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Proteomic profile of visceral adipose tissues between low-fat diet-fed obesity-resistant and obesity-prone C57BL/6 mice

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Abstract. Certain individuals ingesting a low-fat diet are still prone to visceral obesity, and the reasons remain undetermined. This study aimed to investigate differentially expressed proteins of visceral adipose tissues between low-fat diet-fed obesity-resistant and obesity-prone C57BL/6 mice through a proteomics approach and to ascertain potential mechanisms associated with these obesity-prone animals. First, animals were fed low-fat or normal-chow diets for 6 weeks. Dietary intake and body weight were monitored during this period. After 6 weeks of the low-fat diet, obesity-prone and obesity-resistant mice (NOP and NOR groups) were defined as those within the upper and lower quarters for weight gain, respectively. Proteomic analysis showed that ubiquinol-cytochrome c reductase core protein 1 (Uqcrc1) and Enolase 3, β muscle were decreased by 8.1- and 8.8-fold in the visceral adipose tissues of the NOP mice compared to the NOR controls, respectively, while monoglyceride lipase (MGLL) and glucose-6-phosphate dehydrogenase (G6PDH) X-linked were increased by 5.3- and 4.7-fold, respectively. These results indicate that obesity-prone animals fed low-fat diets exhibited differentially expressed proteins, e.g., Uqcrc1, Enolase 3, MGLL and G6PDH involved in energy metabolism, glycolysis and fat synthesis in visceral adipose tissues, which are useful for defining molecular markers and understanding the mechanisms of the development of obesity.

Introduction

Obesity, with its devastating implications for overall health, is a serious medical problem now reaching epidemic proportions worldwide (1). It is associated with an increased risk of numerous comorbidities, such as type 2 diabetes, metabolic syndrome, hypertension, cardiovascular diseases and osteoarthritis (2). However, the prevention and treatment of obesity remains a challenge, since the underlying mechanisms of pathogenesis remain undetermined.

A common idea suggests that interaction between genetic and environmental factors may contribute to the development of obesity (3). A high-fat diet is a necessary catalyst for the observed variability in body weight gain and the development of obesity (4). However, certain individuals are still rendered obese even though they are subjected to low-fat or normal-fat diets. Currently, the approach for treating obesity primarily involves caloric restriction, physical exercise and use of anti-obesity drugs (1). The study of susceptibility to diet-induced obesity may be useful for identifying potential targets for preventing the development of obesity.

Many investigations have searched for candidate factors that render individuals susceptible to diet-induced obesity. Studies comparing obesity-prone and -resistant animals indicate that obesity-prone animals may favor fat storage or show a decreased fatty acid oxidation compared to obesity-resistant controls (5). Different levels of neuropeptide Y and leptin may also contribute to a propensity to obesity resistance (6,7). Taken together, the potential mechanisms involved in obesity resistance may include caloric intake, fat metabolism and storage. However, the exact molecular mechanisms are far from being elucidated.

Visceral adipose tissues are the main markers of obesity. Proteomics is an important method widely used in many research fields and supplies much useful information for our reference. In the present study, we investigated differentially expressed proteins of visceral adipose tissues between obesity-resistant and obesity-prone C57BL/6 mice fed low-fat diets using a proteomics method and tried to identify molecular markers and to understand the potential mechanisms of the development of obesity.

Materials and methods

Animals and diets. Three-week-old male C57BL/6 mice [SPF grade, certification no. SCXK (Guangdong) 2003-0002]

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were obtained from the Guangdong Medical Laboratory Animal Center (Guangzhou, Guangdong, China). Animals were maintained in an environmentally controlled breeding room (temperature 20±2˚C, humidity 60±5%, 12-h dark/light cycle). They were fed standard laboratory chow with water *ad libitum* and fasted from 9:00 am to 3:00 pm prior to the experiments. Our research was conducted in accordance with the Declaration of Helsinki and/or the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health and was approved by the Animal Ethics Committees of Tsinghua University, China. Normal-chow diets were obtained from the Guangdong Medical Laboratory Animal Center. The low-fat or normal-chow diets contained 20% crude protein, 4% crude fat and 60% crude carbohydrates (g/g). Total calorie intake from the normal-chow diets was 15 kJ/g (10% calories in fat).

Experimental procedure. Mice 4 weeks of age had free access to water and chow diets (n=40). Dietary intake and body weight was monitored once a week. Dietary and water intake, urine volume and fecal weight for each mouse were recorded in a metabolic cage (Tecniplast®, Italy) for 24 h, once a week. Total calorie intake was calculated according to the dietary calorie intake and expressed as kJ/g/day. Collected feces were used for lipid assaying. After 6 weeks of low-fat diet feeding, mice ranked in the upper and lower quarters for weight gain during this feeding period were designated as obesity prone and obesity resistant (NOP and NOR, n=10), respectively. At the sixth week, animals were weighed and anesthetized by an intraperitoneal injection of pentobarbital at a dose of 35 mg/kg. Blood was collected from the tail vein prior to and from the orbital plexus after the 6-week treatment, respectively. Serum was isolated by centrifugation at 1,500 x g at 4˚C for 10 min and stored at -80˚C until used for blood glucose and lipid assays. Following blood collection, anesthetized mice were sacrificed by cervical dislocation. Visceral adipose tissues (perigonadal fat, the main part of the internal white adipose tissues) were removed from the animals and immediately weighed. These samples were instantly frozen in liquid nitrogen and then stored at -80˚C until used for biochemical analysis.

Biochemical analysis. Blood glucose and triglycerides, total cholesterol, low-density lipoprotein cholesterol (LDL-c) and high-density lipoprotein cholesterol (HDL-c) (n=10) were estimated using commercial kits (BioSino Bio-Technology and Science Inc., Beijing, China) (8-12). Fecal lipid assays were conducted according to the following protocol. Collected feces were dried at 60˚C to a constant weight. Dried feces (100-200 mg) were combined with 0.2 ml of PBS (0.1 M, pH 7.4) and 1 ml of $CHCl_3$-CH_3OH (2:1) in a 1.5-ml Eppendorf tube and homogenized. The homogenates were centrifuged at 10,000 x g for 5 min. A 0.1-ml aliquot of the lower phase was added to another Eppendorf tube and air-dried. The dried lipids were assayed with commercial clinical diagnosis kits according to the above methods. Data were expressed as μg/mg feces.

2-Dimensional electrophoresis. Freshly prepared protein samples from the visceral adipose tissues of 3 randomly selected animals in each group were analyzed by 2-dimensional electrophoresis (2DE). Protein extraction of adipose tissues, IEF and second dimensional separation of samples, gel staining and image analysis, trypticin-gel digestion, MALDI-TOF/TOF mass spectrometric analysis and database search for protein identification were carried out according to previously described methods (13). Briefly, the cell pellets were re-suspended in lysis buffer (8 M urea, 2 M thiourea, 2% CHAPS, 1% NP-40, 2 mM TBP, 1X Protease Inhibitor Mix, 1X Nuclease Mix, 1 mM PMSF and 2% IPG buffer) and held on ice for 45 min. The lysate was then centrifuged at 16,000 x g for 30 min at 4˚C, and the supernatant was saved and stored at -80˚C until use. IEF was carried out using an IPGphor II apparatus (Ammersham). IPG strips (13 cm, pH 3.0 to 10.0, NL) were used according to the manufacturer's instructions. Samples containing 150 μg protein were diluted to 250 μl in rehydration solution (8 M urea, 2% CHAPS, 0.4% DTT, 0.5% IPG buffer and 0.002% bromophenol blue). The rehydration step was carried out with 13-cm IPG strips for 10 h at a low voltage of 30 V. IEF was run following a step-wise voltage increase procedure: 500 and 1,000 V for 1 h each and 8,000 V for 64 KVh. After IEF, IPG gel strips were placed in an equilibration buffer (6 M urea, 2% SDS, 30% glycerol, 0.002% bromophenol blue, 50 mM Tris-HCl, pH 6.8) containing 1% DTT for 15 min under agitation. The IPG strips were then transferred to the equilibration solution containing 2.5% iodoacetamide and shaken for a further 15 min before applying them to 12.5% uniform polyacrylamide gel slabs (150x158x1.5 mm). Separation in the second dimension was performed in Tris-glycine buffer (25 mM Tris, 0.2 M glycine and 0.1% SDS) at a constant current setting of 15 mA/gel for 30 min and 30 mA/gel thereafter. After 2DE, the gels were stained by a modified silver-staining method which was compatible with the following MS analysis. Spots of interest were manually excised from the 2D gels. Mass spectrometric analysis was carried out using a MALDI-TOF/TOF tandem mass spectrometer ABI 4700 proteomics analyzer (Applied Biosystems, USA). In order to perform protein identification, the MS (peptide-mass-fingerprint approach) and MS/MS (DeNovo sequencing approach) data were loaded into the GPS Explorer™ software v3.5 (Applied Biosystems) and searched against NCBInr 5825255 sequences (released on Jan 10, 2008) by Mascot search engine v1.9.05 (Matrix Science, UK).

Statistical analysis. Data were expressed as the mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA). The Newman-Keuls comparisons were used to determine the source of significant differences where appropriate. P-values of <0.05 were considered statistically significant.

Results

Body weight, visceral fat index, dietary intake and fecal lipid excretion. Following 6 weeks of exposure to low-fat diets, the NOP mice had a significantly increased body weight ($P<0.01$) and visceral adipose tissue ($P<0.01$) compared to the NOR controls (Fig. 1). Caloric intake plays an important role in determining the differences in body weight between individuals. After normalization of body weight, no significant

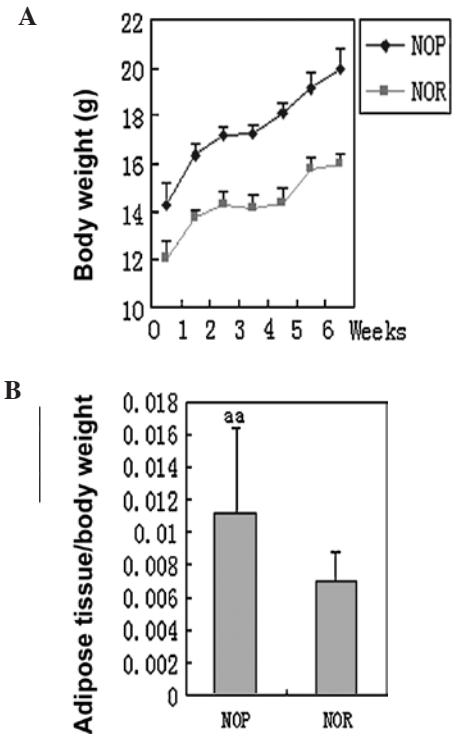

Figure 1. Changes in body weight (A) and visceral fat weight (B) in the obesity-prone and -resistant mice fed normal chow diets for 6 weeks. NOP, low-fat or normal-chow diet-fed obesity-prone mice; NOR, low-fat or normal-chow diet-fed obesity-resistant mice. Data were expressed as the mean ± SD (n=10). $^{aa}P<0.01$ vs. NOR.

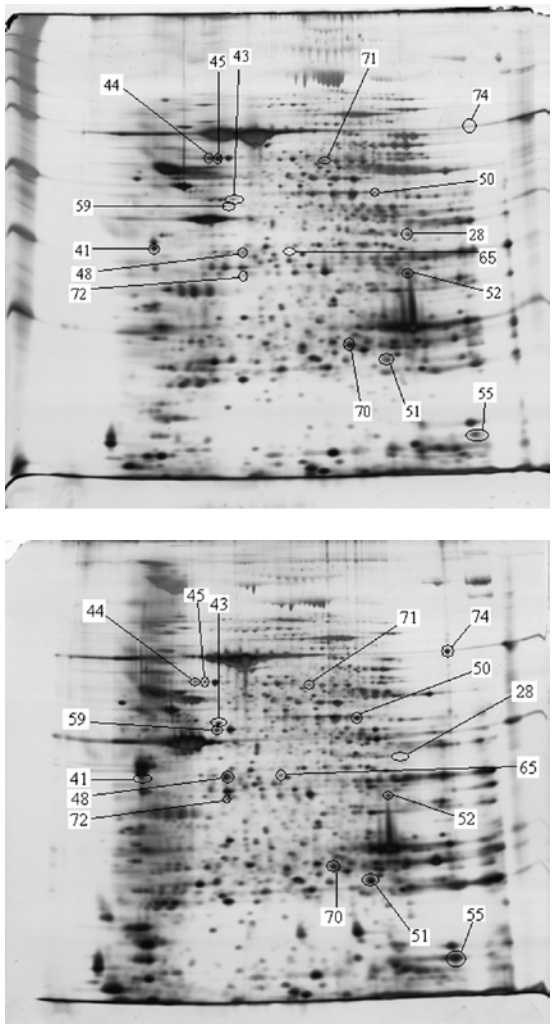

Figure 2. Images of 2DE in visceral adipose tissues in NOP (A) and NOR (B) mouse groups (n=3). Differentially expressed proteins are circled. NOP, low-fat or normal-chow diet-fed obesity-prone mice; NOR, low-fat or normal-chow diet-fed obesity-resistant mice.

difference was observed between the two groups (data not shown). Water intake, fecal and urine excretions showed similar changes to dietary caloric intake between the groups (data not shown). Total calories from fecal fat were crudely calculated (according to fecal weights and fecal triglycerides) and totalled <1% of the total dietary caloric intake (data not shown), which suggested that caloric differences of intestinal lipid absorption were negligible between the mouse groups.

Serum biochemical parameters. No significant changes in blood glucose or lipid levels were observed between the NOP and NOR mice (P>0.05) at any week (data not shown).

Differentially expressed proteins determined through 2-dimensional electrophoresis. Considering that adipose tissues serve as the main marker of obesity, we conducted 2DE in adipose tissues in order to find some direct clues to explain the differences between obesity-prone and -resistant animals. Proteins were regarded as differentially expressed when the magnitude of the difference was >2-fold and the result was reproduced twice. As a result, 16 proteins were found differentially expressed and well-identified in adipose tissues between the NOP and NOR mice (Table I). The NOP mice showed a significant change in the expression of proteins responsible for the cell skeleton, energy metabolism, oxidative stress and undefined functions compared to the NOR controls. Full images of 2DE are shown in Fig. 2. Protein spots of interest (those directly contributing to energy metabolism) are marked and enlarged in Fig. 3. Ubiquinol-cytochrome c reductase core protein 1 (Uqcrc1) expression (related to mitochondrial energy metabolism) and Enolase 3, β muscle were significantly decreased by 8.1- and 8.8-fold, respectively, in the adipose tissues of the NOP mice compared to the NOR controls, while monoglyceride lipase (MGLL) (related to fat synthesis) and glucose-6-phosphate dehydrogenase (G6PDH) X-linked were significantly decreased by 5.3- and 4.7-fold, respectively.

Discussion

High-fat diets play a key role in affecting body weight. However, some individuals are still rendered obese even when they ingest low-fat diets. The potential mechanisms remain unclear. In the present study, inbred C57BL/6 mice, also displayed a differential response to a low-fat diet as obesity-prone and -resistant phenotypes, although these animals are usually used to induce diet-induced obesity (14) by a high-fat diet. After normalization for body weight, no significant difference in caloric intake was observed between the obesity-prone and -resistant animals, suggesting that caloric intake may not be a key factor affecting visceral fat accumulation.

Table I. Differentially expressed proteins in visceral tissues between the obesity-prone and -resistant mice (NOP vs. NOR).

Spot no.	Protein name	Mr/PI	NCBI accession no.	Protein score	Sequence coverage (%)	Change (fold)
41	β-tropmyosin	32.9/4.6	GI 50190	145	35	NOP↓ 3.6x
48	Put. β-actin (aa 27-375)	39.2/5.8	GI 49868	87	34	NOP↓ 4.8x
55	Destrin	18.5/8.1	GI 9790219	70	41	NOP↓ 4.8x
43	Ubiquinol-cytochrome c reductase core protein 1	52.8/5.8	GI 46593021	122	33	NOP↓ 8.1x
50	Enolase 3, β muscle	47.0/6.7	GI 6679651	60	21	NOP↓ 8.8x
52	Monoglyceride lipase	33.3/6.7	GI 6754690	107	43	NOP↑ 5.3x
71	Glucose-6-phosphate dehydrogenase X-linked	59.2/6.1	GI 6996917	196	35	NOP↑ 4.7x
44	60 kDa heat shock protein, mitochondrial precursor (HSp60) (60 kDa chaperonin) (CPN60) (Heat shock)	60.9/5.9	GI 51702230	72	19	NOP↑ 11.4x
45	Heat shock protein 65	60.9/5.9	GI 51455	123	29	NOP↑ 10.0x
59	Creatine kinase, brain	42.7/5.4	GI 10946574	161	46	NOP (-)
65	Protein phosphatase 1, catalytic subunit, β isoform 1	37.2/5.84	GI 4506005	88	37	NOP (-)
51	Chain A, crystal structure of Mgsta4-4 in complex with Gsh conjugate of 4-hydroxynonenal in one Sub	25.4/7.0	GI 6137390	60	36	NOP↓ 4.7x
70	Peroxiredoxin 6	24.8/6.0	GI 6671549	147	61	NOP↑ 3.2x
72	G protein β 2 subunit	37.3/5.6	GI 984551	72	25	NOP (-)
28	Unnamed protein product	38.1/7.1	GI 12850542	61	26	NOP (+)
74	Unnamed protein product	65.0/5.5	GI 26341396	336	56	NOP (-)

↓Up-regulation; ↑down-regulation. NOP, low-fat or normal-chow diet-fed obesity-prone mice; NOR, low-fat or normal-chow diet-fed obesity-resistant mice.

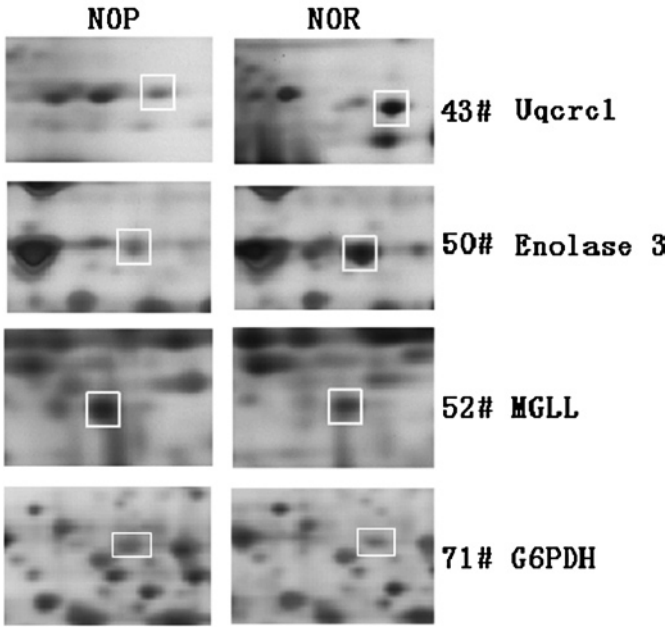

Figure 3. Images of the differentially expressed proteins determined by 2DE in visceral adipose tissues of the normal-chow diet-fed obese-prone and -resistant mice after 6 weeks of treatment. Spots of interest are marked in a rectangle and enlarged (n=3). NOP, low-fat or normal-chow diet-fed obesity-prone mice; NOR, low-fat or normal-chow diet-fed obesity-resistant mice.

Also, the difference in intestinal lipid absorption rate between the obesity-prone and obesity-resistant mice was slight and did not contribute to visceral fat accumulation.

Therefore, we suspected that obesity-prone animals may have an altered energy metabolism and/or lipid synthesis in visceral adipose tissues in this model, since these tissues are an important marker for obesity. However, there are a multitude of factors involved in fat metabolism and/or synthesis, and the selection of specific factors for our research objectives was difficult. The proteomics method is very useful for supplying much information on a disease when the mechanisms involved are unknown.

To our knowledge, this study represents the first use of a proteomics method to investigate protein expression differences in visceral adipose tissues between obesity-resistant and obesity-prone animals fed low-fat diets. Uqcrc1, a subunit of the respiratory chain protein ubiquinol cytochrome c reductase, is responsible for mitochondrial energy metabolism and is associated with the development of obesity (15). Through a proteomics study, a significant decrease in Uqcrc1 expression was found in the visceral adipose tissues of the NOP mice, which may be associated with attenuated mitochondrial energy metabolism. Enolase, also known as phosphopyruvate dehydratase, is a metalloenzyme responsible for the catalysis of the conversion of 2-phosphoglycerate to phosphoenolpyruvate, the ninth and penultimate step of glycolysis. Decreased expression of enolase indicates that glycolysis may be reduced in obesity-prone animals. MGLL functions together with hormone-sensitive lipase (HSL) to hydrolyze intracellular triglyceride stores in adipocytes and other cells to fatty acids and glycerol. MGLL may also complement lipoprotein lipase in completing hydrolysis of monoglycerides resulting from the degradation of lipoprotein triglycerides (16). Despite this, the obesity-prone mice showed a significant increase in visceral adipose tissues. Therefore, it may be speculated that i) MGLL may not play a key role in hydrolyzing triglycerides as HSL and adipose triglyceride lipase do; ii) MGLL may enhance the effects of lipoprotein lipase on fat storing in adipocytes. G6PDH is a cytosolic enzyme in the pentose phosphate pathway. Of greater quantitative importance is the production of NADPH for tissues actively engaged in biosynthesis of fatty acids and/or isoprenoids, such as the liver, mammary glands, adipose tissue and the adrenal glands. Therefore, the increased expression of G6PDH may promote lipid synthesis in visceral adipose tissues of obesity-prone animals.

Taken together, these results indicate that obesity-prone animals fed low-fat diets show differentially expressed proteins, e.g., Uqcrc1, Enolase 3, MGLL and G6PDH involved in energy metabolism, glycolysis and fat synthesis in visceral adipose tissues, which are useful for defining molecular markers and understanding the mechanisms of the development of obesity. Future studies should focus on how these proteins function in the visceral adipose tissues of obesity-prone individuals.

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