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Adipose proteome analysis: focus on mediators of insulin resistance

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Abstract

As is well known, adipose tissue is an important site for lipid metabolism and insulin-responsive glucose uptake. The recent discovery of the endocrine function of adipose tissue and the association of obesity with chronic low-grade inflammation in adipose tissue has reinforced the concept of the central role of adipose tissue in mediating obesity-linked insulin resistance and metabolic dysregulation. The study of adipose cells has provided new insights into the mechanism underlying insulin resistance as well as the therapeutic strategies for diabetes. Numerous efforts have been made in identifying key molecular regulators of insulin action and metabolism, including the utilization of advanced proteomics technology. Various proteomic approaches have been applied to identify the adipose secretome, protein-expression profiling and post-translational modifications in adipose cells in the pathological state. In this review, we summarize the recent advances in the proteomics of adipose tissue, and discuss the identified proteins that potentially play important roles in insulin resistance and diabetes.

Keywords

adipocyte; adipokine; diabetes; insulin resistance; insulin signaling pathway; phosphoproteome; phosphorylation; proteomics; secretome

The prevalence rate of obesity has rapidly and globally increased in the last 20 years and has reached epidemic proportions [1,2]. Obesity is a major risk for developing insulin resistance, Type 2 diabetes, dyslipidemia and cardiovascular diseases. As is well known, adipose tissue is an important site for lipid metabolism and insulin-responsive glucose uptake. For that reason, adipose cells have been extensively used as a model system for the study of insulin signal transduction and regulation of glucose metabolism for many decades. Numerous studies have demonstrated that abnormal development of adipose tissue, leading to either excessive or lacking adipose tissue mass or ectopic lipid accumulation in both animal models and humans,

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results in metabolic disorders that cause severe insulin resistance and Type 2 diabetes and dyslipidemia [3–5]. Adipose tissue is no longer considered as an inert organ for lipid storage and mobilization, but instead is known to play an active role in the regulation of insulin action, whole-body energy metabolism and homeostasis, primarily through its endocrine function. As an endocrine organ, adipose tissue secretes a number of adipokines that exhibit critical functions in metabolic regulation, and adipose tissue is also a major source of inflammatory cytokines mediating obesity-linked metabolic dysregulation [6–9]. In addition to its role as an endocrine organ, adipose tissue is the major site controlling the release of fatty acids that play a critical role in insulin resistance in the pathological states [10,11]. All of these metabolic regulators interplay and contribute to whole-body insulin sensitivity and metabolic homeostasis.

Adipose tissue mass & insulin resistance

Adipose tissue is composed of adipocytes and nonadipocytes. The fraction of nonadipocytes is defined as stromal-vascular cells that comprise heterogeneous cell populations including preadipocytes, fibroblasts, endothelial cells, macrophages and other cells. Adipose tissue is located in stereotypical depots, and white adipocytes from different depots display differential molecular and physiological characteristics [12–14]. For many decades, it was believed that increased adipose tissue in distinct depots could lead to different biological consequences, for example, an increased risk of insulin resistance and metabolic syndrome is closely associated with increased visceral, but not subcutaneous adipose tissue mass. Experiments performed by Tran *et al*. in a recent study documented that transplantation of subcutaneous fat from normal mice into a visceral region showed a beneficial effect on insulin sensitivity [15].

A new concept of the relationship between adipose tissue mass and risk of insulin resistance has emerged; the lipid partitioning to nonadipose tissues such as liver and muscle, as a result of decreased capacity of adipose tissue to store lipids, is considered to be more directly associated with insulin resistance. The following studies are the basis for this concept. First, mice with conditional disruption of PPARγ, a key adipogenic transcription factor, developed no white and brown adipose tissue, but hepatic steatosis and insulin resistance [16]. In the second model of A-ZIP/F mice, overexpression of a dominant-negative protein under the control of aP2 promoter interfered with the function of transcription factor, CCAAT element binding protein, leading to the inhibition of adipogenesis, severe fatty liver, hyperlipidemia and diabetes [4]. Additionally, a line of evidence from another mouse model supports the same concept, for example, knockouts of *Hmgic* gene prevented adipocyte differentiation and obesity, but induced insulin resistance [17,18]. Second, in humans, lipoatrophic diabetes is a relatively rare genetic condition [5,19], but commonly developed in antiretroviral treatment of HIV-infected patients [20]. This condition is associated with gradual loss of subcutaneous adipose tissue, hypertriglyceridemia, hepatic steatosis, severe insulin resistance and early-onset diabetes. Third, in most recent studies, overexpression of leptin receptor-b specifically in adipose tissue of both normal and *db/db* mice resulted in decreased adipogenesis and prevented the development of diet-induced as well as genetic obesity. However, these mice showed an increased lipid accumulation in the liver and heart and accelerated onset and severity of diabetes [21]. On the contrary, *ob/ob* mice overexpressing aP2-adiponectin led to the expansion of subcutaneous fat tissue and greater obesity, but they were more insulin sensitive than *ob/ob* mice [22]. Finally, the improvement of insulin sensitivity by the treatment of PPARγ agonists, thiazolidendiozes (TZD) is primarily associated with increased adipogenesis and decreased lipid accumulation in nonadipose tissue [23]. Thus, the normal functions of adipocytes capable of storing lipids and secreting important metabolic regulators are critical for its role in the control of metabolic homeostasis. In this regard, adipose-derived secretory molecules may play key roles in linking defective adipogenesis to ectopic lipid accumulation and insulin resistance. The discovery and functional characterization of new adipocyte-derived factors is still ongoing.

Adipose tissue & endocrine function

All hormones and peptides or bioactive mediators secreted from adipocytes are collectively known as adipocytokines or adipokines. Leptin was the first adipocyte-derived hormone discovered in 1994 [24]. Thereafter, many other adipokines were identified and reported including adiponectin, resistin, visfatin, retinol binding protein 4 (RBP4), and lipocalin 2 [14,25]. Among the known adipokines, however, only a few have been clearly characterized to be central regulators of insulin sensitivity and whole-body energy metabolism and homeostasis.

Adiponectin is a 30-kDa protein predominantly secreted by adipose cells and present in two forms, a full-length adiponectin and a proteolytic cleavage fragment containing the globular C-terminal domain or so-called globular adiponectin in the circulation [26]. The circulating levels of adiponectin are inversely correlated to insulin sensitivity. For instance, the serum level of adiponectin is significantly decreased in rodent and human obesity [27]. Mice lacking adiponectin develop severe diet-induced insulin resistance, whereas overexpression or pharmacological administration of adiponectin improves insulin sensitivity [28]. All the above studies clearly show that adiponectin plays an important role in regulating systemic insulin sensitivity. Adiponectin, together with leptin, has been demonstrated to stimulate fatty acid oxidation and improve insulin resistance and glucose utilization by activating the AMP-activated protein kinase pathway in the liver and skeletal muscle [29–33]. However, diet-induced insulin resistance in adiponectin-deficient mice was not reversed by TZD as efficiently as in wild-type mice, indicating that adiponectin is an important mediator for PPARγ effects in insulin sensitivity [34]. It is not surprising since adiponectin is one of PPARγ target genes and its gene expression and secretion from adipocytes are regulated by TZD [35].

Resistin is an approximately 10-kDa protein exclusively expressed in adipose cells in the mouse, but expressed highly in macrophages and monocytes in humans [36]. Resistin has been shown to be involved in the regulation of glucose metabolism and insulin resistance in mice [37]. However, the role of resistin in regulation of insulin sensitivity in humans is not well established [38]. Visfatin, also called pre-B cell colony-enhancing factor, a 52-kDa secreted protein, is widely expressed, but adipose visfatin is specifically expressed in the visceral depot. The role of visfatin on insulin action and glucose metabolism has not been determined. RBP4, a new adipokine, was identified by screening differential expressed genes in adipose tissue of insulin resistant and diabetic adipose-specific *GLUT4* knock-out mice [39]. This adipokine was also on the list of adipokines identified by our high-throughput proteomic screen method [40]. The expression of RBP4 in adipose tissue and circulating RBP4 were dramatically upregulated in adipose tissue of $GLUT4^{-/-}$ mice as well as several other models of rodent obesity [39]. Studies in mice with overexpression or genetic deletion of *RBP4* demonstrated that RBP4 functions as an antagonist of insulin action [39]. The circulating levels of RBP4 were also increased in obese humans, and the levels were correlated negatively with insulin sensitivity [41] and positively with percent abdominal fat [42].

Overall, studies indicate that adipokines act as endocrine factors and regulate systemic insulin sensitivity. Endocrine dysfunction of adipose tissue mediates systemic insulin resistance, resulting in hyperglycemia and diabetes. Therefore, understanding the endocrine activity of adipose tissue is crucial for evaluating drug or dietary interventions to improve systemic insulin sensitivity. However, measurement of several adipokines using traditional approaches is not sufficient to explain such a complex metabolic disturbance as obesity. Obviously, alterations in secretion of only one or two adipokines could not be fully responsible for the development of the constellation of metabolic disturbances associated with obesity. An imbalance in the levels of multiple adipokines is more likely attributive to metabolic dysregulation. Thus, identification of adipose secretome and detection of global changes in the secretory proteome

from adipose tissue as a whole are essential and necessary. The recent advance in proteomics technology has greatly forwarded this process. Numerous studies in this regard as described in the late section of this review have provided some insight into the understanding of the role of adipose tissue in insulin resistance and diabetes.

Obesity has been considered to be a chronic low-grade inflammatory state, and adipose tissue is the major source of increased macrophage infiltration and proinflammatory cytokine production in obesity and insulin resistance [43,44]. Both adipocytes and resident macrophages in adipose tissue produce inflammatory cytokines and chemokines such as TNF-α, IL-6, IL-1β, MCP-1, [45–48] that play roles in the regulation of adipocyte metabolic and endocrine functions in an autocrine and/or paracrine fashion, linking inflammation-caused metabolic defects to insulin resistance. TNF-α has been most strongly linked to the pathogenesis of insulin resistance and metabolic dysregulation in obesity. Studies provide the direct evidence that TNFα inhibits insulin-stimulated glucose disposal and induces insulin resistance by inhibiting insulin-dependent tyrosine phosphorylation of insulin receptor and insulin receptor substrate-1 (IRS-1) [49], stimulating serine phosphorylation of IRS-1 protein [50,51] and decreasing GLUT4 [52]. Recently, TNFα has been found to directly suppress AMP-activated protein kinase activation contributing to obesity-associated metabolic dysregulation [53].

Adipocyte fatty acid metabolism & insulin resistance

In addition to adipose-derived adipokines and cytokines, lipids and their derivatives are largely involved in the mechanisms for the development of insulin resistance [10,11]. Free fatty acids have been considered to be the inflammatory stimulus leading to the activation of the NF-κB and PPARγ pathway leading to the serine phosphorylation of IRS protein [54,55]. The proper control of lipid utilization in metabolic pathways is critical for normal functions of adipocytes and this process is regulated by intracellular lipid carriers; fatty acid-binding proteins (FABPs). Adipocyte FABP/aP2 (A-FABP) and epidermal FABP/mal1 (E-FABP), two abundant members of FABPs in adipocytes, are also expressed in macrophages. Mice lacking A-FABP specifically in macrophages showed enhanced PPARγ activity and decreased NF-κB activity in macrophages, resulting in inhibition of inflammatory activity and reduced cytokine production [56]. A series of studies using A-FABP and E-FABP knockout and transgenic mouse models have demonstrated that FABPs are the critical proteins that link lipid metabolism and endocrine action in adipocytes to systemic energy homeostasis [57,58].

Proteomics in adipose tissue & adipocytes

Adipose secretome

In the field of adipocyte biology and obesity, many applications of proteomics have involved the analysis of adipose secretome, adipose subproteome [59] and post-translationally modified proteins such as phosphoproteome and carbonylation of adipose proteins [60]. Characterization of adipose-secreted proteins has been a hot topic since the last decades and it will continue to be an important research area. Multiple proteomics tools have been applied to identify the adipose secretome from immortalized preadipocyte cell lines, primary adipose cells and adipose tissue. We provide the following sections with the detailed description of the techniques, studies and results of proteomics-based research in adipose cells or tissue.

Separation techniques—The complexity of the secretory proteome is generally reduced by separation techniques such as 1D sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) or 2D gel electrophoresis and/or liquid chromatography (LC) techniques prior to mass spectrometry (MS). Historically, 2D gel electrophoresis was the preferred separation technique for complex mixtures. Proteins were first separated by their isoelectric points and then by size. Visualization with an appropriate stain showed a map with

hundreds of spots. Each spot could then be excised and subjected to MS analysis. With a few exceptions of highly specialized laboratories, the lack of automation, together with poor loading capacity and thus poor dynamic range and poor run-to-run reproducibility, have mostly contributed to disfavor this technique in most proteomics laboratories.

Sodium dodecyl sulphate PAGE followed by LC-coupled tandem MS (LC-MS/MS) (also known as GeLCMS) is a standard procedure that is used in many laboratories as a routine method to identify (and quantify) proteins. Proteins are just separated by their size, visualized and the entire gel lane is cut into slices that are subjected to (manual or robotic) tryptic digestion and LC-MS analysis. Most modern mass spectrometers are capable of analyzing a few hundred proteins from one gel band. Disadvantages of this method include the electrical oxidation of proteins during gel electrophoresis and sometimes – depending on the protein's properties – poor recovery from the gel bands. Shotgun proteomics using 1D or 2D liquid separations have been pioneered during recent years and become increasingly popular. In bottom-up proteomics, 1D separation is usually performed at the peptide level; 2D separations can either be carried out at the peptide level (with multidimensional protein identification technology [MudPIT] being the most popular approach) [61] or the first dimension is done on the protein level, followed by digestion and separation of the peptides in the second dimension.

Mass spectrometry techniques—Any mass spectrometer consists of three parts: an ionization source, a mass analyzer and a detector. Depending on the ionization source, either electrospray ionization (ESI), MALDI and/or SELDI techniques have been used in combination with adipose cells and tissue secretomes. As mass analyzers, TOF and quadrupole TOF (QTOF) instruments, ion traps and linear quadrupole ion trap-Fourier transforms (LTQFTs) have been used so far. It is conceivable that the first reports using an Orbitrap will be coming out soon. Typical detectors include electron multipliers (ion traps), microchannel plates (TOFs) and an alternating current image current that is Fourier transformed to an m/z spectrum (LTQFT and Orbitrap). Tandem MS to determine the precursor ion (m/z) and its fragment ions is generally used nowadays, although some MALDI- or SELDI TOF experiments with simple precursor ion detection were performed. It is beyond the scope of this review to discuss the advantages and disadvantages of these instruments and the interested reader is referred to other references [62–69].

Quantification with ^{18}O isotopic labeling—To quantify the differences between treated and untreated adipose cells, $H_2\,^{18}O$ isotopic labeling has been successfully introduced [70–72]. For this, an aliquot of the treated condition sample is being labeled with $H_2\,^{18}O$ during or after trypsinization and then mixed 1:1 with $H_2\,^{16}O$ labeled and trypsinized untreated condition sample. Quantification is achieved on the MS-level, either manually or with advanced bioinformatics tools.

Bioinformatics tools—Once the raw spectra are obtained, the ions of interest are usually extracted and subjected to database searches using a number of search engines (e.g., Sequest or Mascot). The obtained data were then parsed using either in-house scripts or a commercial product such as Scaffold [201]. Protein candidates identified through this have been searched against prediction algorithms using SignalP [202] for signal peptides, and Texas medical algorithm project (TMAP) [203] and transmembrane helix Marcov model (TMHMM) [204] for transmembrane domains. It should be noted that along with the ever improving protocols, these prediction algorithms are also improving.

The first analysis dealt with the 2D gel electrophoresis [73] and SDS-PAGE [74] separation of proteins synthesized during differentiation of preadipocytes to adipocytes. Although the differences were visualized, proteins could not be identified at the time. Kratchmarova *et al.* used a 1D gel electrophoresis and tandem QTOF MS (GeLCMS) and an in-solution tryptic

digestion followed by LC-MS/MS approach to report a total of 20 proteins from 3T3-L1 adipocytes [75]. Of these, 12 were only identified through the in-solution digestion route. The feasibility of this study was determined with a semiquantitative approach to monitor the secretion of ^{35}S-radiolabeled proteins from preadipocytes differentiating into adipocytes over a time course of 9 days. The autoradiograph of the gel-separated proteins clearly showed differences between preadipocytes and adipocytes. In a second experiment, preadipocytes and adipocytes (cells) were grown without the radiolabel and separated by SDS-PAGE. A total of 16 differentially expressed protein bands were excised, reduced, alkylated and digested with trypsin, identifying eight upregulated adipocyte proteins after nanoelectrospray tandem MS **(**Table 1). In a third experiment, in-solution tryptic digestion followed by LC-MS/MS was performed on the supernatant of the adipocytes leading to an additional 12 identifications **(**Table 1). In this early study, no quantitative MS was performed. It was, nevertheless, suggested that stable isotope labeling of amino acids (SILAC) could be performed in cell culture or isotope-coded affinity tags (ICAT) after protein harvest and digestion.

Wang *et al.* selectively blocked the endoplasmatic reticulum/Golgi-dependent and -independent secretion pathways of 3T3-L1 adipocytes to determine 41 secreted proteins from 161 spots by a 2D gel electrophoresis approach followed by MALDI-TOF and LC-MS/MS [76]. Brefeldin A (BFA) was used to block the Golgi-dependent pathway and 20°C treatment was used to block both pathways. Bioinformatic prediction analysis for signal peptides (SignalP) and transmembrane domains (TMAP) were used as an independent means to validate the obtained results. These tools were used with caution as signal peptides of some proteins (e.g., cyclophilin A) were not identified. The majority of the proteins identified in this study belong to extracellular matrix proteins, growth regulation and lipid metabolism **(**Table 1). The differential blockage with 20°C and BFA allowed insights into the secretion mechanism: secretion of galectin-1, cyclophilin A and C, SF20/IL-25 and cofilin could not be blocked by BFA, indicating an endoplasmic reticulum (ER)/Golgi-independent secretion pathway. In this study, quantitative analysis was performed by comparing spot intensity on the 2D gel, which was in some cases limited owing to comigration with high-abundance proteins.

To establish a true quantitative protocol, Chen *et al.* used a 2D LC isotopical labeling approach coupled to tandem MS [40,77]. This study is also the first to report the use of primary adipose cells isolated from adipose tissue rather than immortalized adipose cell line. To address the potential contamination of leakage proteins, the isolated adipose cells were washed four times. Proteins from adipose cell culture medium were separated into eight fractions on a C3 column, digested with LysC and trypsin and analyzed by LC-MS/MS using QTOF [40] and LTQFT [77] MS to identify a total of 183 candidates using Mascot database searches. Leaking proteins were determined in control experiments using potassium cyanide. SignalP predictions reduced the number of proteins that are secreted through the ER/Golgi-dependent pathway to 84 and 53 of those were novel **(**Table 1). The proteins could be categorized as extracellular matrix proteins, immune function and protein degradation. To quantify differentially expressed proteins, samples from condition one (e.g., untreated) and two (e.g., insulin treated) were split and one part was treated with $H_2{}^{18}O$ during trypsinization, mixed 1:1 with a $H_2{}^{16}O$ trypsinized part and analyzed by LC-MS. For quantification of the proteins, the intensity of the ^{18}O (I_4) to ^{16}O (I_0) ratio was determined manually [40] and automatically [77]. This study thus allowed a thorough quantitative analysis of the differential expression of adipokines in adipose tissue.

Although adipose tissue from rodents provides unlimited sources for the studies of adipokines, there exist significant discrepancies in terms of tissue distribution and function of adipokines between rodents and humans [78]. For instance, resistin has been shown to have distinct roles in mouse and human models [78]. Thus, identification and characterization of adipokines in humans would be of great importance. A recent study characterized the human visceral adipose tissue secretome [79]. In contrast to adipocytes, the use of tissue should thus better reflect a

true physiological environment. Proteins were profiled using a weak cation exchange Protein Chip by SELDI-TOF. Peak intensities observed at 15.1 and 15.9 kDa predicted to be the hemoglobin chains and at 66 kDa predicted to be serum albumin were monitored using different culture setups. To reduce the number and quantity of contaminating proteins from serum and intracellular fractions, a minimum of three washes were found to be necessary during the first 24 h of culture. A differential labeling experiment using L-[$^{13}C_6$, $^{15}N_2$] lysine was established to distinguish true adipokines (labeled) from contaminants (unlabeled). Proteins were separated on SDS-PAGE and the entire lane was cut into 28 bands for tryptic digestion. The peptides were analyzed by QTOF MS and identified with ProID software. Whereas 70 out of 108 secreted proteins incorporated the label, 38 did not, most of which had an arginine rather than a lysine at the C-terminus. A few of these proteins such as albumin are believed to be contaminants that could only be reduced but not entirely removed during the culture setup. A quantitative labeling approach has not yet been established.

In two recent studies, primary cultured adipocytes differentiated from human adipose-derived stem cells and S-V cells of rat adipose tissue were used as the model systems [80,81]. The biological characteristics of these adipocytes are different from immortalized cell line and primary isolated mature adipose cells. In the former study, 2D-gel-LC-MS/MS proteomcs approach was applied, while in the later study, 2D reverse phase (RP)/RP-HP LC separation was used to reduce the complexity of protein mixture samples, followed by LC-MS/MS analysis. To address the contribution of the S-V cells to the adipose tissue proteome, in the most recent study from our laboratories, S-V cells were isolated and the secretome of S-V cells were identified using the same 2D-LC-MS/MS proteomic approach as described above [Chen *et al.*; Unpublished Data]. Compared with adipocyte secretome, fewer secreted proteins were detected and most of the proteins possess transmembrane segments, suggesting that they are secreted via a membrane-shedding mechanism and likely function as local regulators of adipocyte differentiation and functions. In addition, we identified the obese adipose tissue secretome and first used quantitative $H^{18}O$ labeling to detect the global changes in adipose tissue secretome in response to TZD treatment in obese rats. We were able to quantify the difference in the secretion levels of total 78 proteins.

Adipose tissue in mammary gland has been demonstrated to influence mammary gland development and tumor growth and metastasis [82], and factors secreted from adipose tissue may play important roles in mediating this effect. In this regard, Celis *etal.* utilized proteomic techniques such as 2D gel-based technology, MS, immunoblotting and antibody-array to identify extracellular and intracellular signaling molecules of mammary adipose cells and interstitial fluid of fresh adipose tissue samples from high-risk breast cancer patients. A total of 359 proteins were identified, including numerous signaling molecules, hormones, cytokines and growth factors that are involved in a variety of biological processes, such as signal transduction and cell communication; energy metabolism; protein metabolism; cell growth and/or maintenance; immune response; transport; regulation of nucleobase, nucleoside and nucleic acid metabolism; and apoptosis.

The secreted proteins identified consistently from different models of adipocytes and adipose tissue by different proteomics platforms better represent the characteristics of adipose secretome. Table 1 shows the list of the proteins consistently identified in multiple studies. The major functional categories of adipocytes/adipose tissue secretome are metabolic regulation, extracellular matrix molecules, immune function and proteolysis, indicating that adipose tissue is a very active endocrine as well as autocrine/paracrine organ that regulates insulin sensitivity and metabolism in distant organs/tissues as well as controls adipocyte differentiation and function in an autocrine and/or a paracrine manner. The later is also an important characteristic of adipocytes as it regulates the secretion of endocrine metabolic regulators of adipocytes, which ultimately influences metabolic homeostasis.

Lipocalin 2 (LCN2)is one of the adipokines consistently identified by our and other laboratories using proteomic screen methods. As a follow-up to the proteomics study, further functional characterization of LCN2 was performed, since it possesses the following interesting characteristics. LCN2 or neutrophil gelatinase-associated lipocalin belongs to the superfamily of lipocalins including retinol-binding protein 4, fatty acid-binding proteins and α_2-microglobulin [83]. In neutrophils, LCN2 secretion is highly regulated by inflammatory mediators, lipopolysaccharide and TNF-α [84]. Studies have demonstrated that LCN2-deficient mice have an increased susceptibility to bacterial infection [85,86]. More intriguingly, LCN2 promoter possesses two key transcription factors NF-κB and CCAAT element binding protein binding sites [87] and glucocorticoid response element [88], suggesting that transcriptional activation of this gene in adipose tissue is associated with inflammation and obesity. Two independent studies have demonstrated that levels of *LCN2* gene expression are significantly increased in adipose tissue of obese rodents; and this increase is reversed by TZD treatment [25,89]. In diabetic humans, the circulating LCN2 levels are positively correlated with insulin resistance [90]. Further characterization *in vivo* in animals is required for the precise role of LCN2 in inflammation and its associated insulin resistance.

In addition to the proteomic analysis of adipose secretome, a comprehensive study of adipose subproteome has recently been conducted [59]. A total of 3287 proteins with a very low false discovery rate have been identified in 3T3-L1 adipocytes. For this, adipose cells were separated into the following organellar fractions: nuclei, mitochondria, membrane and cytosol, and analyzed separately by GeLC-MS and advanced bioinformatics tools. Using stringent data-filtering procedures, a high confidence protein list was generated to give an estimated false-positive probability of $p = 0.0001$. When these proteins were mapped onto the Kyoto Encylopedia of Genes and Genomes pathway, the ribosomal and proteasome complexes were comprehensively identified; whereas proteins from the insulin pathway were approximately half covered. Among the identified adipokines, a 2.5-fold enrichment was found when compared with the complete IPI mouse proteome, indicating the secretory function of adipocytes.

Phosphoproteome of insulin signaling pathway in adipocytes

Adipose tissue is an important site for lipid metabolism and insulin-responsive glucose uptake. Compared with skeletal muscle, the other major insulin-dependent glucose disposal site, adipocytes are readily manipulated and, thus, have been widely used as an insulin-responsive cell model for studying insulin signal transduction and insulin resistance. Most of the studies have been conducted in adipose cell lines such as 3T3-L1 adipocytes and primary adipose cells. Insulin resistance is characterized by decreased sensitivity and response of target tissues to normal levels of insulin, leading to decreased glucose disposal in skeletal muscle and adipose tissues and attenuated insulin suppression of hepatic glucose output [91]. All of these contribute to elevated blood glucose levels and diabetes. In addition, adipocyte insulin resistance causes the increase in lipolysis and release of free fatty acids into the circulation [92]. Furthermore, as described earlier, the insulin signaling pathway may also involve the regulation of adipocyte secretory function. Thus, identifying the role and regulation of insulin signaling transduction in glucose transport and other cellular responses is one of the important steps toward understanding the underlying mechanism for insulin resistance.

Phosphorylation of proteins plays a major role in the regulation of signal transduction events. Upon ligand binding to cell-surface receptors, cellular proteins are phosphorylated or dephosphorylated in multiple pathways and transduce the signals governing cellular responses. Analysis of dynamics of phosphorylation events upon ligand stimulation is of importance for understanding the whole cellular response and regulation. Recently, proteomics approaches have been employed in the identification and quantification of the phosphoproteome and

dynamics of phosphorylation in eukaryotic cells. White and colleagues developed a method using isobaric tags for relative and absolute quantification (iTRAQ) combined with peptide immunoprecipitation and LC-MS/MS, which enables the analysis of time course phosphorylation profiles of EGF stimulation [93].

Isobaric tags for relative and absolute quantification have been applied in many studies as a quantitative proteomics method since it was first described in 2004 [94]. iTRAQ uses a set of four (or even eight) synthetic reagents or isobaric tags that are made to have the same molecular mass of 145 Da by differential isotopic enrichment with ^{13}C, ^{15}N and ^{18}O atoms. The four complete tag molecules comprise a reporter group with molecular mass of 114, 115, 116 and 117, respectively; a balance group (mass range: 31–28); and a peptide-reactive group (N-hydroxysuccinimide ester). The peptide-reactive group is linked to the N termini and lysine side chains of digested peptides. The peptides labeled with four different isobaric tags behave similarly during chromatographic and MS analysis, but differentially in MS/MS mode. The advantage of this method is to compare quantitative differences among multiple samples and, most importantly, it is suitable for identification and quantification of post-translationally modified peptides as all digested peptides can be labeled without bias. Four tryptic peptide samples are differentially labeled with four different iTRAQ reagents, and each of the iTRAQ-labeled peptides are mixed at a certain ratio and analyzed by LC-MS/MS. At the MS/MS stage, the peptides are cleaved at the reporter groups and small fragments of four iTRAQ reagents with mass/charges (m/z) of 114, 115, 116 and 117, respectively, are released for the quantitation. Schmelzle *etal.* applied iTRAQ–LC-MS/MS to the identification and quantitation of tyrosine phosphorylation sites in adipocytes [95]. In the study, digested peptides from the adipocytes with insulin stimulation for a various period of time (0, 5, 15 and 45 min) were labeled with iTRAQ reagents, followed by the analysis of the phosphorytyrosine peptide immunoprecipitation and the enrichment of phosphopeptide by immobilized metal affinity chromatography. After these steps, the peptides that enter the LC-MS/MS system for the final analysis are almost all phosphotyrosine peptides. The sequence identity and phosphorylation sites of the peptides were deduced from the MS/MS spectrum, while the relative levels of phosphorylated peptides were calculated based on the area of the isobaric tag masses 114, 115,116 and 117 m/z at the different time points.

In the study of tyrosin phosphorylation in insulin signaling in 3T3-L1 adipocytes [95], the aforementioned method allows identifying and quantifying tyrosine phosphorylation of insulin stimulation and studying temporal phosphorylation dynamics of insulin signaling pathway. Specifically, the digested peptides from insulin-stimulated adipocytes were labeled with iTRAQ reagents and combined for phosphotyrosine peptide immunoprecipitation, immobilized metal affinity chromatography, and LC-MS/MS. Using this approach, they were able to identify 122 sites in 89 proteins in response to insulin stimulation, including 38 novel sites of tyrosine phosphorylation. Quantitative analysis identified 69 sites that displayed at least 1.3-fold increase after insulin stimulation. A total of 12 phosphotyrosine peptides matching to nine proteins had a greater than tenfold increase following 5 min of insulin stimulation. These nine proteins were insulin receptor, IRS-1, and other insulin signaling components involving the regulation of the trafficking of insulin-responsive glucose transporter GLUT4, such as Munc18C, Gab1 and ERK1/2. Moreover, this approach was capable of detecting quantitative changes of different phosphorylated forms of proteins. For example, singly and doubly phosphorylated forms of the catalytic loop of the kinase domain were increased after 5 min of insulin stimulation. Six tyrosine phosphorylation sites on IRS-2 protein were identified and, for five of them, phosphorylation was increased after insulin stimulation. In addition, increased tyrosine phosphorylation of other substrates of insulin receptor, Gab 1 and APS, and SHC, activator of the MAPK pathway, was detected.

The SILAC method is a stable isotope labeling strategy for the *in vivo* incorporation of specific amino acids into all mammalian proteins. Mammalian cells are cultured in essential amino acid-deficient media supplemented with nonradioactive, isotopically labeled essential amino acids. Labeled essential amino acids are, therefore, incorporated into all newly synthesized proteins or proteome. Compared with chemical methods of isotope labeling such as ICAT, as a metabolic method of stable isotope labeling, SILAC provides more accurate quantitation of protein abundance since it can minimize possible manipulation errors during protein purification. Moreover, ICAT only labels cysteine-containing proteins, which account for approximately 70% in all proteins, while labeled amino acids incorporate into all proteins in SILAC. This method has been applied to many situations, such as the quantification of proteins in different conditions.

Shao-En Ong *etal.* applied this technique to quantify changes in protein-expression profiles during muscle cell differentiation [96]. Deuterated leucine (Leu-d3) was used as a supplemented labeled amino acid to incorporate into the cells. The full incorporation of Leu-d3 was observed after five passages. For the relative quantitative analysis, Ong *et al.* mixed the cell lysates from Leu-d0 and Leu-d3-labeled samples at the ratio of 1:1 and resolved proteins on a 10% SDS-PAGE gel. Gel bands were excised, in-gel digested and subjected to mass spectrometric analysis. Using this approach, several proteins were identified to be upregulated during muscle cell differentiation.

In recent years, SILAC has been employed in quantitative phosphoproteomics in several studies [97–100]. In this review, in-depth description will be given to the studies on phosphorylation of insulin-signaling pathway in adipocytes. Mann and coworkers used a strategy of GeLC-MS combined with SILAC and enrichment of phosphorylated proteins to identify the tyrosine-phosphoproteome in brown adipocytes in response to insulin stimulation [101]. In the experiment, 13C6 15N4-Arg (Arg-10) and 13C6 15N2-Lys (Lys-8) were used as heavy SILAC amino acids in one group of cells with insulin stimulation for 5 min and the other group of control cells without insulin stimulation were grown in an unlabeled medium. The mass difference of single-charged peptides derived from proteins of the two cell populations is either 10 or 8 Da. Tyrosine-phosphorylated proteins were first enriched from the lysates of the mixed cell populations by immunoprecipitation with anti-pY antibodies, separated by 1D gel electrophoresis and digested peptides were analyzed by MS. As a result, a total of 40 proteins were identified to be tyrosine-phosphorylated upon insulin stimulation and tyrosine phosphorylation of 33 proteins were quantitatively changed in response to 5 min of insulin stimulation.

Temporal dynamics of protein activation is also important for evaluating the role of signaling cascade in cellular responses. Toward this end, authors used a triple labeling SILAC strategy to monitor a time course of insulin activation of tyrosine phosphorylation. Three populations of cells were differentially labeled by Arg-0/Lys-0, Arg-6/Lys-D4 and Arg-10/Lys-8, and stimulated by insulin for different periods of times. In this case, a time profile for four different time points could be achieved by combining two of the three different labeled states. This method was capable of dissecting insulin-signaling pathway based on the temporal activation patterns. Insulin receptor was the first tyrosine phosphorylated within the first minute of stimulation and remained phosphorylated at a high level within the next 20 min. The activation kinetics of the substrate protein IRS-1 and Shc was similar to the insulin receptor, while the highest activation of IRS-2 occurred after 1 min of stimulation, but rapidly declined. The data suggests that IRS-1 is physically adjacent to insulin receptor and activation of IRS-1 and IRS-2 plays a different role in insulin signal transduction.

Finally, SILAC was applied to further characterize phosphorylation-dependent protein–protein interactions. For instance, the authors found from the aforementioned temporal dynamic

analysis that the PDZ-containing protein PISP/PDZK11 was highly activated at the early time of insulin stimulation and shares a very similar dynamic profile pattern with IRS-1, suggesting that this protein may be directly involved in the insulin signaling. In an attempt to characterize the proteins that potentially interact with PISP/PDZK11, the authors conducted peptide–protein interaction using the proteomic screen method, as described in an early study [102]. Basically, synthetic peptides in phosphorylated and nonphosphorylated forms were used as baits for affinity pull-down assay. The amount of proteins interacted with phosphorylated or nonphosphorylated peptides were determined by a quantitative proteomics method. In this recent study, phosphorylated and nonphosphorylated peptides derived from PISP/PDZK11 were synthesized and linked to desoxybiotin and the synthesized peptides were incubated with lysates of SILAC light- and heavy-labeled adipocytes, respectively. Peptides and bound proteins were purified by streptavidin-coated beads, and the resulting eluates were analyzed by MS. From this experiment, three proteins were found to specifically interact with the tyrosine-phosphorylated PISP/PDZK11; two of them are calcium-binding proteins SERCA2 and S100 calcium-binding protein A4. This finding suggests that insulin-activated PISP/PDZK11 is involved in calcium homeostasis and provides a possible connection between insulin and calcium signaling.

Carbonylation of adipose proteins in obesity

Oxidative stress is increased in obesity, which may contribute to obesity-linked insulin resistance. Oxidative stress is the condition that results from an imbalance between pro-oxidative and anti-oxidant action. Pro-oxidative conditions lead to lipid peroxidation and the production of reactive aldehydes such as trans-4-hydroxy-2-nonenal (4-HNE) and trans-4-oxo-2-nonenal. Conjugation of reactive aldehydes to glutathione *S*-transferase is one of the detoxifying mechanisms [103]. However, protein levels of glutathione *S*-transferase-4 in adipose tissue of obese mice were significantly decreased, leading to the increased reactive aldehydes and oxidative stress in obesity [60]. Grimsrud *et al.* attempted to address whether the levels of 4-HNE modified proteins are increased in obese adipose tissue and to identify the specific 4-HNE modified or carbonylated proteins in adipose tissue in obesity using proteomics approaches [60]. Biotin hydrazide method was used for the enrichment of aldehyde-modified proteins from soluble adipose proteins of obese mice via immobilized monomeric avidin affinity chromatography. The enriched proteins were then resolved by SDS-PAGE and the results revealed that the carbonylated proteins were approximately two- to threefold higher in obese adipose tissue than lean adipose tissue. Furthermore, the enriched proteins were digested with trypsin and further analyzed by LC-ESI MS/MS for identifying the specific carbonylated proteins. A total of 37 proteins were identified to be carbonylated in adipose tissue of obese mice. These proteins fall into the functional categories of carbohydrate and lipid metabolism, signal transduction, anti-oxidant enzymes/cell stress response, nucleic acid metabolism, protein synthesis/degradation, and structural/motor proteins. A-FABP was consistently identified to be modified with 4-HNE from both proteomics analysis and *in vitro* detection of the direct modification. Finally, authors conducted the experiment to identify the site of 4-HNE modification of A-FABP *in vitro*. His6-tagged A-FABP was incubated with 4-HNE and digested with trypsin or Glu-C. The resulting digested peptides were analyzed for 4-HNE-modified peptides by MALDI-TOF MS/MS. The result showed that A-FABP is modified by 4-HNE on Cys-117 residue *in vitro*. The 4-HNE-modified A-FABP displayed the decreased binding affinity for the hydrophobic probe 1,8-ANS. The information indicates that protein carbonylation may play a role in linking oxidative stress and insulin resistance.

Expert commentary

Dysfunction of adipose cells could be the major contributor for the development of insulin resistance and metabolic syndromes such as Type 2 diabetes. On one hand, the recently

recognized endocrine function of adipose tissue plays a primary role in the regulation of insulin resistance. A variety of proteomics approaches such as 2D-LC-MS/MS, 2D-gel coupled LC-MS/MS and SELDI have been applied to the identification of adipose secretome and the quantification of the changes in adipose secretome in insulin resistance and drug treatment in different adipose cell models and adipose tissue from both rodents and humans. A list of identified adipose secretome is now available, providing very useful information for further functional characterization of individual proteins. These studies have added and will add greatly to our understanding of the precise role of adipose tissue in insulin resistance. On the other hand, adipose tissue is the insulin-responsive tissue accounting for approximately 20% of insulin-dependent glucose uptake. In the insulin-resistant state, insulin signaling transduction in adipose cells and muscle is impaired as demonstrated by decreased insulin-stimulated GLUT4 translocation. Stable isotope labeling of iTRAQ and SILAC in combination with immunoprecipitation enrichment of phosphorylated peptides and LC-MS/MS offered the opportunity to identify and characterize insulin-stimulated protein phosphorylation or insulin signal transduction in adipocytes, which led to the discovery of the novel tyrosine phosphorylation sites and signaling components in insulin signaling pathway. More importantly, from dynamically profiling insulin-stimulated phosphorylation, potential different regulatory roles of components could be dissected. Furthermore, proteomics techniques are the useful tool to characterize carbonylation of adipose proteins in relation to oxidative stress and insulin resistance. All of the information gained from proteomic-based studies on adipocytes has provided the important information for the understanding of adipocyte biology.

Five-year view

The major challenges for the application of proteomics in adipocyte biology in the future exist as follows. First, identifying very-low-abundantce proteins and quantifying very small-fold changes are difficult with currently available proteomics tools. These low-abundance proteins or small changes could be the very critical regulatory events for maintaining normal functions of cells, and the regarded information could help understanding the critical regulatory mechanisms. Second, the information on fast regulation of protein secretion and on–off regulation of signaling transduction is still missing. Although two recent dynamic studies have provided promising results, more studies are needed. Third, the quantitative subproteome analysis of adipocytes for detecting the distinction between disease and nondisease state is lacking in most studies. Finally, knowing the contribution of individual cell populations in adipose tissue to the secretome is critical for better understanding the heterogeneity of adipose tissue as a function of health and disease. Current methods are limited in their ability to distinguish which cells account for the presence of individual secreted proteins. Therefore, cell sorting combined with proteomics will provide the powerful tools to address these issues and improve our understanding of adipocyte biology. These areas would be the focus in the future proteomics research. To achieve the aforementioned goals, improvements in techniques for the enrichment of low-abundance proteins and separation techniques for reducing the complexity of samples will be beneficial. Fortunately, the revolution of proteomics technology has just begun, and with improved protocols, instruments and bioinformatics tools, a global screening is possible, probably soon down to a few molecules per cell. Undoubtedly, proteomics studies are powerful as they offer a global view on the dynamic variations in any given proteome. However, they are not cheap and should be carefully considered.

Key issues

- Adipose tissue is an endocrine organ, secreting key metabolic regulators or adipokines.

- Adipose tissue is an important site for lipid metabolism and insulin-responsive glucose uptake.
- Adipose cells are widely used as a model system for studying insulin regulation of glucose transport activity and insulin resistance.
- The adipose secretome (adipokines) is a very interesting group of proteins that are potential molecular mediators and biomarkers for insulin resistance and diabetes.
- Powerful identification proteomics techniques are the key for identifying the complete list of adipose secretome.
- The use adipose cell models or adipose tissue for proteomics is also critical.
- A list of adipose secretome consistently detected from multiple studies is available for further functional characterization.
- Characterization of insulin signaling pathway in adipocytes is important for understanding the mechanism underlying insulin resistance.
- Analysis of insulin-stimulated phosphoproteome in adipocytes is promising and significant from both biological and proteomic points of view.

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204. TMHMM server www.cbs.dtu.dk/services/TMHMM

Table 1

Adipose secretome consistently identified in multiple studies.

Accession	Secreted protein	Species	Ref.
gi|115312	Collagen α1(IV) chain	Rat, 3T3-L1	[40,76]
gi|6680970	Procollagen, type V, α2	Rat, 3T3-L1	[40,76,80]
gi|115350	Collagen α2(IV) chain	Rat, 3T3-L1	[40,75,76]
gi|6679869	Insulin-like growth factor binding protein 7	Human	[40,79]
gi|817975	Laminin B	Human, rat	[40,79,80]
gi|6981168	Lipoprotein lipase	Rat, 3T3-L1	[40,75,80]
gi|18373301	Adiponectin	Human, rat, 3T3-L1	[40,75,76,79–81]
gi|25008127	Angiopoietin-like protein secreted by fat cells, fasting-induced adipose factor	Rat	[40,80]
gi|19705570	Angiotensinogen	Human, rat, 3T3-L1	[40,75,76,81]
gi|2507169	Antioxidant protein 2 (1-Cys peroxiredoxin)	Rat, 3T3-L1	[40,76]
gi|8392909	Apolipoprotein A-IV	Human, rat	[40,104]
gi|1351954	Apolipoprotein H	Human, rat	[40,104]
gi|2119394	Apolipoprotein J	Human, rat	[40,104]
gi|7549746	β-2-microglobulin	Rat, 3T3-L1	[40,76]
gi|11693172	Calreticulin	Human, rat	[40,79–81]
gi|544413	Cartilage glycoprotein-39	Rat	[40,80]
gi|12018262	Cathepsin B	Human, rat	[40,79,80]
gi|19705441	Cathepsin D	Human, rat	[40,79,81]
gi|11120688	CD14 antigen	Human, rat	[40,79]
gi|92783	Clusterin	Human, rat	[40,79,81]
gi|20302095	Complement component 1, s subcomponent	Human, rat	[40,79,81]
gi|7304937	Complement component 2	Human, rat	[40,79,81]
gi|20348280	Complement component 4	Human, rat	[40,79]
gi|2507246	Adipsin	Human, rat, 3T3-L1	[40,75,76,79,80]
gi|203592	C-reactive protein	Rat, 3T3-L1	[40,75]
gi|118185	Cystatin C	Human, rat, 3T3-L1	[40,75,79]
gi|27465565	Epididymal secretory protein 1	Rat	[40,80]
gi|4504165	Gelsolin	Human, rat, 3T3-L1	[40,75,76,79,80]
gi|25006237	GM2 activator protein	Rat	[40,80]
gi|123513	Haptoglobin	Rat, 3T3-L1	[40,75,76,80]
gi|123036	Hemopexin	Rat, 3T3-L1	[40,75,80]
gi|6996919	Complement component factor B	Human, rat, 3T3-L1	[40,75,79,80]
gi|16758120	Interleukin 12 p35 subunit	Rat	[40,80]
gi|5542285	Macrophage migration inhibitory factor	Rat, 3T3-L1	[40,76]
gi|13591991	Matrix metalloproteinase 2	Human, rat, 3T3-L1	[40,75,76,79,80]
gi|13591993	Matrix metalloproteinase 9	Human, rat, 3T3-L1	[40,75,79]
gi|1174697	Metalloproteinase inhibitor 1 precursor (TIMP-1)	Human, rat	[40,79,80]
gi|1326433	Neu-related lipocalin	Rat, 3T3-L1	[40,75,80]
gi|6754854	Nidogen; entactin 1	Human, rat	[40,79,80]
gi|2493471	Nucleobindin 1	Rat	[40,80]

Accession	Secreted protein	Species	Ref.
gi|7657429	Osteoblast specific factor 2	Human, rat	[40,79]
gi|600381	Osteonectin	Human, rat, 3T3-L1	[40,75,76,79–81]
gi|8394009	Cyclophilin A	Rat, 3T3-L1	[40,76,80]
gi|2500778	Pigment epithelium-derived factor precursor	Human, rat, 3T3-L1	[40,76,79,81]
gi|124096	Plasma protease C1 inhibitor	Human, rat	[40,79,80,81]
gi|129575	Plasminogen activator inhibitor-1	Human, rat, 3T3-L1	[40,75,79,81]
gi|21426805	Resistin	Rat, 3T3-L1	[40,75]
gi|20888903	Retinol-binding protein 4	Human, rat	[40,79,81]
gi|5454034	S100 calcium-binding protein, beta	Rat, 3T3-L1	[40,75]
gi|631854	S45683 metalloproteinase inhibitor 2	Rat, 3T3-L1	[40,76]
gi|13928716	Serine protease inhibitor 2c	Rat, 3T3-L1	[40,75,80]
gi|6175089	Serotransferrin	Human, rat	[40,79]
gi|20825636	Laminin gamma-1 chain	Human, rat	[40,79]
gi|27721871	Tetranectin	Rat	[40,80]
gi|16758644	Thioredoxin	Rat	[40,80]
gi|554390	Thrombospondin	Human, rat	[40,79,80]
gi|18056434	TNF receptor superfamily member	Rat	[40]
gi|1353213	Vimentin	Rat, 3T3-L1	[40,75,80]

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