD (Fig. 1A and B). The majority of the weight difference occurs in fat mass as examined by NMR-based whole body fat quantitation (Fig. 1C). These obese mice have enlarged liver measured as liver weight (Fig. 1D) and drastically increased accumulation of lipid in the liver revealed by Oil Red O staining (Fig. 1E). They were also glucose intolerant, determined by an IGTT (Fig. 1F). These results are consistent with literature [33] and indicate that we have established obesity phenotype.

3.2. Quantitative proteomic comparison of HFD vs. CD using ^{15}N-labeled CD-fed mouse livers

To determine the effects of HFD on global protein expression in liver tissues, we harvested liver tissues from four HFD-fed mice and

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