REVIEW

Adipocyte 1:1, 25–37; January/February/March 2012; © 2012 Landes Bioscience

Proteomics in the characterization of adipose dysfunction in obesity

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Keywords: adipose tissue dysfunction, proteomics, obesity, insulin resistance, type 2 diabetes, adipose secretome, GLUT4 vesicle proteins, lipid-droplet proteins, mitochondrial proteins, phosphoproteome

Adipose tissue plays a central role in body weight homeostasis, inflammation, and insulin resistance via serving as a fat-buffering system, regulating lipid storage and mobilization and releasing a large range of adipokines and cytokines. Adipose tissue is also the major inflammation-initiated site in obesity. Adipose-derived adipokines and cytokines are known to be involved in the modulation of a wide range of important physiological processes, particularly immune response, glucose and lipid homeostasis and insulin resistance. Adipose tissue dysfunction, characterized by an imbalanced secretion of pro- and anti-inflammatory adipokines and cytokines, decreased insulin-stimulated glucose uptake, dysregulation of lipid storage and release and mitochondrial dysfunction, has been linked to obesity and its associated metabolic disorders. Proteomic technology has been a powerful tool for identifying key components of the adipose proteome, which may contribute to the pathogenesis of adipose tissue dysfunction in obesity. In this review, we summarized the recent advances in the proteomic characterization of adipose tissue and discussed the identified proteins that potentially play important roles in insulin resistance and lipid homeostasis.

Introduction

The prevalence of obesity has increased worldwide since 1980 in both rich and poor countries becoming a great health concern. In 2008, an estimated 1.46 billion adults were overweight while 502 million of these were obese.[1] Obesity is characterized by increased hypertrophy and/or hyperplasia of adipocytes due to the incorporation of triglycerides and is closely linked to other metabolic diseases like type 2 diabetes and cardiovascular disease. Once considered only an energy storage organ, it is now established that adipose tissue is a highly active metabolic tissue that produces a number of signaling molecules that regulate metabolism and energy homeostasis.[2,3] During obesity, the ability of the adipocyte to maintain normal secretion of these adipokines is compromised, leading to the development of an inflammatory state that is believed to underlie insulin resistance.[4] Additionally, the expressions and activities of intracellular proteins such as those localized to lipid droplets and in mitochondria are altered during obesity. Proteomic approaches to determine the concentrations of secreted proteins and intracellular proteins, as well as post-translational modifications, can be used to characterize the proteome of adipose tissue in normal and obese states.

White adipose tissue (WAT) is the major site of energy storage for mammals and contains adipocytes that store excess non-esterified fatty acid (NEFA) as triglycerides (TAG) in lipid droplets during nutritional abundance. Lipid droplets are subcellular compartments containing proteins which regulate the storage and hydrolysis of TAG. During times of energy demand, NEFA are cleaved from TAG and released into the circulation for β-oxidation in peripheral tissues. Adipocytes have a large capacity to store and handle TAG as lipid droplets without succumbing to the lipotoxic effects typically experienced by non-adipose tissues. It is believed that lipotoxicity caused by excessive deposition of NEFA in non-adipose tissues is, in part, responsible for the development of insulin resistance. Depending on the nutritional status of the body, adipocytes constantly go through cycles of lipogenesis and lipolysis to maintain energy homeostasis. However, this process can become disrupted during obesity as the production of inflammatory cytokines becomes upregulated, increasing basal lipolysis. Moreover, dysfunctional adipose tissue produces excess extracellular matrix components that restrict adipose mass expansion further contributing to uncontrolled lipolysis.[5] Abnormally high rates of lipolysis in adipocytes will increase the circulating level of NEFA, which are then taken up by the liver and muscle tissue. This, along with the abnormal secretion of adipokines is how adipocyte dysfunction contributes to lipotoxicity and insulin resistance. Therefore, in-depth analyses of the adipocyte proteome are essential for investigating the multitude of human diseases that are linked to the abnormal regulation of adipocyte functions. Characterizing the secretome of dysfunctional adipocytes will aid in the detection of diagnostic or therapeutic biomarkers for obesity-related metabolic diseases.

Proteomics and Adipocyte Dysfunction

Microarray techniques have been used to investigate global gene expression differences in human adipose tissue of obese and lean individuals[6] as well as changes during weight loss.[7] While

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Submitted: 12/19/11; Accepted: 12/20/11
http://dx.doi.org/10.4161/adip.1.1.19129

transcriptome analysis has been useful for examining the mechanisms of adipocyte differentiation during the development of obesity,[6] it is limited when it comes to defining proteome expression and functionality. Gene expression does not correlate with protein concentration in biological samples for many reasons.[8] Some of these include translational efficiency of the mRNA,[9] regulation by sRNA[10] and differences in protein turnover rate.[11] Furthermore, post-translational modifications such as phosphorylation are essential for rapid changes in cell function and cannot be identified measuring the transcriptome. Therefore, to accurately identify key molecular factors that affect lipid homeostasis in adipocytes, methods that characterize the proteome, rather than gene expression alone, are necessary.

While microarrays have made it relatively easy to quickly identify gene expression in extracted mRNA, the process of measuring the proteome in biological tissues is much more difficult, requiring extensive purification techniques and expensive equipment. Mass spectrometers have allowed researchers to quickly determine the amino acid sequence and identify proteins using online databases. However, the use of mass spectrometry (MS) with biological samples requires various preparation strategies depending on sample complexity and objective of the analysis. Due to the enormous complexity of biological samples such as adipose tissue cell lysates, separation techniques prior to analysis are needed to identify low-abundance proteins. Cell lysate samples contain thousands of proteins that may vary up to 5 orders of magnitude, limiting the potential for global proteome analysis by MS.[12] This causes difficulty during identification because high-abundance signals may mask detection of the low-abundance proteins. Therefore gel-based methods like 2-dimensional gel electrophoresis (2D PAGE) and gel free technologies such as HPLC are routinely used to simplify the analyte for more narrow detection.[13,14]

Moreover, many proteins in the adipocyte proteome are post-translationally modified to further complicate protein identification.[15] Prior to MS analysis, protein samples may be processed into plasma membrane, cytosolic, mitochondrial or lipid droplet fractions to reduce the peptide load on the instrument but maintain accuracy.[16] As adipocyte dysfunction during hypertrophy typically involves the dysregulation of proteins from many subcellular locations, fully characterizing the proteome requires extensive preparation and instrument running time. Due to the significance of adipokines and cytokines and their wide spread functions throughout the body, the adipocyte secretome is of particular interest when examining adipocyte dysfunction. Adipocyte-secreted proteins have been extensively studied since the discovery of leptin and may be the first step in the pathogenesis of co-morbidities in other tissues and organs.[17]

Proteomics in Adipose Tissue Secretome

Expansion of adipose tissue alters the secretion profile of adipokines and cytokines which then act in a paracrine or endocrine manner and have positive or negative effects on body weight and insulin resistance. One such adipokine whose increased secretion correlates with adipose mass is leptin. Leptin regulates feeding behavior via receptors in the brain to maintain energy homeostasis. Often during obesity, the increased plasma concentration of leptin does not yield the proper anorexic response, suggesting a degree of leptin resistance.[2] Moreover, monocyte chemotactic protein-1 (MCP-1) and plasminogen activator inhibitor 1 (PAI1) are two inflammatory cytokines that are also upregulated during obesity and have been shown to contribute to insulin resistance.[18,19] Most notably, adipose tissue expression of tumor necrosis factor α (TNFα) is increased during obesity and will interrupt the insulin signaling pathway in adipocytes, indicating one possible link between adipocyte dysfunction and type II diabetes.[20,21] Another adipokine that may be involved with the development of insulin resistance is retinol binding protein 4 (RBP4), possibly again, through interrupting insulin-dependent glucose uptake.[22] While most cytokines are considered harmful, adipose tissue also secretes a smaller number of beneficial proteins.[23] The adipokine adiponectin is inversely associated with adiposity and may improve glucose control through activation of AMPK in muscle tissue.[24] The regulated secretion of these and other adipokines are critical for whole-body metabolic homeostasis however they can also have deleterious effects when adipose tissue becomes dysfunctional. Although these adipokines and cytokines all have individual effects contributing to metabolic dysregulation, it is likely that this condition arises from an altered secretion of a combination of adipokines, therefore, the secretome of adipocytes requires thorough analysis to discover additional adipokines and to determine the extent of their secretion during adipocyte dysfunction. The use of mass spectrometry and improved separation techniques has advanced our understanding of adipose tissue secretory dysfunction and its role in insulin resistance and diabetes. In the past 10 years, various proteomic approaches have been developed and applied to identify and quantify the adipose secretome in the pathophysiological state.[25]

Identifying adipose secretome during adipogenesis. While selectively blocking secretion pathways, Wang et al. screened the secreted proteins during differentiation of 3T3-L1 adipocytes using a 2D PAGE separation combined with matrix-assisted laser desorption ionization (MALDI) detection.[26] Secretion pathways were blocked by incubating cells in either Brefeldin A or 20°C or both, inhibiting ER-to-Golgi vesicular transport and ER/Golgi independent pathways respectively.[27,28] Of the 41 proteins identified as secreted proteins, a majority of them were involved in the extracellular matrix, lipid metabolism or growth regulation. Aside from established adipokines such as adiponection, adipsin and matrix metalloproteinase 2, a total of 13 other proteins had not been previously reported as being secreted by adipocytes. It is important to note that this method was not able to identify some well-known adipokines, including leptin, resistin, plasminogen activator inhibitor-1 and vascular endothelial growth factor. A limitation of 2D PAGE is that it may be difficult to discern spots on the gel from low abundance proteins that may migrate similarly to high abundant or statically expressed proteins. Moreover, 3T3-L1 cells are an immortalized cell line and may not reflect the physiology of primary adipocytes. It also must be noted that adipocytes represent only one fraction of the adipose

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tissue. Cells contained in the stromal-vascular fraction also contribute to the secretome of adipose tissue.[29] Acknowledging the limitations of 2D PAGE, a recent study examining the secretome of primary human adipocytes used not only 2D PAGE MALDI but also 1D PAGE electrospray ionization (ESI) MS/MS and combined the results to achieve a more comprehensive analysis.[30] LC-ESI-MS/MS has the advantage of using the entire extracted protein sample rather then select spots on a 2D gel and allows for a higher throughput and increased sensitivity.[31] In conditioned media from differentiating adipocytes, a total of 263 secreted proteins were identified using MASCOT database searches with 44 of these being novel adipokines that had not been previously described. Selected adipokines were further validated to assess their biological relevance by measuring them in the plasma and adipose tissue of obese and lean patients. Complement factor H, which is involved in the innate immune system[32] and heme oxygenase-1 with the reduction of oxidative stress,[33] were increased in the plasma and adipose tissues of obese patients respectively, suggesting that these may be biomarkers for obesity and metabolic disease. In another study analyzing the secretome of primary human subcutaneous-derived stem cells, Zvonic et al. used 2D PAGE and quadrupole time-of-flight (TOF) mass spectrometry to identify secreted proteins during differentiation.[34] Interestingly, several serum protease inhibitors were increased after differentiation, including pigment epithelium-derived factor (PEDF), PAI1 and protease C1 inhibitor.

Identifying adipose depot-specific secretome. The adipose tissue in the body is segmented into different depots and it is becoming increasingly clear that the anatomical location of adipose tissue can affect the degree of metabolic dysregulation and accompanying diseases such as dyslipidemia and insulin resistance.[35,36] Visceral adipose tissue is considered more metabolically active as it has been shown to secrete increased levels of inflammatory cytokines and less adiponectin compared with subcutaneous tissue.[37] Higher amounts of visceral adipose tissue are associated with an increased risk of obesity-related comorbidities.[38,39] Clearly, determining the secretome of different depots is of great importance. A recent study identified the secretome of visceral, gonadal and subcutaneous adipose tissue using 2D PAGE MALDI-TOF/TOF.[40] The type of identified proteins varied depending on the anatomical location of the tissue and relationships between identified proteins were determined using interaction pathway analysis software to cluster their biological processes for each adipose depot. Using interactive pathway analysis software, visceral adipose tissue secreted proteins were grouped as involved in carbohydrate metabolism and the JAK-STAT and Ras-Raf-ERK signaling pathways while subcutaneous adipose tissue secreted many proteins implicated in free radical scavenging. Lastly, gonadal tissue characterized proteins associated with luteinizing hormone and follicle-stimulating hormone. Importantly, this analysis identified known adipokines such as adiponectin, retinol-binding protein 4, angiotensinogen, collagen and gelectin-1. Surprisingly, leptin was not identified using this proteomics approach, although it was confirmed by antibody using western blot. It must be noted however, that leptin was missing from other proteomics studies using different procedures.[13,41] Nevertheless, this study establishes a location-specific secretome database and illustrates the roles different adipose tissue depots play.

As adipose tissue contains many different populations of cells, each of which may have a unique role contributing to adipose tissue function, Kheterpal et al. profiled the proteome of stromal-vasular (SV) cells and mature adipocytes from human subcutaneous adipose tissue using DIGE, a quantitative 2D gel-LC-MS/MS method.[42] A total of 200 proteins were found to be differentially expressed between adipocytes and SV cells. Bioinformatic analyses of the data revealed that these differentially expressed proteins are involved in cytoskeletal, glycogenic, lipid metabolic and oxidative stress-related pathways. The proteins induced during adipocyte differentiation such as carbonic anhydrase, fatty acid binding protein, peroxiredoxin and superoxide dismutase (SOD) were more abundant in mature adipocytes, while proteins with reduced expression during adipogenesis, for instance annexin, were preferentially expressed in SV cells of the subcutaneous adipose depot. These proteins are potentially valuable biomarkers reflecting the functional state of the cell populations in the subcutaneous adipose depot.

Quantifying adipose secretome changes in obesity and insulin resistance. A key component in large-scale proteomic analysis of biological systems is the ability to quantitate the identified proteins. Quantification allows a more thorough understanding of the contribution of adipocyte dysfunction in the pathogenesis of metabolic disease. Stable isotope labeling using ^{18}O H_2O during proteolytic digestion has been successfully used as a means of quantification during mass spectrometry analysis.[43,44] The hydrolysis of proteins in ^{18}O H_2O during digestion by a protease results in the incorporation of two ^{18}O atoms to the carboxyl terminus of the new peptide fragment. By combining equal amounts of two digested protein samples, one labeled with ^{18}O and another with ^{16}O, prior to mass spectrometry, a ratio can be determined and relative protein concentrations quantified. Chen and colleagues used this method to quantify the secretome of visceral adipose tissue from obese rats with and without thiazolidinedione (TZD) treatment.[43] TZD is a PPARγ agonist that has been shown to restore adipocyte dysfunction-related disorders like elevated inflammatory activity and insulin resistance.[45] By analyzing trypsin-digested, column separated protein fractions by LC-MS/MS with quadrupole TOF and quantification proteomics, 77 proteins were identified to have altered secretion levels after 12 d of in vivo TZD administration. These adipokines include inflammatory molecules, extra cellular matrix proteins, collagens and immune factors and may be candidates partly responsible for the improved adipocyte functionality and insulin sensitivity observed with TZD treatment. In support of this, a separate experiment showed that the majority of TZD-suppressed adipokines were found to be higher in the obese rat model compared with the lean, implying that TZD administration may modify the secretome to resemble adipose tissue in the lean state. Ahmed et al. performed a study identifying the human adipose tissue proteome changes induced by TZD using a 2D gel coupled to LC-ESI-MS/MS approach.[46] In the study, ten moderately obese but otherwise healthy subjects were treated with

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Rosiglitazone (Rosi), a member of the TZD class of drugs, for 14 d; the proteomic analysis demonstrated that there were 122 proteins differentially expressed in Rosi-treated adipose tissue when compared with non-treated controls. Further LC-MS/MS analysis led to the identification of specific proteins that were up or downregualted by Rosi in obese human subjects. Rosi treatment resulted in the upregulation of proteins involved in GLUT4 vesicle transport and fusion, lipid metabolism and storage, signaling, extracellular matrix proteins, redox regulation and adipogenesis. These findings suggest the mechanisms by which Rosi exerts to improve adipose tissue function and insulin sensitivity is through increasing the glucose uptake and inducing the differentiation of insulin sensitive adipocytes to allow normal lipid storage capacity.

In addition to comparing protein samples from obese and lean states, it is also essential to examine the secretome of adipocytes that have shifted from an insulin sensitive to an insulin resistant state. Lim et al. were able to induce insulin resistance in primary rat adipocytes by increasing O-linked *β-N*-acetylglucosamine (O-GlcNAc) modification of intracellular proteins through inhibition of the enzyme O-GlcNAcase.[47] This was achieved by harvesting the culture media of adipocytes in the presence of the O-GlcNAcase inhibitor PUGNAc. A proteomic analysis of secreted proteins was conducted using a shotgun approach where the entire protein sample was digested by trypsin prior to separation and subsequent mass spectrometry analysis. To increase the number of identified adipokines, both 1D LC-MS/MS and 2D RP-LC-MS/MS techniques were used and searched against protein databases using SEQUEST. Twenty proteins were identified that changed secretion levels during insulin resistance. In agreement with previously mentioned studies, many of the altered proteins are involved in the immune response, extracellular tissue remodeling and oxidative stress. For example, complement component 6, metalloproteinase inhibitor 2, cathepsin B, lipoprotein lipase, thioredoxin 1 and gelsolin were also found to be altered in the primary adipocytes from Zucker obese rats during TZD treatment.[43]

Quantifying adipose secretome changes in the inflammatory state. One cellular mechanism believed to underlie the adipocyte dysfunction and inflammatory state during obesity may be the hypoxic response of adipocytes during hypertrophy.[48,49] Hypoxia triggers large changes in gene expression by increasing activity of the transcription factor hypoxia inducible factor 1α (HIF-1α).[50] Increased transcription of inflammatory genes is accompanied by increased protein secretion of IL-6, leptin, macrophage migration inhibitory factor and vascular epithelial growth factor, all of which contribute to metabolic dysfunction.[51] Indeed, glucose transport is attenuated in mouse adipocytes exposed to low oxygen[52] and the phosphorylation of insulin receptor-B and insulin receptor substrate-1 is reduced in human adipocytes via a HIF-1α-dependent mechanism.[52] The significance of HIF-1α on protein expression warrants investigation into the proteomic changes during low oxygen environments. To quantify proteome changes during hypoxia, Choi et al. used isobaric tags for relative and absolute quantification (iTRAQ) combined with 2D MALDI-TOF in 3T3-L1 cells incubated under normal and hypoxic conditions.[53] Quantification by iTRAQ requires protein samples to first be digested into peptides and then incubated with an isotope coded tag that covalently attaches to the N-terminus and side chain amines.[54] Differentially labeled peptide mixtures are pooled and the different tags produce unique reporter ions after fragmentation that can be used for quantification. Hypoxic conditions induced the change of 96 proteins, including those related to stress response and protein elongation. This is the only study examining the cell proteome in adipocytes under induced hypoxia but unfortunately, secretome changes induced by hypoxia are currently not known. As 3T3-L1 cells only represent the adipocyte fraction of adipose tissue, the contribution of the SV fraction to secreted adipokines during hypoxia cannot be ignored.[55]

Functional characterization of novel adipokines. Researchers have taken an important step toward the understanding of adipocyte function by characterizing the role of novel adipokines identified through proteomic approaches in metabolism and insulin resistance. Dipeptidyl peptidase 4 (DPP4) has been recently identified as a new adipokine from a comprehensive proteomic profiling of the human adipocyte proteome.[56] Dipeptidyl peptidase 4 (DPP4) is a serine exopeptidase that cleaves N-terminal dipeptides from a variety of substrates, including growth factors, hormones, neuropeptides and chemokines.[57] It is a ubiquitously expressed transmembrane glycoprotein anchored to the cell membrane by its N-terminal end. Glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) are two substrates of DPP4 that are released from the intestinal mucosa and responsible for 60% of postprandial insulin secretion during the so-called incretin effect.[58] Substantial DPP4 activity is also found in plasma and other body fluids because of a soluble form of DPP4 lacking the cytoplasmic tail and transmembrane region of this protein.[59] Both membrane abundance and circulating activity of DPP4 have been found to be altered in a variety of neurologic and inflammatory diseases.[57] In a recent study, DPP4 expression was reported to be significantly upregulated during the differentiation of human adipocytes.[56] Both insulin and TNFα significantly stimulate the release of DPP4 from human adipocytes, while much lower levels are secreted from adipose tissue-derived macrophages. An in vitro study showed that DPP4 directly acts on adipocytes leading to reduced insulin-stimulated Akt phosphorylation. Studies in humans demonstrated that serum levels of DPP4 were significantly elevated in obesity, suggesting that DPP4 may have an important role in obesity and insulin resistance.

Lipocalin 2 (Lcn2), also known as neutrophil gelatinase-associated lipocalin (NGAL) or 24p3, has been identified as a novel adipokine from high-throughput proteomic[60] and microarray screening analyses.[61] LCN2 is a 25 kDa secreted protein initially identified from human neutrophils[62,63] and other immune cells exposed to microorganisms in the respiratory and gastrointestinal tract. Lipocalins have structural similarity with fatty acid binding proteins (FABPs) and both are members of the multigene family of up and down β-barrel proteins.[64] Both the intracellular FABPs and the extracellular lipocalins have a clearly defined β-barrel motif that forms either an interior cavity (FABP)

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or a deep pit (lipocalins) that constitutes the lipid binding domain.[64] The extracellular lipocalins such as LCN2, RBP4 and α_2-microglobulin use a series of β-strands to form a globular domain with a deep depression resembling the calyx of a flower. Because of their unique structure, the lipocalins function as efficient transporters for a number of different hydrophobic ligands in the extracellular milieu including retinoids, fatty acids, biliverdin, pheromones, porphyrins, odorants, steroids and iron. RBP4, one of the extracellular lipocalins, affects glucose metabolism and insulin sensitivity.[65]

A number of studies have shown that the Lcn2 gene promoter region contains multiple transcription factor binding sites and nuclear receptor response elements including nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and CCAAT-enhancer-binding proteins (C/EBP),[66] retinoic acid response element (RARE)[65,67] and glucocorticoid response element (GRE).[68,69] Additionally, an estrogen response element has also been identified in the promoter region of both the human and mouse lipocalin 2 genes,[70,71] suggesting that Lcn2 is a putative direct target gene of estrogens. LCN2 gene expression is strongly upregulated by the inflammatory inducers lipopolysaccharide (LPS) and TNFα.[63] Lcn2 deficiency results in an increased susceptibility to bacterial infection in mice due to its role as an iron transport protein.[72-74] Lcn2 can restrict the bacterial uptake of iron by sequestrating the iron-laden bacterial siderophores, preventing their transport back into the bacterium.[73,75] Previous studies have demonstrated that LCN2 gene expression is upregulated in the adipose tissue and liver of genetically obese animals.[76,77] Rosiglitazone administration significantly reduces LCN2 expression in adipose tissue of obese animals[76,78] suggesting that the protein may function as a proinflammatory factor.

Most importantly, Lcn2 has been recently characterized as a critical regulator of energy metabolism, glucose and lipid homeostasis and insulin resistance in Lcn2-deficient male mice.[79-81] Lcn2 deficiency led to a significant metabolic phenotype of increased body fat mass, exacerbated adipocyte hypertrophy, dyslipidemia, fatty liver and insulin resistance upon high fat diet feeding. Although the role of Lcn2 in obesity and insulin resistance is still controversial mainly due to the discrepancy of the results between three groups,[82-84] our group additionally discovered that Lcn2 deficiency significantly affects energy metabolism, leading to impaired thermoregulation in mice. It is likely that this thermogenic defect makes $Lcn2^{-/-}$ mice more sensitive to the differences in ambient temperature and diets as discussed by Jun et al.,[83,85] contributing in part to the inconsistent results among different laboratories.

Additionally, our recent studies characterizing the metabolic role of LCN2 in female mice[86] showed that Lcn2 expression in white adipose tissue is gender, depot and age-dependent. In female mice, Lcn2 is predominantly expressed in inguinal adipose tissue but at relatively low levels in the perigonadal depot and the ovary. After 22-week high fat diet (HFD) feeding or at old age, *Lcn2*$^{-/-}$ female mice had significantly reduced levels of serum 17β-estradiol and downregulated expression of estrogen receptor α (ERα) in multiple metabolic tissues. In line with the dysregulated expression of estrogen-regulated genes involved in cholesterol homeostasis such as liver X receptor β (LXRβ) and low-density lipoprotein (LDL) receptor, female *Lcn2*$^{-/-}$ mice developed atherogenic dyslipidemia in response to HFD feeding. Interestingly, when compared with wild-type controls, HFD-fed female *Lcn2*$^{-/-}$ mice had significantly reduced expression levels of aromatase, a key enzyme regulating estradiol biosynthesis, in adipose tissue. Moreover, Lcn2 deficiency markedly blunted the age-related increase in adipose aromatase expression, but had no significant impact on age-related reduction in ovarian aromatase expression. These findings suggest that Lcn2 has a tissue-specific role in adipose estradiol biosynthesis, which may link Lcn2 to obesity- and age-related estradiol production and metabolic complications in females.

Proteomics in GLUT4 Vesicle Proteins

The inefficient action of insulin on glucose uptake into skeletal muscle and adipose tissue is one of the important characteristics of metabolic dysregulation during insulin resistance and type 2 diabetes. The insulin-regulated translocation of GLUT4 from intracellular compartments to the cell surface plays a major role in mediating insulin-stimulated glucose uptake in adipose tissue and muscle. The insulin signal transduction responsible for the regulation of GLUT4 translocation in adipocytes has been well studied over the past 30 years. In the basal state, the majority of GLUT4 resides in a specialized intracellular tubulovesicular compartment, known as 'GLUT4-containing vesicles.' Insulin stimulation induces a rapid subcellular redistribution of GLUT4 from the intracellular pool to the plasma membrane. However, insulin stimulates the translocation of only 50% of the intracellular GLUT4 to the plasma membrane in adipocytes.[87-89] This observation, together with the evidence from kinetic studies of GLUT4, suggests that multiple GLUT4-containing compartments exist in adipocytes.[90-92] Based on these results, it has been proposed that insulin only induces the cell-surface translocation of a specific population of GLUT4 vesicles called GLUT4 storage vesicles (GSVs), while the vesicles that are not responsive to insulin are referred to as precursor GLUT4-rich vesicles. This new concept opens up questions regarding the mechanism of how GLUT4 translocation is regulated. Identifying the protein composition in insulin responsive GLUT4 storage vesicles and characterizing the interaction of GLUT4 storage vesicle proteins with insulin signaling components as well as the consequences of these interactions in regulating GLUT4 translocation are still an important area.

In early studies, several laboratories purified GLUT4-containing vesicles including insulin responsive GSVs and non-responsive precursor GLUT4-rich vesicles and identified several GLUT4 vesicle-associated proteins such as the Rab proteins (Rab10, Rab11 and Rab14),[93] vimentin and α-tubulin;[94] the role of these proteins in insulin-regulated GLUT4 translocation have been characterized. In a recent study, Jedrychowski et al. developed an immunopurification method to specifically purify the insulin responsive GLUT4 vesicles and then analyzed the protein composition using LC-MS/MS.[95] To purify insulin-responsive

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GSVs, the precursor GLUT4-rich vesicles were first immunodepleted using anti-Cellugyrin antibody. Cellugyrin was identified in GLUT4-containing vesicles, but it is not translocated to the plasma membrane in response to insulin stimulation. Thus, cellugyrin is considered only specific to precursor GLUT4-rich vesicles. Using this approach, more than 100 proteins were identified in GSVs and precursor vesicles. After the removal of likely contaminants that also bind to control IgG in significant amount, 50 of the identified proteins are novel proteins in terms of their roles in GLUT4 trafficking. In addition, ICAT labeling was applied to monitor the insulin-regulated translocation of proteins in GVSs, leading to the identification of several insulin-regulated translocation proteins including the new protein low-density lipoprotein receptor-related protein 1 (LRP1). Further confirming studies by conventional methods showed that LRP1 is an important component of GVSs in that it interacts with the lumen domain of GLUT4 and AS160, an insulin-signaling pathway target. Knocking out LRP1 in 3T3-L1 adipocytes and in mice leads to a decreased GLUT4 expression and a reduction in insulin-stimulated glucose uptake in adipocytes. These results suggest that LRP1 potentially plays an important role in the regulation of GLUT4 translocation and insulin resistance in adipocytes.

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Proteomics and Mitochondria Dysfunction

Another factor to consider when defining metabolic deregulation during obesity is the role mitochondria dysfunction can have in the adipocyte. Indeed, WAT from obese mice have roughly 50% less mitochondrial mass compared with lean controls[96] and reduced mitochondrial mass and lower oxidative capacity have been shown in WAT of obese and insulin resistant individuals.[97-99] Interruption of mitochondrial DNA replication by knockdown of the mitochondrial transcription factor A was found to reduce insulin-stimulated glucose uptake.[100] Likewise, glucose uptake was also shown to diminish when decreasing mitochondrial respiratory activity using chemical uncoupling inhibitors.[101] The increased lipolysis in adipocytes during obesity provides fatty acid substrate and increased oxidation in the mitochondria leading to an increased generation of reactive oxygen species (ROS). Although increased ROS appear to have damaging effects on the adipocyte such as increased expression of inflammatory adipokines and insulin resistance, the mechanisms of this process are still unclear.[102,103] While there is a need for more information on the impact mitochondrial dysfunction has in obesity, few proteomics studies have identified proteins from isolated mitochondria specific to adipose tissue.[104,105] To examine the effect of increased lipolysis in adipocytes further, Lee and colleagues increased lipolysis in 3T3-L1 adipocytes by two ways, treatment with either isoproterenol or TNFα, and then identified differences in isolated mitochondrial proteins by 2D PAGE LC-MS/MS.[106] Isoproterenol is a β-adrenergic receptor agonist that will activate lipolysis in adipocytes by increasing intracellular cyclic AMP (cAMP) through increased adenylyl cyclase activity. TNFα also increases cAMP but through activation of mitogen-activated protein kinase kinase (MEK) and extracellular signal-related kinase (ERK). β-adrenergic stimulation increased lipolysis but also had the beneficial effect of increasing the expression of proteins involved in energy production, FA oxidation, TCA cycle and oxidative phosphorylation. Meanwhile, TNFα, a known pro-inflammatory cytokine, also increased lipolysis but did not increase the complementary expression of proteins related to fatty acid oxidation, suggesting a possible link between mitochondrial dysfunction and increased TNFα concentration during obesity. It is important to consider the protein fraction analyzed when attempting to characterize the mitoproteome of cells and its connection to biological processes. An advantage of the aforementioned study is that in addition to analyzing isolated mitochondria, thereby correcting for interference from cellular proteins, functional assays were performed to assess lipolysis and fatty acid oxidation. However, a cell-wide proteomic analysis may in fact be more useful in conjunction with the mitoproteome data considering the complicating effect of mitochondrial biogenesis.[107]

Mitochondria are specialized for different functions depending on the type of tissue they are located in. Mitochondria in the brown adipose tissue (BAT) are able to convert high energy molecules generated during metabolism to heat via uncoupling protein 1 (UCP1).[108] Although once considered metabolically important only in small mammals and infants, advances in technology have shown that adult humans also have areas of metabolically active BAT.[109] It has been suggested that the uncoupling properties of BAT could play an important role in increasing energy expenditure and as a possible treatment for obesity.[110] Proteomics technology has been used to gain an understanding of the BAT mitoproteome and how it is distinctly different from WAT. In a recent study, mitochondria were isolated from BAT and WAT and their mitoproteomes compared quantitatively using stable isotope labeling by amino acids in cell culture (SILAC) technology.[111] The SILAC approach is different than ^{18}O and iTRAQ labeling methods in that instead of labeling proteins during the extraction and digestion process, proteins are labeled during the protein translation process in cell culture. Cells are grown in media that contain heavy labeled amino acids, typically Leu, Arg, Lys or Tyr, which are then incorporated into the newly synthesized proteins.[112,113] In this way, two different cell culture protein fractions can be combined and analyzed by LC-MS/MS for relative quantification. Aside from quantitative differences in protein expression, mitochondria from WAT and BAT contain different isoforms that illustrate the specialized energy management functions of each tissue. An increased protein level of acyl-CoA synthetase isoforms ACSS1 and ACSL5, which activate fatty acids for β-oxidation, as well as the carnitine palmitoyltransferase 1-M isoform, responsible for fatty acyl import into the mitochondria, were at much higher levels in the BAT as compared with the WAT. Moreover, a cluster analysis revealed similar features of the BAT and muscle mitoproteomes supporting findings that BAT and muscle share the same progenitor cell.[114,115] Lastly, enzymes that catalyze the pathway to form triglycerides and phospholipids were found at higher levels in the WAT, perhaps suggesting an increased capacity for TAG synthesis.

Proteomics in Lipid-Droplet Associated Proteins

Adipose tissue is a critical site of fatty acid storage and release. Fatty acids are primarily stored as triglycerides in cytoplasmic lipid droplet organelles in adipose and other types of cells. The traditional view of lipid droplets (LDs) as passive storage structures of lipids has been changed in the last few years. LDs, indeed, are now known to be dynamic organelles that have a key role in the cellular turnover of lipids. The storage and mobilization of lipids in and out of LDs are tightly regulated; dysregulation of this process contributes to an increased fatty acid release from adipose tissue, which is associated with obesity, dyslipidemia, ectopic fat deposition and insulin resistance. Cytoplasmic lipid droplets comprise a core of neutral lipids and a phospholipid monolayer surface containing cholesterol and proteins. LD proteins localized on this surface play crucial roles in the stabilization of LDs and control the lipolytic process in coordination with lipases. Over the last two decades, a number of lipases and lipid droplet proteins have been discovered and the functions of these LD proteins have been characterized as very critical regulators of lipid homeostasis and energy metabolism during obesity.[116-118] Proteomic technology has been a significant contributor in the discovery of the LD proteins and advancing our understanding of LD biology and their role in the regulation of lipid homeostasis, adipocyte function and obesity-related complications.

Proteomics in lipase and LD proteins has been studied in many different tissues and cell types. *Saccharomyces cerevisiae* and Drosophila have been powerful models in proteomic studies for identifying novel LD proteins. Many studies have also been performed in rodent as well as human cells and tissues. Proteomic analyses of LDs from various tissues and cell types have revealed the existence of many LD-associated proteins, including specific marker proteins, structural proteins, enzymes involved in various aspects of cholesterol and fatty acid metabolism and proteins that function as regulators of membrane trafficking.[119-131] Cermelli and colleagues performed a proteomic analysis to identify LD-associated proteins in Drosophila.[130] They purified lipid droplets from Drosophila embryos and analyzed the proteins associated with LDs using capillary LC-MS/MS. This proteomic approach allowed the identification of a number of proteins with important functions in lipid metabolism including enzymes, signaling molecules and membrane trafficking proteins. Surprisingly, the histones H2A, H2Av and H2B were found to be physically associated with lipid droplets. This association appeared during oogenesis and becomes prominent in early embryos, but disappears in later stages of development in Drosophila. The disappearance of histones on lipid droplets is likely due to their transfer from droplets to nuclei as development progresses. Based on these observations, they speculate that lipid droplets may also serve as transient storage sites for sequestrating excess proteins when their binding partners are inadequate in the cell.

Using fluorescently labeled activity tagging combined with analysis by two-dimensional gel electrophoresis and LC-MS/MS Birner-Gruenberger et al. identified and profiled the lipolytic proteome of mouse adipose tissue. A set of fluorescently (NBD) labeled activity tags could mimic substrates for lipases, including NBD-HE-HP (a single-chain carboxylic acid ester), NBD-sn1-TG and NBD-sn3-TG (enantiomeric triacylglycerol analogs) and NBD-CP (a cholesteryl ester).[132] The fluorescent tags react with lipolytic enzymes forming stable probe-protein complexes via a covalent attachment to the nucleophilic serine in the active site of lipolytic enzymes. These stable probe-protein complexes can then be analyzed on the basis of their fluorescence after electrophoretic separation. To achieve a higher resolution, cytoplasmic extracts of white and brown adipose tissue were labeled with the activity tag and separated by 2D PAGE. The visualizations of fluorescently labeled whole proteins of white and brown adipose tissue on the gels stained with SYPRO Ruby showed significantly different patterns. Further LC-MS/MS analysis led to the identification of known lipases including lysophospholipases 1 and 2, MGL, HSL and TGH as well as an additional ten murine lipases and esterases that so far have not been fully characterized. Most importantly, this method allowed identifying a patatin-like phospholipase, which was thereafter characterized as a novel lipase named adipose triglyceride lipase (ATGL).[133-135]

Numerous studies have focused on the proteomic identification of lipid droplet-associated proteins in non-adipocytes, for example, yeast, Chinese hamster ovary fibroblasts, cultured human HuH7 hepatoma cells, cultured human A431 epithelial cells and mouse mammary glands. Interestingly, the PAT family members adipophilin and TIP47 were consistently identified from lipid droplet preparations, suggesting that PAT family members are ubiquitously expressed proteins that are specifically related to the structure and function of lipid droplets.[136] Since adipocytes contain unique regulatory machinery that control lipid storage and mobilization, understanding the biology of adipocyte lipid droplets would be of particular importance. In 2004, Brasaemle et al. published a study on the proteomic analysis of lipid droplet associated proteins in 3T3-L1 adipocytes.[124] In this study, the protein composition of adipocyte lipid droplets was analyzed in the basal and lipolytically stimulated states to capture the rapid changes in proteins on the lipid droplets. The advantage of using isolated lipid droplets is to reduce the complexity of the protein sample and to increase the potential of characterizing a more comprehensive lipid droplet proteome. This will allow for a more thorough understanding of the hormonal regulation of structure reorganization in lipid droplets during the lipolytic process. Their results show that more proteins are associated with lipid droplets after lipolytic stimulation rather than in the basal state. A group of proteins that were selectively identified from lipolytically stimulated lipid droplets include adipophilin, caveolin-1, tubulin, ACSL3 and -4, short chain dehydrogenase/reductase family member 1, aldehyde dehydrogenase ALDH3B1, tumor protein D54, an unstudied protein (expressed sequence AI462440) and several Rab GTPases. Another group of proteins that are consistently present in both basal and lipolytically stimulated preparations of lipid droplets are structural proteins and lipid metabolic enzymes including perilipin, S3–12, vimentin, TIP47, hormone-sensitive lipase, lanosterol synthase, NAD(P)-dependent steroid dehydrogenase-like protein, acyl-CoA

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synthetase, long chain family member (ACSL) 1 and CGI-58. Intriguingly, the accuracy of proteomic identification of lipid droplet localization and lipolytically stimulated mobilization of the proteins was confirmed by immunofluorescence microscopy and immunoblotting. These findings support the concept that the association of proteins with lipid droplets is a dynamic process and that these differentially regulated proteins are likely to be the important regulators of lipolysis.

Kanshin et al. analyzed the stoichiometry of protein phosphorylation in lipid droplets to reveal the role of phosphorylation in the regulation of LD protein activity.[137] The phosphorylation of lipid droplet proteins in WAT of mice in the basal state and after β3-adrenergic stimulation was quantified using isotope tagging and an enzymatic dephosphorylation method. LD proteins were purified from WAT of mice, followed by trypsin digestion. Peptide digests were labeled with light or heavy stable isotope-containing multiplex peptide labels (SIMPL) isotope tags. Half of the labeled peptides were dephosphorylated using bovine intestinal alkaline phosphatase (BIAP). The dephosphorylated samples were then mixed with untreated samples a 1:1 ratio for 2D LC-MS/MS analysis. This approach detected changes in the phosphorylation of six LD-associated proteins from WAT of mice with basal and β3-adrenergic stimulation. Specifically, perilipin was found to be phosphorylated at Ser-410 and Ser-460 during stimulation. Additionally, this method was able to quantitatively estimate the amount of perilipin phosphorylation at different sites. After β3-adrenergic stimulation, ~30% of total perilipin within LDs are phosphorylated at Ser-460 and about 40% at Ser-410.

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Proteomics in Post-Translational Modifications

Protein phosphorylation in adipocytes. Proteomics has been used to explore the global protein expression profiles in adipocytes in order to identify proteins that may contribute to adipocyte dysfunction and insulin resistance. Often, it is not only the protein itself or the concentration of the protein that has an impact on regulating cellular activity; rather it is the post-translational modifications (PTM) that are responsible. Gene expression and protein concentration are usually indicative of long-term regulation by transcription factors and translation machinery and are not useful for quick metabolic changes. Protein kinases control all cellular processes including differentiation, cell division, growth, immune function and energy homeostasis through phosphorylation.[138] Molecular signaling mechanisms, among other functions, are typically transduced using a cascade of PTM such as phosphorylation. Identification and quantification of phosphorylated proteins using proteomic approaches has been useful for understanding the signaling pathways related to obesity.

Schmelzle et al. identified 122 phosphorylation sites on 89 proteins in 3T3-L1 adipocytes and quantified their change during insulin treatment of 0, 5, 15 and 45 min.[139] Using iTRAQ, the four different insulin treatment conditions could be independently labeled then pooled together for relative quantification during MS/MS. For phosphoproteomic analysis, phosphorylated peptides need to be separated from the non-phosphorylated peptides. Phosphorylated digested peptides were enriched by immunopurification using pan-specific anti-tyrosine antibodies to precipitate all peptides with phosphorylated tyrosines. Further purification by immobilized metal affinity chromatography (IMAC) is needed due to non-specific binding of aromatic amino acids to the anti-pTyrosine antibodies.[139] During IMAC, the metal chelator iminodiacetic acid immobilizes positively charged metal ions to a support structure such as a column. The negatively charged phosphate groups in the protein sample interact with the metal ions for a second round of sample purification. Aside from known phosphotyrosine sites, this method identified many sites that had not previously been shown to be modified by insulin treatment including a number of proteins involved in GLUT4 translocation and the MAPK pathway. Of the GLUT4-related proteins, Munc18c, APS and Gab1 had a 10-fold increase in tyrosine phosphorylation following 5 min of insulin stimulation. This technique illustrates the widespread effect of insulin action on tyrosine phosphorylation within adipocytes and identifies many potential sites of dysregulation that may be implicated in insulin resistance.

In another study looking at the phosphoproteome during insulin stimulation, Kruger et al. labeled BAT with the SILAC method.[140] A total of 40 proteins were identified with 33 of them being increased 1.3-fold after 5 min of insulin treatment. For a temporal analysis, protein samples from three different time points were pooled together and enriched with anti-phosphotyrosine antibodies then analyzed by 1D PAGE LC-MS. By using two SILAC experiments, a time profile of 0, 1, 5, 10 and 20 min of insulin stimulation was generated so that the phosphorylation state of proteins can be measured at the initial and late stages of signal transduction. Insulin receptor and IRS-1 quickly became phosphorylated after insulin treatment and continued to stay active after 20 min. Meanwhile, IRS-2 quickly dephosphorylated suggesting that there may be a negative feedback mechanism. The MAP kinase pathway, a downstream target of insulin, achieved maximal activation at 5 min where it then rapidly declined 75% by 20 min, implying a regulatory mechanism as well. SILAC labeling methods provide another way to quantitate and compare signaling pathways without bias and can show the transient effects of ligands on downstream targets.

Protein carbonylation in adipocytes. Reactive oxygen species (ROS) are constantly produced in the cell as a byproduct of the electron transport chain during normal metabolic processes and have been implicated in many chronic diseases ranging from diabetes to Alzheimer.[141–143] The highly reactive ROS, superoxide, can be produced either from uncoupling reactions in the mitochondria or through the enzyme NADPH. It can then undergo peroxidation reactions with the polyunsaturated membrane fatty acids arachidonic and linoleic acid to form many byproducts, one of which is 4-hydroxynonenal (HNE). HNE itself is highly reactive and can diffuse through the cytoplasm and bind to proteins by reacting with the imidazole group of histidine, the amino group of lysine or the thiol group of cysteine via Michael addition.[144] Disrupting the HNE-conjugating enzyme GSTA4–4 in mice allowed HNE to accumulate in the tissues, causing

obesity and insulin resistance.[145] To see if HNE-modified proteins were increased in adipose tissue during obesity, Grimsrud et al. tagged the HNE aldehyde adducts in adipose protein samples using biotin hydrazide and then separated them on a gel for visualization.[146] The adipose tissue of obese mice contained a 2–3-fold increase in protein carbonylation compared with lean controls, implying increased oxidative stress. Additionally, biotin hydrazide-linked proteins were enriched using a monomeric avidin column, digested with trypsin and then identified using LC-ESI-MS/MS. This method identified 37 carbonylated proteins belonging to many different functional categories. Of these proteins, fatty acid binding protein (FABP) was further analyzed due to its role in insulin resistance in mice.[147] The HNE-modified FABP was not able to bind to the hydrophobic probe, 1,8-ANS, implying a loss of functionality and linking oxidative stress to insulin resistance.

Conclusions

Adipose tissue dysfunction is the major contributor to the pathogenesis of obesity-related insulin resistance and the metabolic syndrome, which is featured by a cluster of metabolic deteriorations including an imbalanced secretion of adipokines and inflammatory cytokines, the dysregulation of lipid and glucose homeostasis and mitochondrial dysfunction. Considerable efforts have been put forth in the past decade to identify and characterize the key components involved in the development of the dysfunctional adipose tissue. Various qualitative and quantitative proteomics techniques have been useful tools in identifying and quantifying the changes of the key adipose proteins in obesity and insulin resistance. A list of proteins including adipokines/cytokines, mitochondria- and lipid droplet-associated proteins and GLUT4 vesicle proteins have been identified from the proteomic studies in rodents and humans. This information provides the basis for directing future research in the field. Functional characterization of several novel proteins has been conducted, demonstrating that these proteins play critical roles in glucose and lipid metabolism, glucose uptake and insulin resistance. Additionally, proteomics has significantly advanced our understanding of lipid droplet biology. The applications of proteomics led to the discovery of lipid droplet proteins that control lipolysis and facilitated the understanding of the dynamic nature of LDs during lipolytic stimulation. Stable isotope labeling of iTRAQ and SILAC in combination with immunoprecipitation enrichment of phosphorylated peptides and LC-MS/MS offers the opportunity to identify and detect the dynamic process of post-translational modifications such as phosphorylation. From dynamically profiling insulin-stimulated phosphorylation, different potential regulatory roles of components can be dissected. Furthermore, proteomic techniques allow characterization of other types of modification, for instance, carbonylation of adipose proteins that are altered in oxidative stress and insulin resistance. All of the information acquired from proteomic-based studies on adipocytes provides an important source of the knowledge for understanding adipocyte biology and the contribution of adipose dysfunction to obesity and its related metabolic complications.

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Acknowledgments

X.C. is supported by a NIHR01 Grant (R01DK080743) from the National Institute of Diabetes and Digestive and Kidney Diseases and General Mills Foundation from the University of Minnesota. D.A.B. is supported by a NIHP30 Grant (P30DK050456) (Minnesota Obesity Center) from the National Institute of Diabetes and Digestive and Kidney Diseases.

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