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Original Paper

Differential Protein Expression in White Adipose Tissue from Obesity-Prone and Obesity-Resistant Mice in Response to High Fat Diet and Anti-Obesity Herbal Medicines

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Key Words
Anti-obesity • Obesity susceptibility • Obesity resistance • Proteome • White adipose tissue

Abstract
Background: One of the most interesting issues in obesity research is why certain humans are obesity-prone (OP) while others are obesity-resistant (OR) upon exposure to a high-calorie diet. However, the pathways responsible for these phenotypic differences are still largely unknown. ***Methods:*** In order to discover marker molecules determining susceptibility and/or resistance to obesity in response to high fat diet (HFD) or anti-obesity herbal medicine (TH), we conducted comparative proteomic analysis of white adipose tissue (WAT) from OP, OR, as well as TH-treated mice. ***Results:*** OP mice fed HFD gained approximately 33% more body weight than OR mice, and TH significantly reduced body weight gain in HFD-fed mice by 30%. These mice were further subjected to proteomic analysis using two-dimensional electrophoresis (2-DE) combined with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS). Proteomic data revealed 59 spots that were differentially regulated from a total of 1,045 matched spots, and 57 spots of these were identified as altered WAT proteins between OP and OR mice by peptide mass finger printing. Interestingly, 45 proteins were similarly regulated in OR mice in response to TH treatment. Of these, 10 proteins have already been recognized in the context of obesity; however, other proteins involved in obesity susceptibility or resistance were identified for the first time in the present study. ***Conclusion:*** Our results suggest that TH actively contributed to body weight reduction in HFD-fed obese mice by altering protein regulation in WAT, and it was also found that TH-responsive proteins can be used as potent molecules for obesity treatment.

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Introduction

Obesity is a multifactorial disorder that is influenced by both genetic and environmental factors, leading to physical and external complications as well as progression of various diseases such as cardiovascular risks, hypertension, dyslipidemia, endothelial dysfunction, and type 2 diabetes mellitus [1-3].

Susceptibility to weight gain can vary considerably among individuals due to external influences such as excessive energy intake and low physical activity [4-6]. However, there exist large inter-individual differences in obesity development despite exposure to similar conditions. For example, some may readily gain body weight and become obese (obesity-prone, OP), whereas others may not (obesity-resistant, OR) [7]. However, the pathways responsible for these phenotypic differences are still largely unknown [8].

Nowadays, prevention and management of obesity are major public health challenges and no longer considered to be only cosmetic problems. Although weight loss and weight control drugs are common, their medical effects are far from satisfactory since many pharmaceuticals have unwanted side effects [9]. Due to concerns over currently available Western medicine treatments, some have turned to their interest to alternative medicines, including traditional Oriental medicine, for therapeutic treatment of obesity [10-12].

Recent research has demonstrated that natural products may be an excellent alternative strategy for developing safe anti-obesity drugs. Medicinal plants and their extracts have been verified as beneficial and are the oldest and most widespread form of medication to prevent diet-induced obesity [12-14]. Multiple natural products containing numerous bioactive compounds may result in synergistic activity that increases their bioavailability and action on multiple molecular targets. The anti-obesity effects of these compounds are mediated by regulation of various metabolic processes, including feeding-inhibitory actions, elevation of lipolysis, reduction of lipogenesis, and differentiation of preadipocytes [15-17]. Taeumjowi-tang (designated as "TH") is a traditional Korean medicine that is widely used to treat several diseases [18, 19]. This product consists of multiple compounds and has become the standard treatment regimen for obesity by Korean medical professionals, and its use has been expanded to all types of obesity [20, 21]. In previous studies, TH and TH modified prescription have been evaluated for its inhibitory effects on obesity, suggesting that its modified prescription can clinically be useful as anti-obesity drugs and can be useful for the improvement of hyperlipidemia [22, 23]. Previous clinical results have supported the anti-obesity and hypolipidemic effects of TH, but the results of clinical studies are still insufficient [18].

To discover marker molecules that could be helpful in elucidating the mechanisms underlying obesity susceptibility, various animal tissues have been studied and their whole proteomes were analyzed in order to characterize protein functions and post-translational modifications [24, 25]. Separation, identification, and characterization of proteins as well as their interactions with other proteins are the essential goals of proteomic analysis. Two-dimensional electrophoresis (2-DE) coupled with MALDI-TOF-MS is considered a powerful tool for the separation of thousands of adipose tissue proteins [26, 27]. This approach enables comparison between normal and disease samples revealing differentially expressed proteins.

Obesity-related factors in adipose tissue proteins play major roles in the development of metabolic disorders [26, 28]. Adipose tissue acts as an active endocrine organ, which secretes adipokines contributing to the regulation of physiological processes such as energy homeostasis, reproduction, and inflammation. Several studies based on gene profiling have focused on WAT reprogramming after weight loss [29, 30]. Although DNA array is a powerful tool for this purpose, the predictive value of mRNA expression is limited with respect to cellular physiology. Expression levels of mRNA often do not parallel protein expression levels of a particular gene [31, 32]. Further, protein turnover and post-translational modifications, which are essential for cellular behavior, are not covered by the information obtained from DNA data [33]. Consequently, a broader understanding of the effects of diet on WAT requires

independent examination of protein expression and function in conjunction with mRNA expression analyses.

In the present study, we addressed the anti-obesity effects of natural traditional herbal medicines based on their abilities to alter expression of WAT proteins in HFD-induced obese mice. To the best of our knowledge, this is the first proteomics study profiling WAT protein modulation by traditional herbal medicines in an obese animal model.

Materials and Methods

Animals and breeding conditions

Four-week-C57BL/6J male mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All mice were provided water and standard chow *ad libitum* for 1 week for acclimatization before the start of any experimental procedures. The mice were then randomly divided into two groups, viz. normal diet group (ND, *n*=10) and HFD-fed group (60% fat, *n*=30), and bred for 12 weeks. HFD-fed mice were further subdivided into three groups; OP mice showed the highest body weight gain (OP group, *n*=10) while OR mice showed the lowest body weight gain (OR group, *n*=10). Among 20 HFD-fed mice, 10 mice were randomly selected and treated with TH (TH group, *n*=10). Taeumjowi-tang (TH) was purchased from I-world Pharm. Co. Ltd. (Incheon, Korea). TH is a decoction consisting of eight herbal ingredients, and the ingredients of TH are *Semen Coicis* (11.25g), *Semen Castaneae* (11.25g), *Semen Raphani* (7.5g), *Schisandrae Fructus* (3.75g), *Liriopis Tuber* (3.75g), *Herba Ephedrae* (3.75g), *Radix Platycodi* (3.75g), and *Acori Tatarinowii Rhizoma* (3.75g). Dietary compositions used in this study are presented in Table 1. Mice had free access to food and distilled water during the experimental period. Food intakes and body weights were measured daily and weekly, respectively. On the 12th week, mice were anaesthetized with diethyl ether and sacrificed after 12 h of fasting. Blood was taken from the inferior vena cava and then centrifuged at 1,000×*g* for 15 min at 4°C, after which plasma was isolated to characterize plasma biochemistry. After blood collection, epididymal adipose tissues were promptly removed, rinsed, weighed, frozen in liquid nitrogen, and stored at -80°C. This animal study was approved by the Ethics Committee for Animal Studies at Kyungpook National University, Republic of Korea.

Preparation of protein samples

WAT were excised from mice immediately following anesthetization with diethyl ether after overnight fasting. The resulting tissues were then washed with cold saline solution and pulverized under liquid nitrogen and stored at -80°C. Tissues were lysed in 200 mL of rehydration buffer solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT, 1 mM PMSF, 2% IPG buffer (Ampholyte 3/10, Bio-Rad), and a trace of bromophenol blue. Lysed tissues were then homogenized by a homogenizer (PT 1200E, Kinematica, Luzern, Switzerland) on ice, after which extracts from homogenized WAT tissues were centrifuged at 13,000×*g* for 20 min. The supernatant was then stored at -80°C until analysis. Protein content of whole WAT tissue was determined using RC DC™ protein assay (Bio-Rad).

Table 1. Dietary compositions of normal diet (ND), high-fat diet (HFD), and high-fat diet with Taeumjowi-tang (TH)

	ND	HFD	TH
Composition by weight, g/kg			
Casein	200	265	265
Corn starch	397.486	0	0
Sucrose	100	90	60
Maltodextrin	132	160	160
Cellulose	50	65.6	65.6
Soybean oil	70	30	30
Lard	0	310	310
Mineral mix	35	48	48
Calcium phosphate, dibasic	0	3.4	3.4
Vitamin mix	10	21	21
TBHQ antioxidant	0.014	0	0
L-cystine	3	4	4
Choline bitartrate	2.5	3	3
Taeumjowi-tang			30
Total (kcal/kg)	3,800	5,100	4,980

Two-dimensional electrophoresis (2-DE)

2-DE was performed in duplicate using WAT protein samples from six mice per group, which consisted of ND, OP, OR, and TH mice. 2-DE experiments were conducted using the previous methods outlined by our laboratory [26, 27, 34]. Briefly, immobilized pH gradient (IPG)-isoelectric focusing (IEF) of WAT samples was performed on pH 3-10 and 18 cm IPG DryStrips (GE Healthcare, Buckinghamshire, UK) in a PROTEIN IEF cell (Bio-Rad) using the protocol recommended by the manufacturer. IPG strips were rehydrated passively for 12 h in strip holders with 350 μL of rehydration solution, which included 7 M urea (Bio Basic, Ontario, Canada), 2 M thiourea (Sigma, St. Louis, MO, USA), 4% CHAPS (Bio Basic), 1 mM PMSF (Sigma), 20 mM DTT (GE Healthcare), 2% IPG buffer (Bio-Rad), and 150 μg of WAT proteins. IEF was executed as follows: 15 min at 250 V, 3 h at 250-10,000 V, 6 h at 10,000 V, and then held at 500 V until ready to run in the second dimension. After focusing, the gel strips were equilibrated in a solution containing 6 M urea, 2% SDS (Generay Biotech, Shanghai, China), 1% DTT, 30% glycerol (Bio Basic), and 50 mM Tris-HCl (pH 6.8) for 15 min, followed by further incubation in the same solution, except for replacement of DTT with 2.5% iodoacetamide (Bio-Rad) for an additional 15 min period. Gel strips were then placed onto a 20 × 20 cm 12% polyacrylamide gel for resolution in the second dimension. Fractionation was performed using the Laemmli SDS discontinuous system at a constant voltage of 15 mA per gel for 14 h. For image analysis and peptide mass fingerprinting (PMF), a total of 48 gels, including two gels per group with separated proteins, were visualized by silver staining. Silver staining was performed as follows: gels were fixed over a period of 2 h in 50% ethanol and 5% acetic acid, followed by 10 min in 30% ethanol and washing in water for 5 min three times. Gels were sensitized for 10-15 min in 0.02% sodium thiosulfate (Sigma), followed by washing in water for 0.5 min three times and incubation for 1 h in 0.3% silver nitrate (Kojima Chemicals, Sayama, Japan). After washing in water two times for 1 min, proteins were visualized with developing solution containing 3% sodium carbonate, 0.02% sodium thiosulfate, and 0.05% formalin and then stopped using 6% acetic acid.

Image acquisition and data analysis

Gels were imaged on a UMAX PowerLook 1120 (Maxium Technologies, Akron, OH, USA), and modified ImageMaster 2-D software V4.95 (GE Healthcare) was used for comparison of images. A reference gel was selected from gels of the normal group, and detected spots from other gels were matched with those in the reference gel. Relative optical density and relative volume were calculated in order to correct differences in gel staining. Each spot intensity volume was processed by background subtraction and total spot volume normalization, and the resulting spot volume percentage was used for comparison.

Protein identification

For protein identification by PMF, protein spots were excised, digested with trypsin (Promega, Madison, WI, USA), mixed with CHCA in 50% ACN/0.1% TFA, and subjected to MALDI-TOF analysis (Microflex LRF 20, Bruker Daltonics). Spectra were collected from 300 shots per spectrum over an m/z range of 600-3000 and calibrated by two-point internal calibration using trypsin auto-digestion peaks (m/z 842.5099, 2211.1046). Peak list was generated using Flex Analysis (ver 3.0). The threshold used for selecting peaks was as follows: 500 for minimum resolution of monoisotopic mass, 5.0 for S/N. Peptide masses were matched with the theoretical peptides of all proteins in the NCBI database using MASCOT developed by Matrixscience (http://www.matrixscience.com). The following parameters were used for the database search: trypsin as the cleaving enzyme, a maximum of one missed cleavage, iodoacetamide (Cys) as a complete modification, oxidation (Met) as a partial modification, monoisotopic masses, and a mass tolerance of ± 0.1 Da. Protein score is -10 * Log (p), where p is the probability that the observed match is a random event, and greater than 61 is significant ($p<0.05$).

Immunoblot analysis

Tissue lysates were prepared with RIPA buffer (Sigma), homogenized, and centrifuged at 12,000×g for 20 min. The extract was diluted in sample buffer (50 mM Tris of pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% β-mercaptoethanol) and heated for 5 min in a boiling bath. Samples were then subjected to SDS-polyacrylamide gel electrophoresis and transferred to Poly-Screen membranes (NEN, Boston, MA, USA). Membranes were subsequently blocked with 5% nonfat dry milk in TBS (10 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 0.1% Tween-20 (TBS-T). After washing with TBS-T, membranes were

Table 2. List of RT-PCR primers. [a] F, sequence from sense strands; [b] R, sequence from anti-sense strands

Genes		Primer sequence (5' - 3')
Anxa1	F[a]	ACCAACCCCATTGCTTCCAT
	R[b]	AAGCCAGCCTCAATGGTCTC
Fabp4	F	GACCAGCAGGAGCTTTCCTC
	R	TGGCACACTTCACGATGGTT
Idh1	F	ATCGACTTGGGGACCACCTA
	R	CAATCAGACGCTCCCCTTCA
Internal control		
Gusb	F	AGAACAACAGCCTTCCACCTT
	R	GGCTGTGGAGTAAGTCCTGT

incubated for 2 h with a 1:1000 dilution of primary polyclonal mice antibody (anti-CEBPα, anti-CEBPβ, anti-PPARγ, anti-LPL, anti-FABP4, and anti-β-actin; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by HRP-conjugated anti-rabbit IgG, anti-goat IgG, and anti-mouse IgG secondary antibody (1:1000; Santa Cruz Biotechnology) for 1 h and development using enhanced chemiluminescence (iNtRON Biotechnology, Seoul, Korea). Western blot was analyzed by scanning with UMAX PowerLook 1120 (Maxium Technologies) and digitalization using image analysis software (KODAK 1D, Eastman Kodak, Rochester, NY, USA).

Network analysis

Associations of differentially expressed genes with broadly defined molecular networks were carried out using IPA (Ingenuity Pathway Analysis, http://www.ingenuity.com) tools. Molecular networks were predicted based on the direct and indirect relationships between differentially expressed molecules and members of the networks in the IPA database. When using IPA, we analyzed up-regulated and down-regulated proteins for each group as separate groups. Only values that were significant at $p<0.01$ showing FDR correction and at least a 2-fold change in protein levels were included in the analysis.

Real-time RT-PCR

Transcription levels of genes were quantitatively determined by real-time RT-PCR (Stratagene Mx 3000p QPCR System, Santa Clara, CA, USA). Total RNA was isolated from WAT tissues using an easy-spin TM (DNA-free) Total RNA Extraction kit, and reverse-transcription was carried out using a Maxime RT Premix kit (iNtRON Biotechnology). We employed FastStart Universal SYBR Green Master (Roche Diagnostics, Indianapolis, IN, USA) for real-time RT-PCR. Transcription levels of each gene (*Anxa1*, *Fabp4*, and *Idh1*) were normalized to *Gusb* transcription levels. Oligonucleotide sequences are shown in Table 2.

Statistical analysis

All experimental results were compared by One-way Analysis of Variance (ANOVA) using the Statistical Package of Social Science (SPSS, version 14.0K) program; data are expressed as the mean±SEM. A protected least-significant difference (LSD) test, which is a method for multiple comparison consisting of single-step procedures in One-way ANOVA, was used to demonstrate significant differences between means ($p<0.05$).

Results

Effects of TH treatment in HFD-fed obese mice

Changes in body weight among individuals during the experimental period are shown in Fig. 1A. Body weights of mice were the same among all groups at the beginning of this study. However, after inducing obesity with HFD for 4 weeks, OP mice were heavier ($p<0.01$) than normal controls and OR mice at all subsequent time points (data not shown). Accordingly, body weight gain in OP mice was approximately 33% higher than that in OR mice (Fig. 1A). No significant difference was observed in energy intake (total food intake) per total body weight between OP, OR, and TH mice (Fig. 1B). Moreover, total WAT weight in OP mice was higher ($p<0.01$) than those in normal, OR, and TH mice (Fig. 1C). After TH treatment for 12 weeks, total WAT per body weight was significantly reduced by up to 15% ($p<0.01$) compared to the

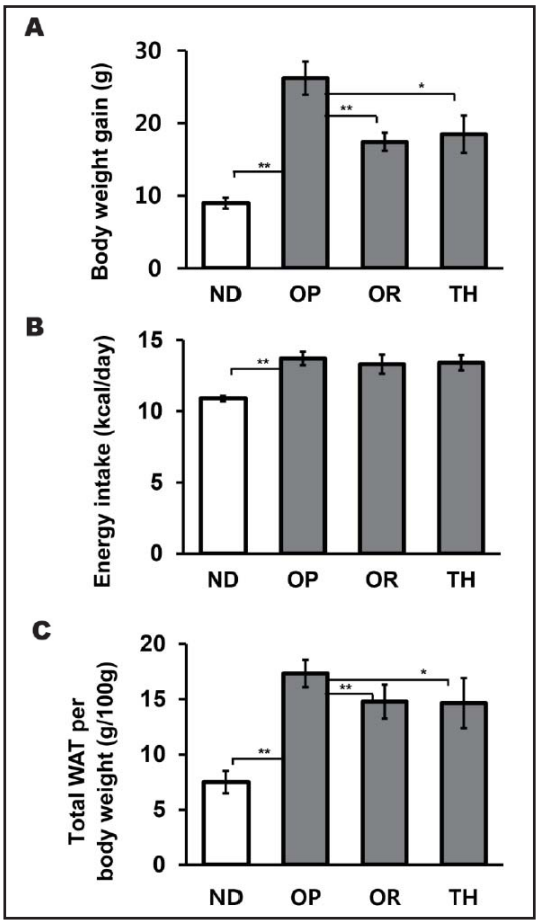

Fig. 1. (A) Average body weight gain, (B) energy intake, (C) average weight of WAT per body weight in mice fed normal diet (ND), HFD-fed obesity-prone mice (OP), HFD-fed obesity-resistant mice (OR), and HFD-fed obese mice treated with the herbal medicine Taeumjowi-tang (TH). Statistical significance was determined by One-way ANOVA test, where *p* values are **p*<0.05 and ***p*<0.01 for significance. Error bars represent means ± S.E.M (*n*=6).

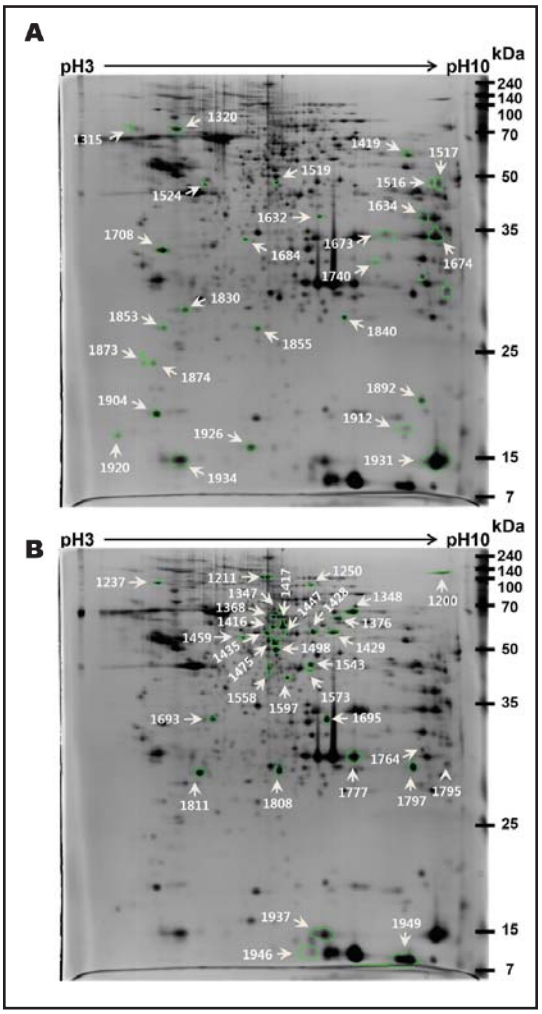

Fig. 2. Representative silver-stained 2-DE gel images of mice WAT proteome. (A) Up-regulated and (B) down-regulated proteins in OP mice compared to ND-fed mice. Differentially regulated proteins and proteins of interest are marked with circles and arrows. The numbers in gels are listed in Table 3.

OP group. Collectively, these results suggest that TH inhibited fat accumulation in HFD-fed obese mice, prompting us to perform further proteomic studies.

Proteomic analysis of WAT

For identification of differentially expressed proteins among the four groups (ND, OP, OR, and TH group), WAT protein samples were separated by 2-DE (Fig. 2), protein spots were identified by MALDI-TOF-MS (Table 3), and database searches were performed with high confidence based on high score and sequence coverage. For the results, a total of 1,045 individual matched spots ranging in mass from 6 to 240 kDa between pH 3-10 were detected, and obtained WAT protein maps represented patterns that were similar to our previous results [26-28, 34]. Fifty-nine spots were identified with statistically different intensities between the ND and OP groups. These differentially altered spots dramatically changed in response to TH, suggesting that TH played a pivotal role in obesity prevention in HFD-fed obese mice.

Table 3. List of identified proteins showing differential expression (2≥fold change) in white adipose tissue (WAT) between mice fed normal diet or high fat diet (OP, OR, and TH groups). [a] NCBInr/SWISS database accession number. [b] The nominal mass is the integer mass of the most abundant naturally occurring stable isotope of an element. [c] MASCOT probability-based molecular-weight search score calculated for PMF. Protein score is – 10 x log(*P*), where *P* is the probability that the observed match is a random event; it is based on the NCBInr database using the MASCOT searching program as MS/MS data and protein scores >61 are significant ($P<0.05$). [d] ND means not detected. Spot Id is same numbers in gel image of Fig. 2

Spot ID	Description	Acc. No.[a]	Nominal mass (M_r)[b]	Calculated PI	Score [c]
1200	Alanyl-tRNA editing protein Aarsd1	AASD1_MOUSE	45228	6.00	47
1211	Pyruvate carboxylase, mitochondrial isoform 2	gi|251823978	129618	6.25	154
1237	Heat shock protein 90, beta (Grp94), member 1	gi|14714615	92432	4.74	97
1250	Elongation factor 2	gi|33859482	95253	6.41	65
1315	PHD and RING finger domain-containing protein 1	PHRF1_MOUSE	185622	8.87	30
1320	78 kDa glucose-regulated protein precursor	gi|254540166	72378	5.01	207
1347	PREDICTED: WD repeat-containing protein 1 isoform X1	gi|568934035	58889	6.52	98
1348	Transketolase	gi|11066098	60545	6.54	73
1368	EH domain-containing protein 2	gi|55742711	61136	6.08	142
1376	Me1 protein	gi|13096987	63799	6.87	133
1416	Bicaudal D-related protein 2	BICR2_MOUSE	57607	4.93	39
1417	Triacylglycerol hydrolase	gi|14269427	61848	6.30	156
1419	PREDICTED: zinc finger protein 787 isoform X1	gi|568946454	49505	9.15	69
1428	Aldehyde dehydrogenase family 6, subfamily A1	gi|23271115	57924	8.25	102
1429	Katanin p60 ATPase-containing subunit A-like 1	KATL1_MOUSE	55472	6.67	52
1435	ND [d]	ND	ND	ND	ND
1447	Endonuclease 8-like 2	NEIL2_MOUSE	37210	8.76	36
1459	mRNA turnover protein 4 homolog	MRT4_MOUSE	27642	8.63	34
1475	Aldehyde dehydrogenase, mitochondrial precursor	gi|6753036	56502	7.53	103
1498	Enolase 1B, retrotransposed	gi|70794816	47111	6.37	89
1516	Serine (or cysteine) peptidase inhibitor, clade H, member 1, isoform CRA_a	gi|148684430	44954	9.01	87
1517	Serine (or cysteine) peptidase inhibitor, clade H, member 1, isoform CRA_a	gi|148684430	44954	9.01	159
1519	Exosome complex component RRP40	EXOS3_MOUSE	30041	8.35	55
1524	PACRG-like protein	PACRL_MOUSE	27615	9.92	58
1543	NADP-dependent isocitrate dehydrogenase	gi|3641400	46630	6.48	79
1558	Dual specificity phosphatase 28	DUS28_MOUSE	17779	7.66	48
1573	Cell division control protein 6 homolog	CDC6_MOUSE	63658	9.43	50
1597	Conserved oligomeric Golgi complex subunit 4	COG4_MOUSE	89574	5.06	40
1632	Aldo-keto reductase family 1, member A4	gi|148698648	30208	6.35	110
1634	Aldolase A	gi|7548322	39526	8.55	137
1673	Centrosomal protein of 85 kDa	CEP85_MOUSE	86085	5.86	46
1674	Glyceraldehyde-3-phosphate dehydrogenase	gi|55153885	35751	7.59	126
1684	DNA-directed RNA polymerases I and III subunit RPAC1	RPAC1_MOUSE	39310	5.09	44
1693	Transcription elongation factor A protein 2	TCEA2_MOUSE	34325	9.32	32
1695	Monoglyceride lipase isoform a	gi|261878516	35234	7.21	103
1708	Annexin A5	gi|6753060	35730	4.83	186
1740	Annexin A1	gi|124517663	38710	6.97	68
1764	Parp3 protein	gi|15928823	60003	6.53	38
1777	mRNA turnover protein 4 homolog	MRT4_MOUSE	27642	8.63	46
1795	GTP:AMP phosphotransferase AK3, mitochondrial	gi|23956104	25467	8.87	68
1797	Glutathione S-transferase Mu 1	gi|6754084	25953	7.71	103
1808	Unnamed protein product	gi|74191773	38749	6.22	115
1811	PREDICTED: serine/threonine-protein kinase 25 isoform X2	gi|568909449	29514	5.51	73
1830	Major vault protein	MVP_MOUSE	96150	5.43	38
1840	Galectin-7	gi|31543120	15250	6.37	42
1853	Sdccag1 protein	gi|54887337	47900	6.21	53
1855	Calcium-binding mitochondrial carrier protein SCaMC-3	SCMC3_MOUSE	52692	7.19	46
1873	Cytochrome b5 type B precursor	gi|31542438	16308	4.79	70
1874	Cytochrome b5 type B precursor	gi|31542438	16308	4.79	103
1892	Cofilin-1	gi|6680924	18548	8.22	96
1904	Cytochrome b5	gi|13385268	15232	4.96	143
1912	Peroxisomal membrane protein 20	gi|6746357	17004	7.71	175
1920	Adenylate kinase 8	gi|152963553	55437	6.62	46
1926	Alpha-(1,3)-fucosyltransferase 11	FUT11_MOUSE	56068	5.81	38
1931	Fatty acid-binding protein, adipocyte	gi|14149635	14641	8.53	139
1934	ND	ND	ND	ND	ND
1937	FYVE, RhoGEF and PH domain-containing protein 6	FGD6_MOUSE	156954	8.09	45
1946	Hemoglobin, beta adult t chain	gi|31982300	15738	7.14	85
1949	Hemoglobin alpha, adult chain 2	gi|145301549	15103	7.96	71

Differential expression of obesity-associated proteins in WAT

Prior to comparison of differentially altered proteins between OP and OR mice, we first compared WAT protein levels among ND, OP, and OR mice. Most of the identified proteins showed significantly altered protein expression between OP and ND-fed mice in response

Fig. 3. Differentially regulated proteins in WAT from OP mice compared with ND/OR/TH mice. (A) Up-regulated and (B) down-regulated proteins in OP mice. Each bar shows an average volume density (%) in 2-DE analysis. Statistical significances between ND and OP were determined by One-way ANOVA test, where $p<0.05$.

A

Spot ID	Identified proteins	Expression levels ND OP OR TH
1315	PHD and RING finger domain-containing protein 1	
1320	78 kDa glucose-regulated protein precursor	
1419	PREDICTED: zinc finger protein 787 isoform X1	
1516	Serine (or cysteine) peptidase inhibitor, clade H, member 1, isoform CRA_a	
1517	Serine (or cysteine) peptidase inhibitor, clade H, member 1, isoform CRA_a	
1519	Exosome complex component RRP40	
1524	PACRG-like protein	
1632	Aldo-keto reductase family 1, member A4	
1634	Aldolase A	
1673	Centrosomal protein of 85 kDa	
1674	Glyceraldehyde-3-phosphate dehydrogenase	
1684	DNA-directed RNA polymerases I and III subunit RPAC1	
1708	Annexin A5	
1740	Annexin A1	
1830	Major vault protein	
1840	Galectin-7	
1853	Sdccag1 protein	
1855	Calcium-binding mitochondrial carrier protein SCaMC-3	
1873	Cytochrome b5 type B precursor	
1874	Cytochrome b5 type B precursor	
1892	Cofilin-1	
1904	Cytochrome b5	
1912	Peroxisomal membrane protein 20	
1920	Adenylate kinase 8	
1926	Alpha-(1,3)-fucosyltransferase 11	
1931	Fatty acid-binding protein, adipocyte	

B

Spot ID	Identified proteins	Expression levels ND OP OR TH
1200	Alanyl-tRNA editing protein Aarsd1	
1211	Pyruvate carboxylase, mitochondrial isoform 2	
1237	Heat shock protein 90, beta (Grp94), member 1	
1250	Elongation factor 2	
1347	PREDICTED: WD repeat-containing protein 1 isoform X1	
1348	Transketolase	
1368	EH domain-containing protein 2	
1376	Me1 protein	
1416	Bicaudal D-related protein 2	
1417	Triacylglycerol hydrolase	
1428	Aldehyde dehydrogenase family 6, subfamily A1	
1429	Katanin p60 ATPase-containing subunit A-like 1	
1447	Endonuclease 8-like 2	
1459	mRNA turnover protein 4 homolog	
1475	Aldehyde dehydrogenase, mitochondrial precursor	
1498	Enolase 1B, retrotransposed	
1543	NADP-dependent isocitrate dehydrogenase	
1558	Dual specificity phosphatase 28	
1573	Cell division control protein 6 homolog	
1597	Conserved oligomeric Golgi complex subunit 4	
1693	Transcription elongation factor A protein 2	
1695	Monoglyceride lipase isoform a	
1764	Parp3 protein	
1777	mRNA turnover protein 4 homolog	
1795	GTP:AMP phosphotransferase AK3, mitochondrial	
1797	Glutathione S-transferase Mu 1	
1808	Unnamed protein product	
1811	PREDICTED: serine/threonine-protein kinase 25 isoform X2	
1937	FYVE, RhoGEF and PH domain-containing protein 6	
1946	Hemoglobin, beta adult t chain	
1949	Hemoglobin alpha, adult chain 2	

to TH treatment, whereas 45 proteins showed similar levels in OR mice (Fig. 3). A total of 26 proteins were up-regulated in OP mice while also maintained at low levels in ND and OR mice. Furthermore, analysis of WAT samples identified 31 proteins that were down-regulated in HFD-fed OP mice but up-regulated in normal and OR mice. Until now, most of these proteins have not been shown as being differentially expressed in WAT in response to HFD. Therefore, these proteins can be considered as potential marker proteins for determination of phenotypic differences in WAT between OP and OR mice.

We also investigated differential expression of WAT proteins between OP and TH mice based on the above results for ND and OP mice. A total of 10 proteins showed significantly altered protein expression upon TH treatment between OP and TH mice. Among these, five proteins were up-regulated while five proteins were down-regulated in OP mice. Interestingly, all of these proteins showed opposite expression patterns between OP as well as OR and TH mice (Fig. 4), thereby confirming a role for TH in metabolic balance in adipose tissue.

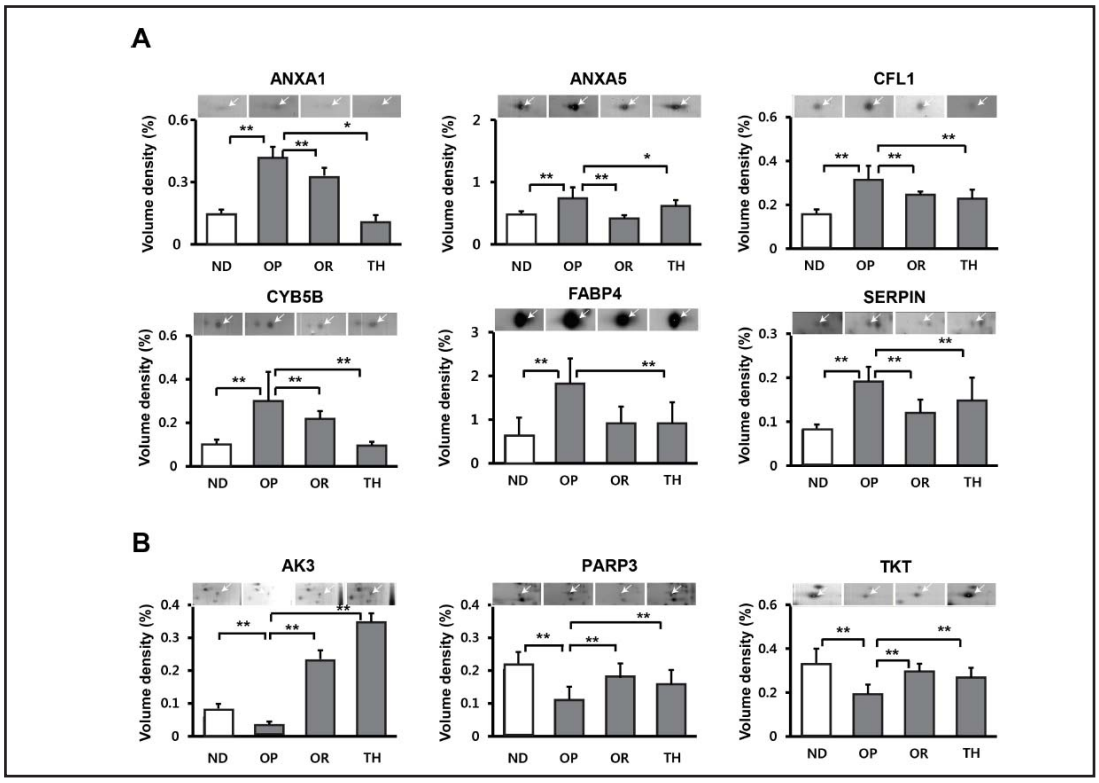

Fig. 4. Comparison of expression patterns of obesity-related proteins between OP and ND/OR/TH mice. (A) Up-regulated and (B) down-regulated proteins in OP mice. For full name of each protein, see Abbreviations. Band intensity was calculated by ImageMaster 2D software version 4.95, and relative intensity (%) refers to the values of target proteins normalized to those of β-actin. Statistical significance was determined by One-way ANOVA test, where *p* values are *$p<0.05$ and **$p<0.01$.

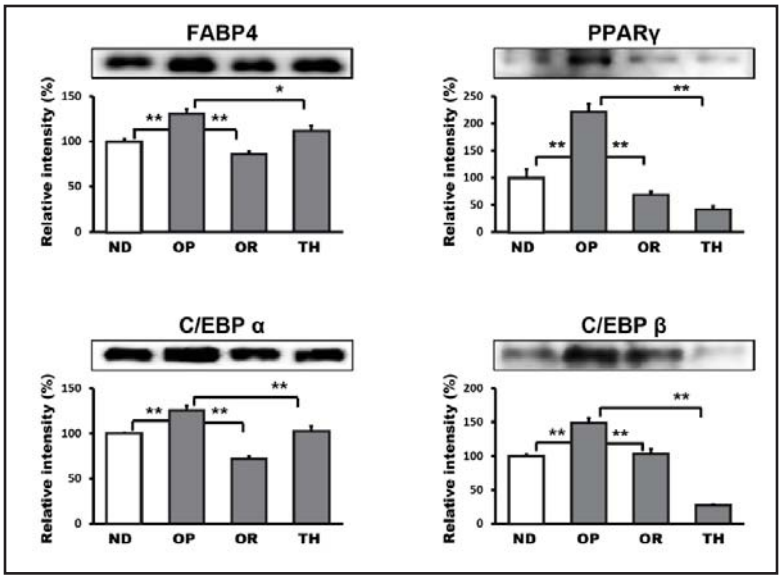

Fig. 5. Differentially regulated adipogenic factors in WAT as determined by immunoblot analysis. For full name of each protein, see Abbreviations. Data are representative of three independent experiments. Statistical significance was determined by One-way ANOVA test, where *p* values were *$p<0.05$ and **$p<0.01$.

Distinct expression of adipogenic factors between OP and OR mice

We also examined differential expression patterns of adipogenic factors that could not be detected by 2-DE analysis in WAT from each group of mice. It is well known that C/EBPα, C/EBPβ, and PPARγ are master regulators of adipogenesis that induce expression of lipid metabolic proteins such as FABP4 and LPL. Immunoblot analysis was performed on five

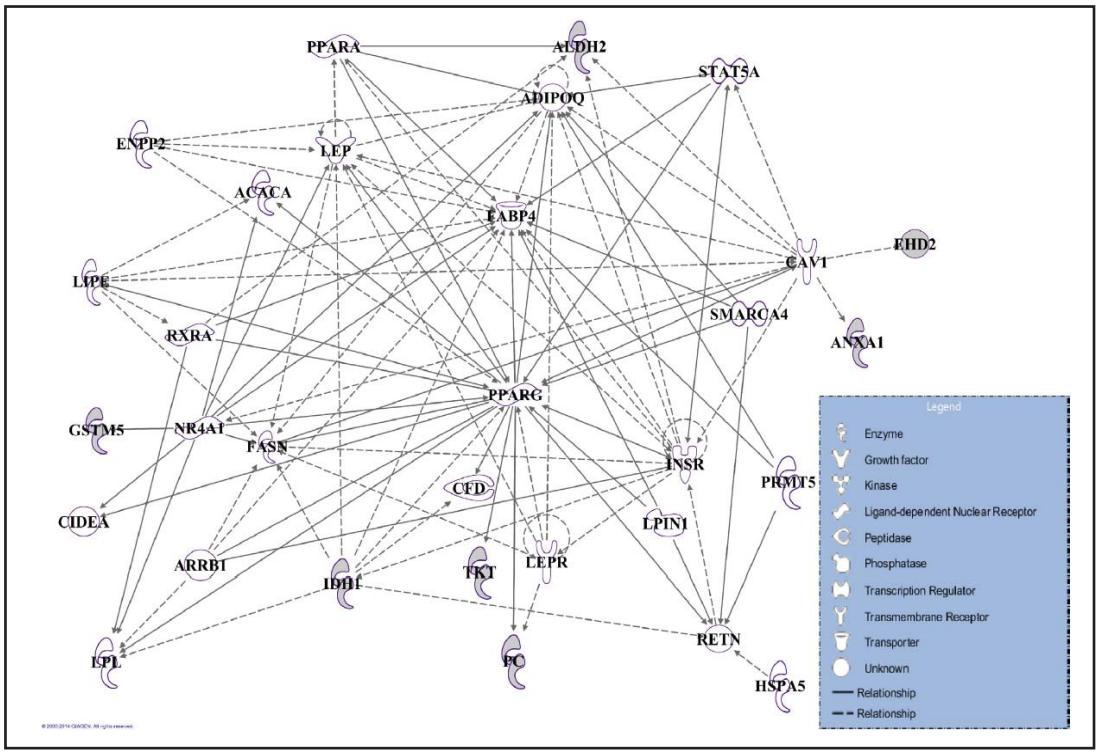

Fig. 6. Networks generated by Ingenuity Pathway Analysis using up- or down-regulated proteins in response to HFD and TH treatments. Network was constructed using human homologues of identified mouse proteins, where connections represent direct (solid lines) or indirect interactions (dashed lines).

WAT proteins of interest, including FABP4, PPARγ, C/EBPα, C/EBPβ, and LPL. Four proteins (FABP4, PPARγ, C/EBPα, and C/EBPβ) were markedly up-regulated in OP mice compared to OR and ND mice, whereas LPL levels were not altered upon HFD feeding (data not shown) or TH treatment (Fig. 5). Most proteins were also down-regulated in response to TH treatment in HFD-induced obese mice.

Network analysis of proteomic data

To further investigate the biological roles of molecules identified in this study, we performed network analysis using Ingenuity Pathway Analysis (IPA) based on the proteomic data. The representative network in WAT is shown in Fig. 6, and these genes were found to be associated with lipid metabolism, molecular transport, and small molecule biochemistry when ranked by *p*-value significance. IPA network analysis predicted possible interactions between *Fabp4*, *Pparγ*, *Anxa1*, and *Idh1*. Unfortunately, PPARγ could not be detected using 2-DE, although it was activated by TH treatment in the network analysis.

Validation of results of IPA network analysis using real-time RT-PCR

To evaluate the results of the IPA network analysis, the predicted target genes (*Anxa1*, *Fabp4*, and *Idh1*) were further confirmed by determining their mRNA levels using real-time RT-PCR (Fig. 7). HFD treatment remarkably activated these predicted targets. Moreover, expression levels of these genes were markedly reduced by TH treatment.

Discussion

In the present study, we performed a comparative analysis of WAT protein expression profiles in HFD-induced obese mice treated with anti-obesity herbal medicine by 2-DE

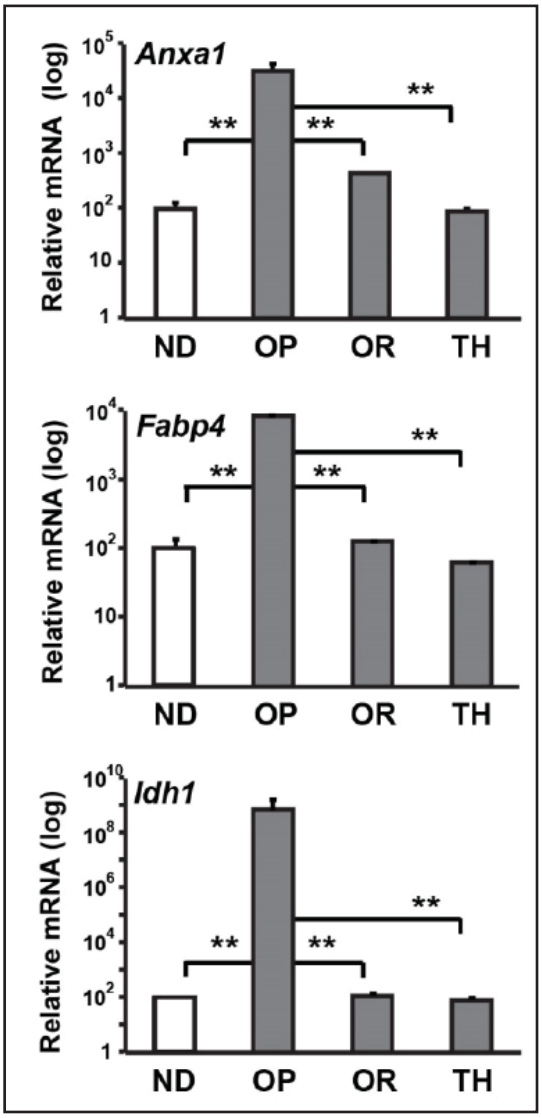

Fig. 7. Validation of proteomic data and predicted targets generated by IPA analysis using real-time RT-PCR. mRNA expression patterns of three targets from WAT of experimental groups by real-time RT-PCR analysis. Real-time PCR results are expressed as relative intensity normalized to *Gusb* gene expression and represent the mean ± S.E.M of triplicate independent experiments. Statistical significance was determined by One-way ANOVA test, where $^{**}p<0.01$.

combined with MALDI-TOF-MS. Our results firmly establish that TH, a traditional herbal medicine, significantly reduced body weight gain and significantly altered regulation of WAT proteins. From the current proteomic study, we identified a total of 57 proteins showing differential expression among the experimental groups, and some were previously unrecognized in the context of obesity. Among differentially regulated proteins between OP as well as OR and TH mice, a total of 12 proteins are discussed below due to space limitations.

Importantly, there were higher levels of Annexin A1 (ANXA1) and Annexin A5 (ANXA5) in OP mice, which may be associated with elevated lipid synthesis in WAT. ANXA1 is a glucocorticoid-regulated protein that has been implicated in cell signaling and proliferation, and several isoforms of ANXA1 in 2-DE have been identified in adipose tissue of rodents [35]. Significant reduction of ANXA1 protein levels in both OR and TH mice may be due to decreased lipid formation. Similarly, ANXA5 was also up-regulated in OP mice but markedly down-regulated in OR and TH mice. ANXA5 is known to exhibit high affinity for anionic phospholipids in lipid membranes and protects the lipid membrane barrier against damage caused by inflammation [36]. Moreover, there is an association between inflammatory molecule levels and visceral obesity [37, 38]. A recent study demonstrated that SNPs of ANXA5 are associated with susceptibility to being overweight or obese in Koreans [39]. Taken together, both ANXA1 and ANXA5 may play important roles in lipid metabolism and may be associated with obesity development in HFD-fed mice.

Cofilin-1 (CFL1) showed increased expression in WAT from obese mice along with reduced expression in OR and TH groups. CFL1 is a well known actin cytoskeleton-regulating protein [40]. There are limited reports concerning differential expression of CFL1 in the context of obesity. Recently, it was demonstrated that protein levels of CFL1 are increased during differentiation of 3T3-L1 preadipocytes due to PKA activity [41]. Although the physiological role of CFL1 in WAT has not yet been demonstrated, CFL1 overexpression is known to inhibit brown fat deposition as well as repress expression of browning marker genes such as UCP1, PRDM16, and PGC-1α [42]. Therefore, altered protein expression of

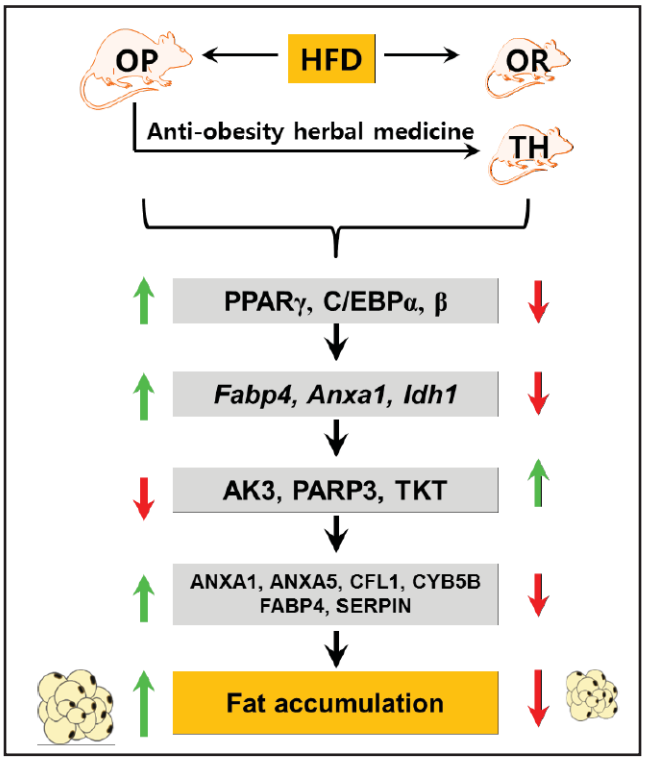

Fig. 8. Summary on differentially regulated proteins/genes determining phenotypic differences between obesity susceptibility (OP) and resistance (OR) in mice fed HFD. Effects of anti-obesity herbal medicine are also described. (↑) Up-regulation, (↓) down-regulation. For abbreviated protein names, see Abbreviations.

CFL1 in OR and TH mice is likely associated with possible browning characteristics of WAT, thereby affecting obesity resistance [43].

Elevated expression levels of cytochrome b5 type B (CYB5B) in obese mice also drew our attention. There are contradictory results concerning expression patterns of genes involved in mitochondrial oxidative metabolism in WAT from obese subjects. For instance, Marrades et al. [44] showed that orchestrated down-regulation of genes involved in oxidative metabolic pathways, including the cytochrome family, contributes to weight gain susceptibility in humans. In contrast, a recent study reported a 3.83-fold increase in cytochrome P450 expression in obese mice [45]. Our proteomic data also indicate increased protein expression of CYB5B in HFD-fed obese mice as well as reduced expression in OR and TH-treated mice. Taken together, other mechanisms seem to be responsible for these differences and thus further studies are required to clarify this issue.

As another interesting result, fatty acid binding protein 4 (FABP4) was highly expressed in OP mice. FABP4 is one of the most abundant proteins in mature adipocytes and is also detected at high concentrations in human serum. Although the biological role of FABP4 is not fully understood, it has been linked to insulin sensitivity, lipid metabolism, and inflammation [46]. Previously, we have shown that FABP4 levels are elevated in obese and diabetic individuals, and such increased levels are closely correlated with adverse lipid profiles, insulin resistance, and vascular smooth muscle cell proliferation [47-50]. In this study, protein and mRNA expression of FABP4 were reduced in OR and TH mice, which suggests alleviation of fat accumulation in adipocytes. In support of this, data from previous studies have shown that FABPs are involved in systemic regulation of lipid and glucose metabolism, and blood levels of FABP4 are positively associated with body weight and fat mass [51, 52].

One of the most important findings in the current proteomic study was significant up-regulation of serine/cysteine peptidase inhibitor (SERPIN) in obese mice, whereas its expression was reduced in both OR and TH-treated mice. Data from our previous study showed that SERPIN expression is significantly higher in males than in females in both ND and HFD rats [26]. Other studies have also demonstrated that SERPINs could play an important role in obesity development [53, 54]. For example, SERPIN family vaspin is highly

expressed in WAT from obese rats at the peak of obesity, and elevated blood vaspin levels are associated with obesity and insulin sensitivity [53]. However, long-term physical training combined with weight loss has been shown to increase blood vaspin levels, suggesting there is no clear link between vaspin and fat accumulation [53]. As OR mice showed decreased SERPIN levels in WAT, this protein may be associated with obesity resistance.

As another important finding, GTP:AMP phosphotransferase AK3 (AK3) and PARP3 showed lower expression levels in HFD-induced obese mice. One of the main functions of AK3 is to maintain homeostasis of cellular nucleotides by catalyzing the interconversion of nucleoside phosphates, and it is a central player in cellular energy metabolism [55]. PARP3 is a member of the PARP family catalyzing ADP ribosylation, which is a key post-translational modification step for proteins involved in various signaling pathways such as DNA damage and energy metabolism [56]. However, to date, no evidence has linked AK3 or PARP3 to lipid metabolism and obesity. Thus, higher expression of AK3 and PARP3 in response to TH treatment may regulate obesity by maintaining homeostasis of cellular nucleotides and energy metabolism. This is the first report demonstrating possible roles for AK3 and PARP3 in HFD-induced obesity.

Similarly, reduced protein expression of transketolase (TKT) was also observed in obese mice. Indeed, TKT is a ubiquitous protein used in multiple metabolic pathways, and disruption of a single TKT allele can slow growth of adipose tissue [57]. A previous study demonstrated that TKT-null mice display preferential reduction of adipose tissue, suggesting that obesity may be treated by inhibition of TKT in adipose tissue [57].

In this study, we also determined expression levels of key players during adipocyte differentiation such as peroxisome proliferator-activated receptor-gamma (PPARγ) and CCAAT/enhancer binding proteins (C/EBPs) [58], which were not detected by our proteomic analysis. In addition to adipose tissue development, members of the C/EBP and PPAR family are involved in regulation of lipid metabolism [59-61]. C/EBPβ is the first transcription factor induced following exposure of preadipocytes to differentiation, and is thus postulated to be involved in directing the differentiation process. In accordance with this opinion, expression of C/EBPβ in preadipocytes accelerates the rate of C/EBPα induction and adipogenesis in response to hormonal inducers, indicating its stimulatory factor in adipogenesis [62]. Interestingly, protein levels of PPARγ and C/EBPs were reduced in OR and TH groups, suggesting that TH played a crucial role in alleviating adiposity in HFD-fed obese mice. Similarly, numerous natural compounds such as catethins, capsaicin, and berberine have been shown to act as inhibitors of adipogenesis by reducing expression of adipogenic factors [63-65].

IPA network analysis predicted interactions between *Fabp4*, *Pparγ*, *Anxa1*, and *Idh1*. One important network was identified involving *Fabp4*. However, the mechanisms by which FABP4 promote insulin resistance and inflammation are not fully understood. *Pparγ* has been reported to be a potent modulator of adipogenesis, acting as a transcription factor of *Fabp4* expressed in mature adipocytes [66]. A recent study found that FABP4 is up-regulated while PPARγ is down-regulated in human visceral fat and mouse epididymal fat compared to subcutaneous fat [49]. Furthermore, suppression of PPARγ by FABP4 in visceral fat may explain the reported role of FABP4 in the development of obesity-related morbidities such as insulin resistance and diabetes.

In addition to our proteomic data, we observed elevated expression of *Idh1* encoding isocitrate dehydrogenase (IDH) in HFD-induced obese mice. Elevated transcription and protein expression of *Idh1* can increase cellular levels of NADPH, which causes lipid deposition in adipocytes as well as obesity and fatty liver [67]. Reduction of cellular NADPH levels resulting from suppression of *Idh1* expression in OR and TH mice was associated with less lipid deposition in adipocytes. Therefore, by taking advantage of the suppressive or inhibitory effects of IDH inhibitors, pharmaceutically effective materials for the treatment of obesity, hyperlipidemia, and fatty liver can be developed [68].

In conclusion, the current study demonstrated that TH, a traditional herbal medicine, shows promising anti-obesity effects through alteration of WAT protein expression in HFD-

fed obese mice. Further, several of the identified proteins are recognized for the first time in the context of obesity. Multi-components from TH might regulate numerous genes/proteins involved in adipogenesis and lipid metabolism, which was further confirmed by IPA network analysis. This study is the first to link TH, a natural anti-obesity medicine, to its regulatory actions (Fig. 8). However, more research should be carried out to further unravel the cellular pathways behind the multi-components of TH.

Abbreviations

2-DE (two-dimensional electrophoresis); AK3 (GTP:AMP phosphotransferase AK3 mitochondrial); ANXA1/*Anxa1* (annexin A1); ANXA5 (annexin A5); C/EBP (CCAAT/enhancer binding protein); CFL1 (cofilin 1); CYB5b (cytochrome b5); FABP4/*Fabp4* (fatty acid binding protein 4); Gusb (glucuronidase beta); HFD (high fat diet); IDH1/*Idh1* (isocitrate dehydrogenase 1); LPL (lipoprotein lipase); OP (obesity-prone); OR (obesity-resistant); PARP3 (poly (ADP-ribose) polymerase 3); PPARγ (peroxisome proliferator–activated receptor γ); SERPIN (serine/cysteine peptidase inhibitor); TKT (transketolase).

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Disclosure Statement

The authors have declared no conflict of interest.

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