Analysis of Main Proteins Associated with Lipid Droplets from Peri-Adrenal Adipose Tissue of Patients with Cushing's Syndrome

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Abstract: The Cushing's syndrome results from chronic exposure to excess glucocorticoids produced by the adrenal cortex. The most common feature of patients with Cushing's syndrome is central obesity associated with dyslipidaemia and insulin resistance leading to type 2 diabetes and cardiovascular diseases. In the adipocytes, triacylglycerol and cholesterol esters are stored within an intracellular lipid droplet covered by a monolayer of phospholipids and surrounded by proteins involved in the regulation of lipolysis. Since the protein composition of adipocyte lipid droplets from patients with Cushing's syndrome has not yet been reported, we used two-dimensional gel electrophoresis followed by mass spectrometry to identify the main lipid droplet-associated proteins from peri-adrenal adipose cell of patients with this syndrome.

The lipid droplet proteome of peri-adrenal adipose cell of patients with Cushing's syndrome is highly complex and contains more than 500 proteins. MALDI-TOF MS analysis of silver-stained protein spots revealed the identity of 27 proteins clustered into 7 groups according to their known function. Interestingly, proteins involved in response to oxidative stress and endoplasmic reticulum stress were found on the lipid droplets of adipose cell from patients with Cushing's syndrome.

Keywords: Adipocyte, Cushing's syndrome, endoplasmic reticulum, lipid droplet, two-dimensional gel electrophoresis.

1. INTRODUCTION

The Cushing's syndrome (CS) results from chronic exposure to excess glucocorticoids produced by the adrenal cortex [1]. It may be caused by excessive ACTH production from the pituitary gland, ectopic ACTH secretion by a non-pituitary tumor, or more rarely, by a tumor secreting CRH (ectopic CRH secretion) [2, 3]. CS can also be ACTH-independent when it results from excess secretion of glucocorticoids by adrenocortical tumors or adrenal hyperplasia [4]. The most common feature of patients with CS is progressive central obesity with omental fat accumulation [5, 6] usually involving the abdomen, face, neck and trunk [7]. The central obesity is associated with glucose intolerance, hyperglycemia, dyslipidaemia and insulin resistance leading to type 2 diabetes and cardiovascular diseases [7-9].

White adipose tissue has traditionally been considered as an energy storage organ. Different hormones, in particular catecholamines, insulin and glucocorticoids, mediate the storage and use of energy [7, 10, 11]. Catecholamines, *via* beta-adrenergic receptors, mediate a cascade of reactions including elevation of cAMP levels, stimulation of protein kinase A and activation of hormone sensitive lipase (HSL), which hydrolyzes triglycerides to glycerol and FFA. Insulin inhibits lipolysis by stimulating phosphodiesterase 3B activity, leading to decreased activation of protein kinase A and HSL and thus inhibition of hydrolysis of triglycerides. Glucocorticoids are also involved in the regulation of energy storage. Glucocorticoids are known to induce *in vitro* differentiation of human adipocytes [12, 13]. Moreover, elevated glucocorticoid levels in humans are associated with redistribution of fat from peripheral to central depots [7]. The specific increase in abdominal fat stores is a consequence not only of elevated glucocorticoids but also of their interaction with insulin [14, 15]. Indeed, liver insulin resistance due to anti-insulin effect of glucocorticoids is associated with increased insulin concentrations [7], which may also favor adipocyte differentiation. Therefore, the interaction between glucocorticoids and insulin may contribute to intra-abdominal fat accumulation and the development of the metabolic syndrome, i.e. lipodystrophy, hypertension, hyperglycemia, dyslipidaemia.

In adipocytes, triacylglycerol (TG), as well as excess of cholesterol and cholesterol esters are stored within intracellular lipid droplets covered by a monolayer of phospholipids,

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free cholesterol and proteins. In times of energy need, such as fasting and exercise, catecholamines activate cAMP-dependent protein kinase (PKA), leading to activation of HSL, which hydrolyzes TG at the surface of intracellular lipid droplets into glycerol and FFA [16]. A number of proteins specifically located at the surface of the lipid droplet have been involved in the regulation of lipolysis [17, 18]. The first lipid droplet-associated proteins to be identified were the PAT proteins including perilipins, adipophilin, TIP47 and S3-12 [17, 19-22]. Interestingly, perilipin knock-out mice have markedly reduced adipose tissue mass and high levels of basal lipolysis [23]. In contrast, ectopic expression of perilipin in 3T3-L1 cells increased the half-life of stored TG [24]. These findings indicate that lipid droplet-associated proteins may play a crucial role in the control of the storage of TG and appropriate hydrolysis in times of energy demand.

The mechanisms of fat accumulation in patients with CS remain unclear. A few studies, using techniques of proteomic analysis have shown that lipid droplets from several cells include proteins involved in lipid metabolism [25, 26]. Protein composition of adipocyte lipid droplets from patients with CS had not been reported previously. Taking advantage of surgical ablation of the tumor in patients with CS, we prepared adipocytes from the tissue surrounding the tumor and used a 2D gel electrophoresis followed by mass spectrometry approaches to identify major lipid droplet-associated proteins from adipose tissue of these patients.

2. MATERIALS AND METHODOLOGY

2.1. Patients

Clinical data and hormonal status were evaluated as previously described [27]. Briefly, for CS diagnosis, increases in 24-h urinary cortisol excretion, alteration of plasma cortisol circadian rhythm and lack of cortisol suppression under a dexamethasone test (2 mg/day for 2 days) were observed in all patients with CS. After ablation of the tumors, peri-adrenal fat was immediately dissected in sterile condition by the pathologist and processed for the proteomic analysis as described below. Four patients were included in the study. Informed consent was obtained from the patients and tissue collection approved by an institutional review board (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale, Cochin Hospital, Paris, France).

2.2. Isolation of Mature Adipocytes

Immediately after surgery, 6 – 16 g of peri-adrenal adipose tissue was collected and processed to produce isolated mature adipocytes, according to Rodbell [28] as modified by Honnor *et al.* [29]. All processing of adipocyte preparation was performed in Krebs-Ringer solution buffered with 30 mM Hepes at pH 7.4 and containing 200 nM adenosine (KRH medium). The tissues were washed 4 times in KRH medium to remove any remaining blood. Digestion was performed during 1 hour at 37°C in KRH medium containing 1 mg/ml collagenase (386 U/mg, Sigma) and 20 mg/ml BSA fraction V (Sigma). The ratio of adipose tissue mass to incubation solution was approximately 1/4 (w/v). After dispersion, the cells were filtered through a nylon mesh (pore size, 250 µm). Collagenase digestion of adipose tissue results in the formation of two distinct fractions: the floating mature fat cells and the sedimented stromal–vascular cells. The latter was removed by aspiration, and the fat cells were washed by resuspending them in warm KRH medium (37°C). This procedure was repeated three times.

2.3. Preparation of the Adipocyte Lipid Droplets

1 ml of isolated fat cells was lysed in 4 ml of 5 mM Tris-HCl buffer (pH 7.4) by osmotic shock. The suspension was mixed by slowly inverting the centrifuge tube three times and then centrifuged at 200 x g for 3 min at room temperature. The fat layer was mixed with 4 ml of 5 mM Tris-HCl buffer (pH 7.4), containing 0.025% Triton X-100, by slowly swinging the tube three times. The mixture was centrifuged at 200 x g for 3 min at room temperature. The fat layer was washed once with 25 mM TES, pH 7.4, containing 135 mM NaCl, 5mM KCl, 1mM $MgCl_2$, incubated with the same buffer at 37°C for 10 min, and centrifuged at 200 x g for 3 min [30].

2.4. Delipidation of Lipid Droplets and Preparation of Component Proteins

To each ml of fat cake, 5 ml of an ice-cold extraction solution containing chloroform/methanol (2/2.4) were added. The suspension was mixed vigorously for 30 sec every 5 min during 1 hour, at 4°C. The suspension was then centrifuged at 4°C for 20 min at 20000 x g. After elimination of the supernatant, a second volume of extraction medium, equivalent to the first, was added to the protein-containing pellet. The pellet was suspended by vigorous mixing at room temperature. This mixture was re-centrifuged, and the resulting pellet was washed with ice-cold acetone. The supernatant was removed and the protein-containing pellet was stored at -80°C prior to analysis.

2.5. Immunoblotting of Lipid Droplet Protein Preparations

To determine whether perilipin, a well-known lipid droplet associated protein, was indeed recovered in our lipid droplet preparations from peri-adrenal adipose cells, immunoblotting experiments were performed using antiserum raised against amino terminus of perilipin (guinea pig polyclonal antiserum, Progen Immuno-Diagnostika, Progen Biotechnik, Heidelberg, GERMANY).

2.6. Analysis of Proteins by 2-D PAGE

Stored samples from chloroform/methanol extraction were resuspended in 420 µl of rehydration solution (6 M urea, 2 M thiourea, 4% CHAPS, 15 mM DTT, 0.5% IPG buffer pH 3-10 and traces of bromophenol blue) and centrifuged at 10 000 g for 30 min at 22°C. Proteins were quantified using the Amersham assay method (Amersham Biosciences, Uppsala, SWEDEN). 300 µg of proteins diluted in 410 µl of rehydration solution were applied on non-linear pH 3-10 IPG 24 cm strips (Biorad Laboratories, Hercules, CA, USA) using the in-gel rehydration method. The strips were focused on the Multiphor II IEF (isoelectric focusing) system (Amersham, Biosciences) at 15°C during 2 days. The run was stopped when 100 kVh were reached.

After focusing, the strips were first equilibrated for 15 min in 15 ml SDS equilibration buffer (50mM Tris-HCl, pH 8.8, 6 M Urea, 2% SDS, 30% glycerol, and 0.002% bromophenol blue) containing 100 mM DTT then followed by equilibration buffer containing 250 mM iodoacetamide for 15 min. The strips were layered on top of a vertical 12% SDS-PAGE for the second dimension separation using Duracryl acrylamide (Proteomic Solutions, Saint-Marcel, FRANCE). Migration in the second dimension was performed using an EttanDalt 6 system (Amersham Biosciences). The separation was performed using 2.5W par gel for 25 min followed by 9W per gel at 4°C during 5 hours. After SDS-PAGE, gels were fixed overnight and silver stained during 5 min using the protocol described previously [31]. Spots were chosen according to two main criteria: (1) presence of the spot in most patients and (2) sufficient silver intensity of the spot to allow identification of the protein.

2.7. In-gel Trypsin Digestion

Spots of interest were excised from the gel, washed with 200 mM ammonium bicarbonate (ABC) and immediately destained according to Gharahdaghi *et al.* [32]. In-gel trypsin digestion was carried out as described in a protocol based on ZipPlate (Millipore Billerica, MA, USA) with minor variations. After destaining, spots were rinsed three times with water and shrunk with 50 mM ABC/50% acetonitrile (ACN) for 20 min at ambient temperature. Gel pieces were then dried using 100% ACN for 15 min. Protein spots were then incubated in 50 mM ABC containing 10 mM DTT for 1 hour at 56°C. The solution was then replaced by 55 mM iodoacetamide in 50 mM ABC and incubated 30 min in the dark at ambient temperature. The protein spots were washed twice with 50 mM ABC and finally shrunk with 25 mM ABC/50% ACN for 30 min and dried using 100% ACN for 10 min. Spots were rehydrated with 20 µl of 12.5 µg/ml Sequencing Grade Modified Trypsin (Promega, Madison, WI, USA) in 40 mM ABC/10% ACN pH 8.0. Proteins were digested overnight at 37°C. After digestion, the spots were shrunk with 100% ACN and peptides were extracted with 0.2% TFA. Peptides were then desalted using C_{18} phase on ZipPlate. Two elutions were performed to recover products from C18 phase first with 50% ACN/0.1% TFA and then using 90% ACN/0.1% TFA. Pooled eluates were concentrated using a vacuum centrifuge (Eppendorf) and generated peptides were re-suspended in 3 µl of 1% formic acid before mass spectrometry analysis.

2.8. MALDI-TOF Mass Spectrometry Analysis

Digested samples were spotted directly onto a 96x2 MALDI Plate (Applied Biosystems, Foster City, CA, USA) using the dried-droplet method (0.5 µl of the sample are mixed on a plate with an equal volume of a solution containing 5 mg/ml of alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix in ACN/water/trifluoroacetic acid (50/50/0.1%)). Droplets were allowed to dry at ambient temperature. Mass spectrometry analysis were performed on a matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF, Voyager DE-PRO mass spectrometer, Applied Biosystems) and acquired in positive ion using reflector mode. Spectra were obtained in a reflectron mode with in source delayed extraction over a mass range of 500-3500 Dalton. Monoisotopic masses were calculated after internal calibration using auto-digestion tryptic peptides or using external calibration using a mixture containing five external standard peptides (PepMix 1, LaserBio Labs, Sophia-Antipolis, FRANCE). Peptide were searched against a comprehensive non-redundant protein sequence database (NCBInr). Four different algorithms were used for protein identification: Profound (version 2005, Proteometrics), Mascot (version 2.1, Matrix Sciences), MS-Fit (version 3.2.1, ProteinProspector) and AlDente (version 2006, Expasy). A combination of results from different algorithms offers the option to cross-validate and consolidate the identification through the complementary use of several packages.

Allowed variable modifications were oxidation of methionine, acrylamide modified cysteine and carbamidomethylation of cysteine. Up to one missed tryptic cleavage was considered and a mass accuracy in the range of 25 to 50 ppm was used for all tryptic mass searches. Identified proteins are listed in Table **1**.

3. RESULTS AND DISCUSSION

Studies have revealed that perilipins, adipophilin, TIP47 and S3-12 are the major structural proteins of lipid droplets [19, 22, 25]. Although adipophilin (ADRP) and TIP47 are ubiquitously expressed [20], perilipins and S3-12 are selectively expressed in adipocytes [17, 19]. Immunoblotting of our protein preparations with an antibody that recognizes perilipin was used to confirm the presence of this protein in the lipid droplets from peri-adrenal adipose cell of patients with CS. Representative results obtained with preparations from the patients are shown (Fig. **1**). Perilipin was detected as a band at 63 kDa (Fig. **1**). In addition the alternative RNA splicing isoform of perilipin was also detected as a band of 47 kDa (Fig. **1**).

Lipid Droplet-Associated Proteins

Lipid droplet-associated proteins were separated by two-dimensional gel electrophoresis. Fig. (**2**) shows that two-dimensional gel electrophoresis patterns obtained with preparations from 4 different patients are very similar, indicating that the lipid droplet preparation procedure is highly reproducible.

MALDI-TOF MS analysis of silver-stained protein spots revealed the identity of 27 proteins representing 4.2-6% of all detected spots (548-388 respectively). Numbers of the protein spots on the gel images correspond with the proteins listed in Table **1**. The identified proteins were clustered into 6 groups according to their known function:

Known Lipid-Droplet Associated Proteins

The lipid droplet-associated protein, perilipin (spot 1) was identified, consistent with the previous localization of these proteins to lipid droplets in cultured 3T3-L1 adipocytes [19, 25] and with western-blotting results shown in Fig. (**1**). Perilipins represent the most abundant proteins at the surface of adipocyte lipid droplets [18, 33]. Perilipins are located at the periphery of lipid storage droplets and are known to regulate lipolysis and energy balance. Indeed, in the basal state, perilipin coats the lipid droplet and prevents HSL access to the droplet. Lipolytic activation of adipocytes is

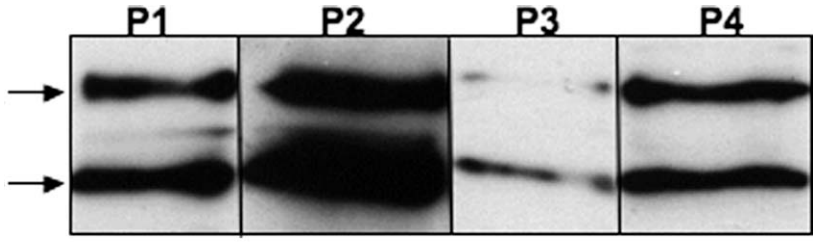

Fig. (1). Western-Blotting of lipid droplet fractions from 4 patients (P1-P4) with Cushing's syndrome. Perilipin antibody recognizes perilipin and its alternative RNA splicing isoform (arrows) yielding polypeptides of 63 and 47 kDa.

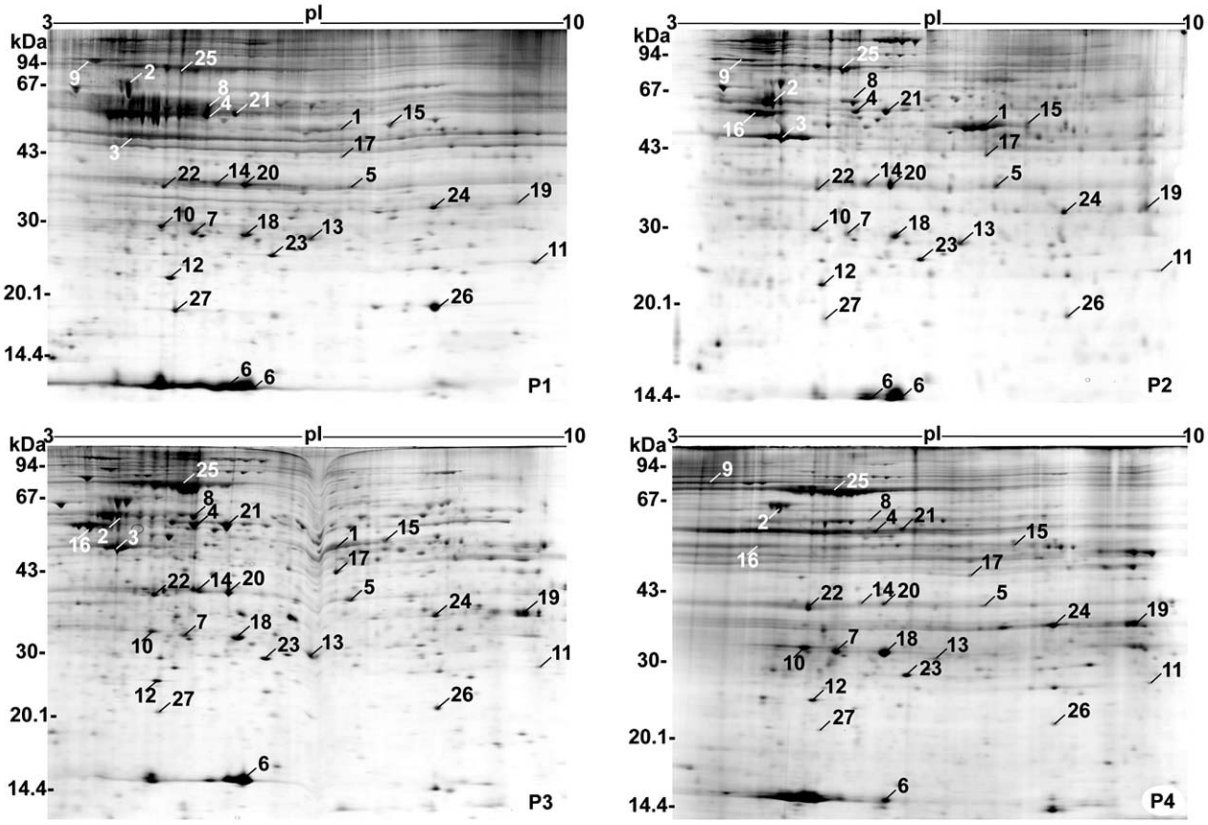

Fig. (2). Silver nitrate 2-D gel of lipid droplet associated protein patterns from peri-adrenal adipose cells of 4 patients (**P1**-**P4**) with Cushing's syndrome. Protein extracts were subjected to IEF on a non linear IPG strip 3-10 followed by a second dimensional run performed on an homogenous 12% SDS-PAGE. The labeled and numbered spots indicate the identified proteins as listed in Table **1**.

associated with polyphosphorylation of perilipin by protein kinase A, which induces changes in the lipid droplet surface allowing HSL access to triacylglycerol stored in the lipid droplet. Perilipin is expressed in human adipose tissue and obese subjects have increased levels of perilipin expression [34]. This emphasizes the importance of perilipins in lipid homeostasis and energy metabolism.

Vimentin (spot 2) is a member of the intermediate filament family of proteins, which correspond to an important component of cell cytoskeleton. During the differentiation of 3T3-L1 pre-adipocytes, vimentin intermediate filaments are reorganized to form cage-like structures around the nascent lipid droplets [35]. Perturbation of the vimentin filament network in 3T3-L1 cells significantly decreased the lipid-droplet forming capacity of these cells during adipose conversion. Indeed, expression of a dominant negative mutant of vimentin in 3T3-L1 cells was associated with an increased rate of triglyceride turnover, resulting impaired capacity of these cells to accumulate lipid droplets [35].

Cytoskeleton Proteins

Two other cytoskeleton proteins (beta-actin, spot 3) and (beta-tubulin, spot 4) were identified in lipid droplets of peri-adrenal adipose cells of CS patients. Two-dimensional gel electrophoresis followed by immunoblot analysis previously indicated that globular beta-actin was associated with intracellular lipid droplets from rat adrenocortical cell and adipocytes [36]. This association of actin with surface of lipid droplets suggested that beta-actin might be involved in the regulation of intracellular lipid metabolism, particularly in the transport of lipid constituents. In contrast, to the best of our knowledge, beta-tubulin had not yet been described associated with lipid droplets.

Calcium Binding Proteins

Annexin 1 (spot 5), a member of the calcium and phospholipid-binding proteins, was also identified in each lipid droplet preparation. Annexin 1 (lipocortin 1) is a steroid-

Table 1. Main Proteins Associated with Lipid Droplets from Peri-Adrenal Adipose Tissue of 4 Patients with Cushing's Syndrome Identified by MALDI-TOF Mass Spectrometry. (Spots Indicated in Fig. (2))

Protein Groups	Spot no.	Protein Identity	Accesion no.	Coverage %	Peptide Number[$]	Experimental Mr (kDa)	Theoretical Mr (kDa)	Experimental pI	Theoretical *pI*	Profound Score*
Known lipid droplet-associated proteins	1	perilipin	4505885	30%	11/65	50	44	7.5	5.5	1 / 2.21
	2	vimentin	2119204	26%	11/52	63	61	5.5	6.0	1 / 2.32
Cytoskeleton proteins	3	beta actin	4501885	52%	14/78	45	41	5.5	6.0	1 / 2.4
	4	beta tubulin	27368062	29%	8/66	51	49	5.9	5.0	1 / 1.34
Calcium binding proteins	5	annexin 1	4502101	32%	8/71	35	39	7.5	6.5	1 / 1.26
Stress/Chaperon proteins	8	glucose regulated protein grp58	21361657	40%	15/60	52	57	5.5	6.0	1 / 2.41
	9	glucose regulated protein BiP75	16507237	20%	9/69	75	72	4.5	5.5	1 / 2.24
	10	prohibitin	4505773	58%	11/77	23	30	5.5	5.5	1 / 2.39
Redox proteins	11	peroxiredoxin 1	4505591	48%	8/58	22	22	9.5	8.0	1 / 2.43
	12	peroxiredoxin 2	16117118	57%	12/54	21	22	5.5	5.7	1 / 2.1
	13	peroxiredoxin 6	4758638	52%	12/64	26	25	7.5	6.0	1 / 2.41
	14	thioredoxin-like	5730104	30%	11/61	38	38	5.5	5.0	0.97 / 0.9
Proteins involved in energy metabolism	15	alpha 1 enolase	45003571	24%	8/59	47	47	7.5	7.0	1 / 1.62
	16	ATP-synthase	32189394	48%	19/69	61	57	4.5	5.3	1 / 2.04
	17	acyl-CoA dehydrogenase	19684166	51%	11/72	40	44	6.5	8.1	1 / 2.2
	18	enoyl-CoA hydratase	12707570	52%	12/69	19	25	6.5	6.0	1 / 2.3
	19	3-hydroxyacyl-CoA dehydrogenase	2078327	21%	8/54	31	22	9.5	8.5	0.97 / 0.76
	6	FABP adipocytaire	4557579	53%	7/44	11	15	6.5	6.6	1 / 0.87
	20	glycerol 3-phosphate dehydrogenase	33695088	37%	10/64	34	38	6.5	6.0	1 / 1.93
	21	aldehyde dehydrogenase	25777732	31%	12/72	53	56	6.5	7.0	1 / 2.37
	22	pantothenate kinase	23510398	30%	8/73	34	36	5.5	8.0	0.92 / 0.48
Other proteins	7	RAB 2B	26892279	32%	7/63	27	17	6.5	5.5	1 / 2.14
	23	urokinase plasminogen activator receptor	4335704	61%	8/61	23	28	7.0	6.0	1/2.43
	24	spermatogenesis associated Ser/Thr kinase 22D	14042947	33%	7/42	35	42	7.5	8.0	0.99 / 0.86
	25	human serum albumin precursor	6013427	22%	12/68	80	70	6.5	6.0	1 / 2.32
	26	alpha beta-cristallin	4503057	51%	9/64	17	20	5.5	7.0	1 / 2.38
	27	ferritin light polypeptide	4557579	45%	6/51	18	15	5.5	6.6	1 / 1.69

*Searches were carried out employing four different algorithms for protein identification allowing cross-validation against the human NCBI datbase. The profound score is given in the table as identification probability/Z-score.
$Number of peptides matched peaks *vs.* Peptides submitted

regulated protein and has been implicated in some of the effects of glucocorticoids, including inhibition of cell proliferation, anti-inflammatory effects, regulation of cell growth and differentiation, and membrane trafficking [37-39]. Annexin 1 levels are augmented in response to glucocorticoid and cytokines such as TNF-alpha, IL-1 and IL-6 [37, 39]. Annexin 1 is also a substrate for protein kinase C and protein-tyrosine kinases, and it specifically modulates the ERK signaling cascade [40].

Stress/Chaperon Proteins

Our proteomic analysis demonstrated the expression of chaperon proteins, such as grp58 (spot 8), BiP 75 (spot 9) and prohibitin (spot 10) in the lipid droplets.

The glucose regulated protein 58 (grp58), also known as protein-disulfide isomerase (PDI) is an abundant, soluble protein found in the lumen of the endoplasmic reticulum of eukaryotic cells. The major role for protein disulfide isomerase is thought to be the formation and rearrangement of disulfide bonds of secretory proteins in the endoplasmic reticulum. Interestingly, protein-disulfide isomerase was also shown to be a component of microsomal triacylglycerol transfer protein (a heterodimer of PDI and a 97kDa subunit), that is believed to catalyze the transfer of neutral lipid onto nascent lipoprotein particles [41]. When complexed with the microsomal triacylglycerol transfer protein, protein disulfide isomerase does not show isomerase activity, suggesting that it may be acting to stabilize the complexes [42]. A proteomic analysis has also shown a significant increase of gpr58 expression in lung adenocarcinomas [43].

BiP, the glucose regulated protein 78 (grp78), is a chaperon protein also involved in the folding and assembly of proteins in the endoplasmic reticulum [44]. BiP is a member of the ATP–binding family of stress proteins. It has an ATPase domain and a peptide-binding domain and it is involved in the quality control of newly synthesised proteins in the endoplasmic reticulum [45].

The prohibitins (Phb1 and Phb2) are present in the inner mitochondrial membrane and have long been considered as chaperones for mitochondrial proteins [46, 47]. A role in the regulation of mitochondrial metabolism through inhibition of pyruvate carboxylase has also been described [48]. Both Phb1 and Phb2 are members of a superfamily of molecules containing a PHB domain, which may constitute a lipid recognition motif and may be important in facilitating membrane association [47]. In this regard, it is of note that Phb1 and Phb2 have been identified on the plasma membrane, on lipid raft preparations and on adipocyte lipid droplets [25, 47, 49]. Interestingly, immunohistochemical analysis showed expression of prohibitin in the membrane of endothelial cells in the white adipose tissue and recent study established prohibitin as a vascular marker of adipose tissue. Targeting a proapoptotic peptide to prohibitin in the adipose vasculature caused ablation of white fat, leading to resorption of adipose tissue, normalization of metabolism and rapid weight loss [49].

Redox Proteins

The proteins associated with oxidation/reduction pathways such as peroxiredoxins 1, 2 and 6 (spots 11, 12 and 13 respectively) and thioredoxin-like (spot 14) were also identified in lipid droplet fractions. The peroxiredoxins are a family of thiol-specific antioxidant enzymes [50]. Their protective antioxydant role in cells is exerted by reduction of hydrogen peroxide or alkyl peroxide to water or corresponding alcohol and is dependent upon the presence of a conserved cysteine [51]. Peroxiredoxins are produced at high levels in cells and had a large range of cellular stress responses, including the modulation of growth factor and cytokine-induced hydrogen peroxide levels, cell proliferation, differentiation and apoptosis [52]. Detailed analysis of their sequences indicated that Peroxiredoxins possess a thioredoxin-like fold and consequently are homologues of both glutathione peroxidase and thioredoxin. Spot 14 (PKC-interacting cousin of thioredoxin) also displays an N-terminal homology domain and appears to inhibit PKCθ–induced JNK activation [53].

A role of thioredoxins in regulation of energy metabolism was revealed by the demonstration that a mutation in the thioredoxin-interacting protein gene was responsible for a form of combined hyperlipidemia in mice [54]. More recently, it was shown that thioredoxin-interacting protein deficiency disrupts the fasting-feeding metabolic transition, resulting in mice that exhibit in the fed state a metabolic profile similar to that of fasted animals, including increased levels of free fatty acids, decreased glucose and increased hepatic expression of PPARγ coactivator-1α [55].

Proteins Involved in Energy Metabolism

The function of mature adipocyte is to store excess energy in the form of lipids in lipid droplets. Consequently, adipogenesis is accompanied by the induction of proteins associated with glycolysis and fatty acid metabolism.

α-1 enolase (spot 15) was found in lipid droplets. This enzyme is involved in glycolysis and catalyzed the conversion of 2-phophoglycerate into phosphoenolpyruvate, a high-energy intermediate which dephosphorylation into pyruvate produces ATP. α-1 enolase is induced by hypoxia [56] and is found to be up-regulated in a number of tumors [57, 58].

A subunit of ATP synthase (spot 16) was also identified in our protein droplet preparations. Mitochondrial ATP synthase catalyzes ATP synthesis, utilizing an electrochemical gradient of protons across the inner membrane during oxidative phosphorylation [59].

In respect to fatty acid metabolism, one fatty acid binding protein, FABP4 (spot 6), and three of the four enzymes involved in the beta-oxidation pathway, acyl-CoA dehydrogenase (spot 17), enoyl-CoA hydratase (spot 18), 3-hydroxyacyl Co-A dehydrogenase (spot 19) are also found in lipid droplet preparations.

The FABP4 (spot 6), an adipose-specific isoform (aP2), is a cytoplasmic protein involved in intracellular free fatty acid transport and metabolism [60]. It has been shown that adipocyte FABP associates with hormone sensitive lipase in the basal state and the complex translocates to the surface of the lipid droplets under lipolysis conditions [61]. The FABP4 is regulated by fatty acids [62], thiazolidinediones [63, 64], and hormones such as glucocorticoids. Indeed, dexamethasone induces a rapid and dose-dependent increase in the expression of the aP2 gene in rodent [65] and human adipocytes [13].

The glycerol-3-phosphate dehydrogenase (G3PDH), an enzyme that formed an important link between carbohydrate and lipid metabolism, was also detected in our proteomic analysis (spot 20). This NADH-dependent enzyme catalyzed the conversion of dihydroxyacetone phosphate to glycerol-3-phosphate. The G3PDH activity in adipose tissue is stimulated by dexamethasone [66] and cortisol [12] and has been associated with obesity. Indeed, a positive correlation between adipose tissue G3PDH activity and body mass index has been found [67].

The aldehyde dehydrogenase (spot 21) was also found associated with lipid droplets. This enzyme is considered to be important for the detoxification of both exogenous and

endogenous aldehydes such as those derived from lipid peroxidation of membrane phospholipids [68]. This enzyme has been shown to be expressed in an insulin dependent manner in rodent adipose tissue [68].

Finally, pantothenate kinase (spot 22) a key regulator of coenzyme A (CoA) biosynthesis was also detected. The biosynthesis of CoA, an essential cofactor in energy metabolic pathways, is governed by the activity of pantothenate kinase [69].

Other Proteins

The Rab2B (spot 7), was also found associated to lipid droplets of peri-adrenal adipose cells. Rab proteins are small-molecular-weight guanosine triphosphatases (GTPases) that control vesicular traffic in eukaryotic cells. Rab 2B is highly homologous to Rab2, a small GTPase protein resident of pre-Golgi intermediates, that is required for protein transport from the endoplasmic reticulum to the Golgi complex [70].

Several relatively unstudied proteins were identified on lipid droplets. The significance of the isolation of urokinase plasminogen activator receptor (spot 23), spermatogenesis serine-threonine kinase (spot 24), serum albumin precursor (spot 25), alpha-crystallin (spot 26) and ferritin light polypeptide (spot 27) on lipid droplet preparations is unknown.

4. CONCLUDING REMARKS

The present study is the first proteomic approach to characterize lipid droplet-associated proteins from adipose cell of patients with CS. Using two-dimensional gel electrophoresis we found that the lipid droplet proteome is highly complex and contains more than 500 proteins. Mass spectrometry identification showed proteins already known to be associated with lipid droplets and new proteins identified for the first time associated with lipid droplets.

In the present study, several proteins usually associated with the endoplasmic reticulum were found in the lipid droplet. Interestingly, it has been shown that lipid droplets are formed by accumulation of neutral lipids between the two leaflets of the endoplasmic reticulum membrane [71, 72]. Obesity and diabetes are associated with several stress conditions, including oxidative stress and endoplasmic reticulum stress [73, 74]. It recently became clear that obesity is associated with cellular stress response, particularly in the endoplasmic reticulum [75, 76]. Notably, expression of ER chaperone proteins, such as BiP, were found increased in liver of diet-induced or genetically obese mice [75]. We found that chaperones and redox proteins are present on adipose tissue lipid droplets of patients with CS, and a common feature of these patients is progressive central obesity. These observations comfort the hypothesis that oxidative stress and ER stress may be considered as major hallmarks of obesity. Further determinations of the biological function of other identified lipid droplet proteins should improve our understanding of the lipid droplet proteome. Our study should also provide a useful reference for future comparative proteomic studies.

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5. ABBREVIATIONS

CS	=	Cushing's syndrome
ACTH	=	Adrenocorticotropin hormone
CRH	=	Corticotropin-releasing hormone
cAMP	=	Cyclic 3',5' adenosine monophosphate
HSL	=	Hormone sensitive lipase
FFA	=	Free fatty acids
TG	=	Triacylglycerol
PKA	=	Protein kinase A
PAT	=	Perilipins,adipophilin and TIP47
PHB	=	Prohibitin

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