Research Article

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Food and Nutritional Proteomics

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Abstract

Objective: *I introduce a new technical and conceptual term "nutritional proteomics" by identifying and quantifying the proteins and their changes in a certain organ or tissue dependent on the food intake by utilizing a mass spectrometry based proteomics technique.*

Purpose: *Food intake is essentially important for every life on earth to sustain the physical as well as mental functions. The outcome of food intake will be manifested in the health state and its dysfunction. The molecular information about the protein expression change caused by diets will assist us to understand the significance of functional foods. We wish to develop nutritional proteomics to promote a new area in functional food studies for a better Understanding of the role o1'firnctional foods in health and disease.*

Introduction

Food intake is essentially important for every life on earth to sustain the physical as well as mental functions. In a classical sense, the functions of foods are to replenish the molecules that construct our body as well as to be consumed as energy source for our everyday activity. From a modern point of view, however, foods can do more than these conventional functions; foods could actively be involved in the healthy maintenance of our body, through controlling molecular and cellular signaling pathways [1]. Therefore, investigating the molecular and cellular pathways on which food intake influences will open a fruitful area in functional foods studies. There are at least two ways how to investigate the changes in molecular and cellular pathways caused by diets, one by the genomics method and the other by the proteomics. Genomics measures mRNAs, whereas proteomics measures proteins. These two sets of parameters do not always correlate each other by many unknown reasons [2, 3]. Moreover, proteins, but not mRNAs, are the physical entities that function in cellular events. Therefore, it is essentially important to obtain protein information to evaluate the effect of functional foods on the alteration of cellular and molecular signaling. In this report we will introduce a potential subarea in functional food studies; studies of protein expression and its change by diets. We designate the new discipline "nutritional proteomics." In this report, we will use a class of essential fatty acids, omega-3 polyunsaturated and omega-6 PUFAs, as an example of nutritional proteomics study.

Materials and Methods

I chose two classes of food ingredients to show the feasibility of nutritional proteomics, omega-3 polyunsaturated fatty acids and omega-6 polyunsaturated fatty acids both of which are involved in the inflammation/anti-inflammation axis. Each class of the polyunsaturated fatty acids was mixed in mouse chow respectively. The liver tissue of mice fed, with omega-3 diet or omega-3 diet was analyzed by the state-of-the-art shotgun proteomics using nano -HPLC-ESI-MS/MS. The data were analyzed by the number of differentially expressed proteins that were guaranteed by l% false discovery rate for protein identification and by the statistical significance of variance evaluated by p-value in two-tailed distribution analysis better than 0.05 (n=4). The differential pattern of protein expression was characterized with Gene Ontology designation

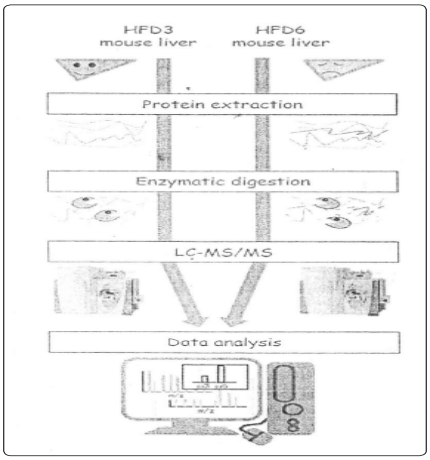


Figure l: Schematically illustrated protocol for nutritional proteomics. The starting materials are livers from HFD3 (omega-3 diet-fed) or HFD6 (ourega-6 diet-fed) C51BL|6 mice. Protein extraction, enzymatic digestion, LC-MS/MS, and data analysis steps are described in Materials and Methods

C57BL/6 mice: Weight-matched males of C57BL|6 mice at the age of 4 weeks were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in the OUHSC Rodent

Barrier and maintained on a normal mouse chow. After adjustment to the environment for a week, the mice were divided into two groups; HFD6, mouse chow supplemented with 10% Safflower oil that is rich with omega-6 polyunsaturated fatty acids, and HFD3, mouse chow supplemented with 10% Menhaden oil that is rich with omega-3 polyunsaturated fatty acids. Both HFD6 and HFD3 diets were adjusted to the, energy content to 54%o fat calories with hydrogenated coconut oil in order to accelerate the dietary effects of PUFAs. The rodent barrier had been maintained under temperature-and moisture-controlled pathogen-free environment under a 12 hour light /12 hour dark c1, cle. Afier4 weeks on each diet, we killed the mice (n=5 for each group) by guillotining. The livers were collected, and stored at -80 "C until use. The IACUC at OUHSC had approved the animal protocol used in the experiment.

Protein extraction and trypsin digestion

Liver tissues (about 10 mg each dissected from four individual mice; n=4) were processed individually for shotgun proteomics analysis. The samples were homogenized using an ultrasonic processor in 100 volumes of 12 mm sodium Nlauroylsarcosinate, 12 mm sodium lauryl sulfate, and 200 mm triethyl ammonium bicarbonate containing protease inhibitors (complete mini EDTA-free; Roche Diagnostics GmbH, Mannheim, Germany) according to the published literature [4]. The homogenate was centrifuged at 19,000g at 4 °C for 20 min, and the clear supernatant containing proteins was collected. Twenty Pl of the supernatant were reduced by the addition of 2 LIL of 200 mm *tries* (2-carboxyethyl) phosphine for 30 min at 55 °C. After cooling to room temperature, reduced disulfides were then alkylated by the addition of 2 Pl of 375 mm iodoacetamide for 30 min at room temperature in the dark. The alkylated sample was enzymatically digested by the addition of 2 Pl of 100 ng/pl Lys-C (Wako, Osaka, Japan) and 2 pL- of 100 ng/pl Trypsin (Promega, Madison. WI) for 18h at 31°C. The digested sample was acidified by 5% trifluoroacetic acid, and the detergents were precipitated. After centrifugation, the supernatant was collected followed by lyophilization. The lyophilized sample was dissolved in 100 Pl of 1x lnvitrosol (Invitrogen, Carlsbad, CA, USA) in order to enhance the recovery of hydrophobic peptides [5].

Nano-LC-MS/MS analysis

The peptide digests (Z Pl each) were injected into a trap column: Crs0.075x50 mm (MonoCap C18 Trap Column; GL Sciences, Tokyo, Japan) followed by an analytical column: C_{18} 0.075x 120 mm (Nano HPLC Capillary Column; Nikkyo Technos, Tokyo, Japan), which was interfaced to the EASY-nLC 1000 (Thermo Fisher Scientific, Bremen, Germany). The flow rate of the mobile phase was 300 nL/min, and the mobile phases consisted of (A) 0.1% formic acid and (B) 0.1% formic acid and 90% acetonitrile. The mobile phase was programmed as follows: 0-8% B (0-2 min), 8olo-32%B (2-132 min), 32-45% B (132-152 min), 45-100% B (152-153 min), and 100% B (153-165 min). Separated peptides were introduced from HPLC to Q-Exactive mass spectrometer (Thermo Fisher Scientific). The Q Exactive instrument was operated in the data dependent mode to automatically switch between full scan MS and MS/MS acquisition. Full scan MS spectra (m/z 350-1500) were acquired in the Orbit rap with 70,000 resolutions at m/z 200 after accumulation of ions to a 3 x 10^6 target value. The ten most intense peaks with charge state >2 from the full scan were selected with an isolation window of 2.0 Da and fragmented in the HCD collision cell with normalized collision energy of 25%. Tandem mass spectra were acquired in the Orbit rap mass analyzer with a mass resolution of 17,500 at m/z 200 after accumulation of ions to a 2 x 10^5 target value. The ion selection threshold was 1 x 10^5 counts, and the maximum allowed ion accumulation times were 60 ms for full MS scans and 60 ms for tandem mass spectra. Typical mass spectrometric conditions were as follows: spray voltage, 2 kV; no sheath and auxiliary gas flow; heated capillary temperature, 250 "C; dynamic exclusion time, 60 s.

Data Analysis

Mass spectral data were processed, exported, and searched against a UniProtKB mouse protein database using MASCOT (version2.3, Matrixscience, London, UK) by Proteome Discoverer (version 1.3; Thermo Fisher Scientific). Database search parameters were: peptide mass tolerance, 6 ppm; fragment tolerance, 0.02 Da; enzyme was set to trypsin, allowing up to two missed cleavage; fixed modifcations, carbamidornethyl (cysteine); variable modifications, oxidation (methionine). False discovery rate (FDR) was calculated by enabling the peptide sequence analysis using a decoy database (http://www.matrixscience.com/help/decoy_help.html) [6, 7]. We used 1% FDR as a cut-off to export results from the analysis. Among over 5,000 proteins identified, 2,810 proteins were validated with better than 1% FDR. Quantitative analysis of shotgun proteomics data was achieved by label-free quantification of four independent liver samples from each group [8, 9]. For this, we used spectral counting after incorporating the mass data to Scaffold (Proteome Software, Inc., Portland, Oregon). The statistical significance of protein expression difference between the two diets (omega-3 and omega-6, respectively) was validated by two-tailed distribution analysis (n=4) with the cut off limit of better than $p<0.05$. The total of 125 proteins passed both the 1% FDR and the t-test [10]. Assumed molecular functions, cellular localization and biological process of the proteins were defined using Proteome Discoverer according to the Gene Ontology (GO) annotations (http://www.geneontology.org).

Results and Discussion

The data analysis of the shotgun nutritional proteomics identified 2,810 proteins that are validated with l% FDR. Among these 2,810 proteins, 125 were characterized with statistical significance of variance ($p<0.05$; n: 4) between the omega-3 diet and the omega-6 diet by two tailed distribution analysis. The results illustrate that the dietary influence of omega-3 and omega-6 on protein expression is eminent with the proteins directly responsible for catalytic activity in the "Molecular Function" category, totaling 192 proteins, of Gene Ontology designation. In a similar analysis with regard to the "Cellular Localization" category, protein expression changed the most in the sub-categories of "Cytoplasm", "Membrane", "Nucleus", and "Mitochondrion", totaling 22l proteins. The same analysis with regard to "Biological Process" -considering the top four categories, i.e., "Metabolic process", "Regulation of biological (process)", "Response to stimulus", and "Transport" also indicated significant alteration of 182 proteins. These results illustrate a robust influence of dietary elements, omega-3 or omega-6 polyunsaturated fatty acids, on the protein expression in mouse liver.

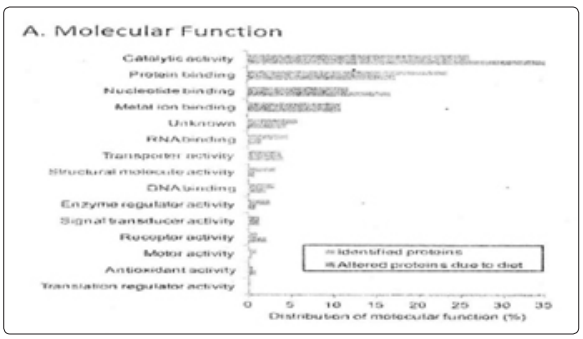
Novel protocol for nutritional proteomics

The overall protocol of nutritional proteomics using shotgun analysis applied to liver samples is illustrated in (Figure l). In the cohort of C57BL|6 mice we are interested in how protein expression differs between omega-3-enriched diet (HFD3) and onega-6-enriched diet (HFD6) on the high fat (54% fat calories) background. We put the mice on the high fat background because high fat diets have propensity to induce inflammatory biological response, thereby

would illustrate better the two opposing effects of ontega-3 and ornega-6, i.e., the former anti-inflammatory and the latter pro-inflammatory [11]. Reliability of protein identification was validated with 1% FDR; after the shotgun data analysis we identified 2,810 proteins that were validated by I% FDR for all the liver proteins. Based on the spectral count data, the differential ratio of each protein between HFD6 and I-IFD3 was calculated. It should be noted that the same protocol could be applied to any organ and tissue samples to investigate differences in protein expression caused by different dietary intake [12-15].

Alteration of the cellular signaling pathways caused by dietary intake enriched with omega-3 or omega-6

The data analysis of the shotgun nutritional proteomics outlined in Figure 1 identified 2,810 proteins that are validated with 1% FDR. Among these 2,810 proteins, 125 were characterized with significant statistical parameters ($p<0.05$; n: 4). All the 2,810 liver proteins were sorted into three categorical groups, i.e., protein function, cellular localization, and biological process based on the GO annotations (Figure 2). The numbers of proteins that belong to the top four sub-classes arc sumrrarized in Table 1. It should be noted that, since many proteins can be counted more than once because one protein could belong to multiple GO classes, the total number of proteins is larger than 2, 8 l0 that are validated by FDR scores better than 1%. In the protein function (Figure 2A and Table 1), the proteins in the "Catalytic activity", "Protein binding", "Nucleotide binding", and "Metal ion binding" are counted 3, 858. Among these 3, 858 proteins, 192 (=85+41+40+26) changed their expression levels including both up- and down regulation in the top four categories with significant p-values ($p<0.05$). These results illustrate that the dietary influence of omega-3 and omega-6 on protein expression is eminent with the proteins directly responsible for catalytic activity. We interpret that the follow-ups, i.e. the protein groups classified in "Protein binding", "Nucleotide binding", or "Metal ion binding", are entities supporting the functions of the proteins responsible for the catalytic activity. In a similar analysis with regard to cellular localization (Figure 2C and Table l), protein expression changed the most between the two diets in the GO categories of "Cytoplasm", "Membrane", "Nucleus", and "Mitochondrion", totaling to 221(=101+54+22+44) proteins. The same analysis with regard to biological process (Figure 2C and Table l) considering the top four categories, i.e., "Metabolic process", "Regulation of biological (process)", "Response to stimulus", and "Transport" indicated that 182 (95+31+31+25) proteins showed $p<0.05$. These results clearly illustrate a robust influence of dietary elements, omega-3 or omega-6 polyunsaturated fatty acids, on the protein expression. It is anticipated that the substantial alteration of protein expression is a reflection of the complex chains of cellular signaling processes involving the omega-3 and onrega-6 molecules and their *in vivo* prod.



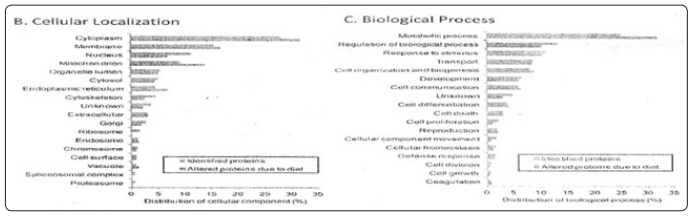


Figure 2: Identified liver proteins and their distribution in categorized groups

In A, B, and C, the identified proteins are shown in blue by the percentage of the total proteins and the proteins that altered expression dependent on diets are shown in red by the percentage of the total changed proteins. Note that the percentage was calculated on different bases between "identified proteins" shown in blue and "altered proteins due to diets" shown in red.

A. Categorized by molecular function.
B. Categorized by cellular localization.
C. Categorized by biological process.

Almost all available literatures suggest the role of these cellular signaling processes to be associated directly or indirectly, with inflammation/anti-inflammation events. The result implies that detailed dissection of such complex cascades could be possible using nutritional proteomics approaches.

Table 1: The difference of protein expression between HFD3 and HFD6 is manifested in distinct GO annotation categories

	Number of identified Proteins	Number of Altered Proteins	% of Identified Proteins	% of Altered Proteins
Molecular Function				
Catalytic activity	1387	85	26.0	35.9
Protein binding	1255	41	23.6	17.3
Nucleotide binding	633	40	11.9	16.9
Metal ion binding	583	26	10.9	11.0
Cellular Localization				
Cytoplasm	2065	101	28.1	31.8
Membrane	1142	54	15.6	17.0
Nucleus	824	22	11 2	6.9
Mitochondrion	672	44	9 2	13.8
Biological Process				
Metabolic process	1912	95	25.1	32.0
Regulation of biological	1006	31	13.2	10.4
Response to stimulus	843	31	11.0	10.4
Transport	665	25	8.7	8.4

Conclusions

We showed in this report that the omega-3 diet induced a unique set of liver proteome compared to the one on the ornega-6 diet. Such biochemical phenotypes represented by differential patterns of protein expression are likely to reflect the changes of complex cellular signaling process modulated by the two different types of polyunsaturated fatty acids, omega-3 and omega-6. Since all the available information points to the role of omega-3 and omega-6 in anti-inflammatory and inflammatory signaling, the changes in

protein expression must reflect the alteration of cellular signaling directly involved in inflammatory/anti-inflammatory processes or of the secondary consequence of such processes. Using nutritional proteomics technique, dissecting the time-dependent sequence of the signaling steps associated with inflammation and modified by diets will be possible. Furthermore, we may be able to extend our observation into more general context; the nutritional proteomics approach will bring in a significant body of information that would help us to decipher the altered cellular signaling process by diets. Also, the nutritional proteomics approach will be applicable to the studies of other functional foods and this new discipline could teach us molecular mechanisms how functional foods will affect our health.

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