Quantitative secretome and glycome of primary human adipocytes during insulin resistance

Jae-Min Lim1,2,5, Edith E Wollaston-Hayden1,3, Chin Fen Teo1,3, Dorothy Hausman4 and Lance Wells1,2,3*

Abstract

Adipose tissue is both an energy storage depot and an endocrine organ. The impaired regulation of the secreted proteins of adipose tissue, known as adipocytokines, observed during obesity contributes to the onset of whole-body insulin resistance and the pathobiology of type 2 diabetes mellitus (T2DM). In addition, the global elevation of the intracellular glycosylation of proteins by O-linked β-N-acetylglucosamine (O-GlcNAc) via either genetic or pharmacological methods is sufficient to induce insulin resistance in both cultured cells and animal models. The elevation of global O-GlcNAc levels is associated with the altered expression of many adipocytokines. We have previously characterized the rodent adipocyte secretome during insulin sensitive and insulin resistant conditions. Here, we characterize and quantify the secretome and glycome of primary human adipocytes during insulin responsive and insulin resistant conditions generated by the classical method of hyperglycemia and hyperinsulinemia or by the pharmacological manipulation of O-GlcNAc levels. Using a proteomic approach, we identify 190 secreted proteins and report a total of 20 up-regulated and 6 down-regulated proteins that are detected in both insulin resistant conditions. Moreover, we apply glycomic techniques to examine (1) the sites of N-glycosylation on secreted proteins, (2) the structures of complex N- and O-glycans, and (3) the relative abundance of complex N- and O-glycans structures in insulin responsive and insulin resistant conditions. We identify 91 N-glycosylation sites derived from 51 secreted proteins, as well as 155 and 29 released N- and O-glycans respectively. We go on to quantify many of the N- and O-glycan structures between insulin responsive and insulin resistance conditions demonstrating no significant changes in complex glycosylation in the time frame for the induction of insulin resistance. Thus, our data support that the O-GlcNAc modification is involved in the regulation of adipocytokine secretion upon the induction of insulin resistance in human adipocytes.

Keywords: O-GlcNAc, Insulin resistance, Type 2 diabetes, Adipocytokine, Tandem mass spectrometry, Shotgun proteomics, Glycomics, N-linked, O-linked

Introduction

Type 2 diabetes mellitus (T2DM) is a rapidly growing problem in the industrialized world. In the United States, it is estimated that 8.3% of the population has diabetes [1]. T2DM results from a combination of insulin resistance and pancreatic beta-cell dysfunction [2]. The development of T2DM depends on both genetic and environmental risk factors [3]. The major environmental risk factor for the development of insulin resistance and T2DM is obesity. Increased white adipose tissue (WAT) mass during obesity causes a variety of problems related to the development of insulin resistance. It is now established that WAT acts as a lipid metabolism modulator as well as an endocrine organ [4].

The secreted proteins of adipose tissue, known as adipocytokines, can act in a paracrine or endocrine manner to modulate a variety of processes such as inflammation and whole-body energy homeostasis. During obesity, adipocytokine secretion is altered, which can lead to the development of whole-body insulin resistance and T2DM [5,6]. Although many adipocytokines have been identified using methods such as mass spectrometry-based proteomics, the alteration in the adipose tissue secretome for normal vs. insulin resistant human adipose tissue has not been well-defined [7]. Several adipocytokines identified in rodents have different expression patterns in humans highlighting the need for quantitative proteomic studies...
in human adipose tissue [8,9]. To achieve a more physiological representation of the adipose tissue secretome, we use primary human pre-adipocytes derived from human adipose tissue rather than an adipocyte cell line [10].

The regulation of adipocytokine expression during insulin resistance is still unclear in many cases. One possible way for cells to sense nutrient abundance and modulate insulin sensitivity and adipocytokine expression is through the hexosamine biosynthetic pathway (HBP). Excessive flux through the HBP results in insulin resistance, thereby limiting the amount of glucose that enters the cell and the resulting toxicity [11,12]. Flux through the HBP also modulates the expression of several adipocytokines [13-16]. The final product of the HBP is UDP-GlcNAc, which is the sugar donor for the enzyme O-GlcNAc transferase (OGT) that adds the O-linked β-N-acetylglucosamine (O-GlcNAc) modification onto the serine and threonine residues of nucleocytoplasmic proteins [17,18]. It has been shown in multiple systems that the elevation of O-GlcNAc levels is sufficient to cause insulin resistance [19-24]. Furthermore, the overexpression of OGT in the peripheral tissues of mice results in both glucose disposal defects and hyperleptinemia providing further evidence that the O-GlcNAc modification causes insulin resistance and the dysregulation of adipocytokine expression [21]. We have previously characterized and quantified the secretome of rodent adipocytes during insulin resistance generated by either directly or indirectly modulating O-GlcNAc levels [25]. We use a similar approach in this study. Insulin resistance is induced in primary human adipocytes by either the classical method of chronic hyperinsulinemia and hyperglycemia or by directly elevating O-GlcNAc levels using O-GlcNAcase (OGA) pharmacological inhibitors. To identify and quantify the secretome we use a proteomic approach with reverse phase (RP) liquid chromatography-nanospray-tandem mass spectrometry (LC-NS-MS/MS). We identify a total of 190 secreted proteins from primary human adipocytes. We compare the relative abundance of the adipocytokines during insulin responsive versus insulin resistant conditions using spectral counts. We report that 20 proteins are upregulated and 4 are downregulated when primary human adipocytes are shifted from insulin sensitive to insulin resistant conditions.

Glycosylation is one of the most common post-translational modifications (PTMs) of proteins, especially of secreted proteins [26-34]. In certain disease states, the glycome and the enzymes responsible for the glycan complexity can be altered [35,36]. It has been suggested that the increased pool of UDP-GlcNAc during excessive nutrient flux alters glycosyltransferase activity and the degree of N-glycan branching [37]; however, another study reported no change in complex glycosylation during elevated HBP flux [38]. Determining the glycome in different disease states is important for the future identification of structure-function relationships and to discover targets for diagnostic biomarkers [25,39,40]. The glycome of human adipose tissue has not been previously described. Glycan analysis is challenging due to the diversity of possible structures and their low abundance [35,41-49]. We use several strategies to identify, site-map, and quantify glycans from adipose tissue using mass spectrometry (MS). We characterize a total of 155 N-linked glycans and 29 O-linked glycans using MS/MS spectra by total ion mapping (TIM) scan. Predominant N-linked and O-linked glycans can be quantified by 13C isotopic labeling by permethylation using heavy or light iodomethane (13CH3I and 12CH3I) and isobaric pairs of iodomethane (13CH3I or 12CH2DI) (48, 49). A total of 48 N-linked glycans and 12 O-linked glycans are compared between the insulin responsive condition and both insulin resistance conditions by calculating the 13C/12C ratios from the sum of peak areas and from non-labeled prevalence rates. We also identify 91 N-glycosylation sites derived from 51 secreted proteins using the PNGase F digestion in 18O water using a parent mass list method.

Herein, we describe the secretome and glycome of primary human adipocytes during insulin sensitive and insulin resistant condition. Given the important physiological roles of adipocytokines in maintaining whole-body energy homeostasis, the characterization of the primary human adipocyte secretome during insulin resistance could provide novel markers and/or therapeutic targets for the onset of insulin resistance.

**Experimental procedures**

**Tissue culture and conditioned cell treatments**

Cryopreserved human subcutaneous preadipocytes (number of donors: 6–7, gender of donors: female, average age: 39, and average BMI: 27.32) were purchased from Zen-Bio, Inc. (Research Triangle Park, NC). The adipose tissue culture protocols of the maintenance and differentiation from preadipocytes to adipocytes were based on Zen-Bio instruction manual (ZBM0001.01). Briefly approximately 6.7 × 10^5 cells were cultured in a T-75 cm^2 flask using preadipocyte medium (PM-1, Zen-Bio, Inc.), under a humidified atmosphere containing 5% CO2 in air at 37°C until they were 85-90% confluent, and then were trypsinized for 5 minutes at 37°C. After neutralization and centrifugation steps, the cell pellet was resuspended in PM-1 and seeded at an average density of 2.67 × 10^5 cells/cm^2 in 10-cm cell culture dishes for differentiation. 2 days after the cells reached confluence (referred to as day 0), the medium was replaced with adipocyte differentiation medium (DM-2, Zen-Bio, Inc.). On day 7, the DM-2 was removed and the cells were maintained in adipocyte maintenance medium (AM-1, Zen-Bio, Inc.). The medium was changed every 3 days. On day 15, at which the majority of the cells contained large lipid droplets, the AM-1 medium
was replaced with low glucose DMEM (Cellgro, Mediatech, Inc.) containing 10% FBS (GIBCO, Invitrogen) and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin (P/S), Cellgro, Mediatech, Inc.). The medium was changed every 2 days until treatments were applied. On day 20, adipocytes were treated with different conditions, either (1) low glucose (LG) (LG, DMEM containing 10% FBS and P/S), (2) insulin (100 nM, human, Roche) in high glucose (HG + INS) (HG, DMEM containing 10% FBS and P/S), (3) PUGNAc (100 μM, TRC, Inc.) in low glucose (LG + PUGNAc) (LG, DMEM containing 10% FBS and P/S), or (4) GlcNAcstatin (20 nM, kind gift from Dr. Daan van Aalten) in low glucose (LG + PUGNAc) (LG, DMEM containing 10% FBS and P/S). After the first 24 h of incubation, the cells were washed five times (for mass spectrometry analysis) or 3 times (for immunoblotting) with low or high glucose serum-free DMEM without antibiotics and incubated for 15 min during the last rinse. After the final wash, the treatment conditions were added as above except no serum was added and the HG + INS treatment was changed to 1nM Insulin. The cells and media were harvested after 16 h of incubation.

**Secreted protein sample preparation**

The conditioned media was harvested with extreme care not to disrupt underlying cells and then centrifuged once at 1800 rpm, at 4°C for 7 min. The supernatant was filtered using 1 μm syringe filters (PALL). The samples were then centrifuged again at 1800 × g at 4°C for 30 min. The samples were then transferred to equilibrated spin columns (Centriprep YM-3, Amicon, Millipore) and buffer-exchanged at 2800 × g, at 4°C into 40 mM ammonium bicarbonate (NH₄HCO₃) in the presence of 1 mM di-thiothreitol (DTT, Fisher Scientific) and concentrated. The samples were either quantified and prepared for immunoblotting or were denatured with 1 M urea (Sigma), reduced with 10 mM DTT for 1 h at 56°C, carboxymethylated with 55 mM iodoacetamide (ICH₂CONH₂, Sigma) in the dark for 45 min, and then digested with 4 μg of trypsin (Promega) in 40 mM NH₄HCO₃ overnight at 37°C. After digestion, the peptides were acidified with 200 μL of 1% trifluoroacetic acid (TFA). Desalting was subsequently performed with C18 spin columns (Vydac silica C18, The Nest Group, Inc.) and the resulting peptides were dried down in a Speed Vac and stored at −20°C until analysis. For the subset of samples to be analyzed for N-linked glycosylation, peptides were resuspended in 19 μL of 18O water (H₂¹⁸O, 95%, Cambridge Isotope Laboratories, Inc.) and 1 μL of N-Glycosidase F (PNGase F, Prozyme) and allowed to incubate for 18 h at 37°C. Peptides were dried back down and resuspended in 50 μL of 40 mM NH₄HCO₃ with 1 μg of trypsin for 4 h, to remove any possible C-terminal incorporation of ¹⁸O from residual trypsin activity, and then dried down and stored at −20°C until analysis.

**Whole cell extracts and western blots**

After culture medium was removed for secreted proteins analysis, the cell monolayer was washed twice with 10 ml of ice-cold PBS and scraped in the presence of 1 ml PBS plus protease inhibitors. After removing PBS by centrifugation at 6,000 × g for 5 min at 4°C, the pellet was snap frozen and stored at −80°C. To prepare lysate for immunoblotting, the pellets were lysed in 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1:100 protease inhibitor cocktail set V, EDTA-free (Calbiochem), and 1 μM PUGNAc. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific) and samples were boiled in Laemli sample buffer. The immunoblots were performed essentially as described using CTD 110.6 (for O-GlcNAc modified proteins) and ERK-2 (as a positive control for loading) antibodies. For secreted protein immunoblotting, the media concentration was quantified using the Bradford method and verified by Coomassie staining. Equal amounts of protein were separated by SDS-PAGE with Tris–HCl precast minigels (Bio-Rad) and transferred to nitrocellulose membranes for Western blot analysis. After blocking for at least 1 hour, membranes were incubated with the appropriate primary antibody, anti-SPARC (Abcam) or anti-Chitinase-3-like protein 1 (R&D Systems) overnight at 4°C. Membranes were incubated with the appropriate horseradish peroxidase-coupled secondary antibodies for 1 hour, followed by extensive washing and Pierce ECL detection.

**Protein extracts for glycan analysis**

After the conditioned media was collected, the adipocytes were immediately washed twice with ice-cold PBS and harvested by scraping for glycan analysis. The cells were centrifuged at 12,000 × g, at 4°C for 15 min to remove the supernatant and debris, then snap frozen and stored at −80°C until analysis. After the cell pellets thawed on ice, the samples were subjected to Dounce homogenization in ice-cold 100% methanol. The homogenized samples were delipidated by two solvent extractions using a mixture of chloroform/methanol/water (4:8:3, v/v/v) and rocking for 3 h at room temperature as described previously. The emulsion was centrifuged at 2800 × g for 15 min at 4°C to remove the supernatant. The pellets were resuspended in an acetone/water (10:1, v/v) mixture and incubated on ice for 15 min for washing. The protein pellets were collected by centrifugation and dried on a heating module at 45°C under a mild nitrogen stream (Reacti-Therm™ and Reacti-Vap™, Pierce). The dried protein powder was weighed and stored at −20°C until analysis.

**Preparation of N-linked glycans**

Three mg of the protein powder was resuspended in 200 μL of 40 mM NH₄HCO₃ by sonication followed by
boiling at 100°C for 5 min. After cooling to room temperature, 25 μL of trypsin (2 mg/mL in 40 mM NH4HCO3, Sigma) and chymotrypsin (2 mg/mL in 40 mM NH4HCO3, Sigma) were added. The samples were denatured with 250 μL of 2 M urea in 40 mM NH4HCO3, leading to a final concentration of 1 M urea, and incubated overnight (18 h) at 37°C. After digestion, the peptide samples were centrifuged and 10 μL of the supernatant was collected for protein quantification. The peptide concentrations were measured using a Pierce BCA Protein Assay Kit (Thermo Scientific). The samples were boiled at 100°C for 5 min and then acidified by adding 500 μL of 10% acetic acid (AcOH) to deactivate proteases. The samples were loaded onto an equilibrated C18 extraction column (BakerBond™, J.T.Baker), washed with 1 mL of 5% AcOH three times, and then eluted stepwise using 1 mL of 20% isopropanol in 5% AcOH, 40% isopropanol in 5% AcOH, and 100% isopropanol. The resulting glycopeptides were dried in a Speed Vac, resuspended in 48 μL 1x PNGase F reaction buffer and 2 μL PNGase F and incubated for 18 h at 37°C. Following PNGase F digestion, released oligosaccharides were separated by the C18 extraction column. The mixture was reconstituted in 5% AcOH and loaded onto an equilibrated C18 extraction column. The N-linked oligosaccharides were eluted using 1 mL of 5% AcOH three times and then collected and dried using a Speed Vac for subsequent permethylation.

Preparation of O-linked glycans
O-linked oligosaccharides were released by reductive β-elimination then purified by a cation exchange resin. Three mg of delipidated protein powder was weighed and transferred into a clean glass tube. 500 μL of 50 mM sodium hydroxide (NaOH) and 500 μL of alkaline borohydride solution (a mixture of 2 M sodium borohydride (NaBH4, Sigma-Aldrich) in 50 mM NaOH leading to a final concentration of 1 M NaBH4) were added to the tube. The mixture was incubated for 16 h at 45°C on the heating block and the reaction was stopped by the addition of 10% AcOH with vortexing. The acidified mixture was loaded onto an equilibrated cation exchange resin cartridge (AG 50 W-X8, Bio-Rad) with 5% AcOH. O-linked glycans were eluted with 6 mL of 5% AcOH and then dried in a Speed Vac. The sample was resuspended in 1 mL methanol/glacial acetic acid (9:1, v/v) solution and dried on the heating module at 45°C with a mild nitrogen stream. For secretome analysis, a full ITMS (ion trap mass spectrometry) spectrum, typically recorded at 60000 resolution in positive ion and profile mode, was acquired at 300–2000 m/z followed by 8 MS/MS events on the 8 most intense peaks with enabled dynamic exclusion using a repeat count of 2, a maximum exclusion list size of 100, and an exclusion duration of 30 s. Each MS/MS scan event was followed by CID (34% normalized collision energy), 0.25 activation Q, and 30.0 ms activation time. For N-liked glycosylation site mapping, a full FTMS (Fourier transform mass spectrometry) spectrum, typically recorded at 60000 resolution in positive ion and profile mode, was acquired at 300–2000 m/z followed by 5 data dependent MS/MS spectra of ITMS on the most intense ion peaks from parent mass list following CID (36% normalized collision energy), 0.25 activation Q, and 30.0 ms activation time. Dynamic

Analysis using mass spectrometry
For proteome analysis of secreted proteins by liquid chromatography tandem mass spectrometry (LC-MS/MS), the peptides were resuspended with 19.5 μL of mobile phase A (0.1% formic acid, FA, in water) and 0.5 μL of mobile phase B (80% acetonitrile, ACN, and 0.1% formic acid in water) and filtered with 0.2 μm filters (Nanosep, Pall). The samples were loaded off-line onto a nanospray tapered capillary column/emitter (360 × 75 × 15 μm, PicoFrit*, New Objective) that was self-packed with C18 reverse phase (RP) resin (8.5 cm, Waters) in a nitrogen pressure bomb for 10 min at 1000 psi (~5 μL load). The samples were then separated via a 160 min linear gradient of increasing mobile phase B at a flow rate of ~200 nL/min directly into the mass spectrometer. One-dimensional LC-MS/MS analysis was performed using a linear ion trap and an Orbitrap mass spectrometer (LTQ and LTQ Orbitrap XL, Thermo Fisher Scientific Inc.), equipped with a nanoelectrospray ion source at 2.0 kV capillary voltage and 200°C capillary temperature. For secretome analysis, a full ITMS (ion trap mass spectrometry) spectrum in positive ion and profile mode was collected at 300–2000 m/z followed by 8 MS/MS events on the 8 most intense peaks with enabled dynamic exclusion using a repeat count of 2, a maximum exclusion list size of 100, and an exclusion duration of 30 s. Each MS/MS scan event was followed by CID (34% normalized collision energy), 0.25 activation Q, and 30.0 ms activation time. For N-liked glycosylation site mapping, a full FTMS (Fourier transform mass spectrometry) spectrum, typically recorded at 60000 resolution in positive ion and profile mode, was acquired at 300–2000 m/z followed by 5 data dependent MS/MS spectra of ITMS on the most intense ion peaks from parent mass list following CID (36% normalized collision energy), 0.25 activation Q, and 30.0 ms activation time. Dynamic
exclusion was set at a repeat count of 2, a maximum exclusion list of 100, and an exclusion duration of 60 s.

For glycome analysis by direct infusion nanospray MS, permethylated glycans were dissolved by combining 15 μL of the isotopically mixed sample in 100% methanol plus 35 μL 1 mM NaOH in 50% methanol. They were infused directly into a linear ion trap and an Orbitrap mass spectrometer using a nanospray ion source with a fused-silica emitter (360 × 75 × 30 μm, SilicaTip™, New Objective) at 2.0 kV capillary voltage, 200°C capillary temperature, and a syringe flow rate of 0.4 μL/min. The full ITMS spectra and FTMS spectra, typically recorded at 60000 resolution in positive ion and profile mode, were collected at 400–2000 m/z for 30 s with 5 microscans and 150 maximum injection times (ms). The centroid MS/MS spectra following collision-induced dissociation (CID) were obtained from 400 to 2000 m/z at 34% and 28% normalized collision energy for N- and O-linked glycans, respectively, 0.25 ms activation Q, and 30.0 ms activation time by total ion mapping (TIM). Parent mass step size and isolation width were set at 2.0 m/z and 2.8 m/z, respectively, for automated MS/MS spectra with TIM scans.

Data analysis
The resulting proteomic data was searched against a target nonredundant human (Homo sapiens, 5-2-07) database including the common contaminants database obtained from the human International Protein Index (IPI) protein sequence database (European Bioinformatics Institute, ftp://ftp.ebi.ac.uk/pub/databases/IPI) using the TurboSequest algorithm (BioWorks 3.3.1 SP1, Thermo Fisher Scientific Inc.) [52,53]. It was also searched against a decoy human database to determine statistically relevant peptide identification. DTA files were generated for spectra with a threshold of 15 ions and a TIC of 2e3 over a range of [MH]+ = 600–4000 for only IT data. The SEQUEST parameters were set to allow 2.0 Da (20 ppm for FT) of precursor ion mass tolerance and 0.5 Da of fragment ion tolerance with monoisotopic mass. Only strict tryptic peptides were allowed with up to two missed internal cleavage sites. Dynamic mass increases of 15.99 and 57.02 Da (15.9949 and 57.0215 Da for FT) were allowed for oxidized methionine and alkylated cysteine, respectively. The cases where sites of N-linked glycosylation were investigated with PNGase F and 18O water, a dynamic mass increase of 3.0 Da (2.9883 Da for FT) was allowed for Asn residues [54]. The masses were selected between 300–2000 m/z at each charge state and 7352 total masses were obtained for parent mass list. Each sample was analyzed by four LC-MS/MS runs with different parent mass list up to 2000 because of maximum number of parent masses for FTMS and mass complexity. The results from shotgun method and parent mass list method were combined and filtered at ≥ 0.60 Final Score (SF). For relative protein quantification, the identified peptides and proteins were statistically validated between the target and decoy database search results, and protein ratios were determined by normalized spectral counts using ProteoIQ 1.1 (Premiere BioSoft). Proteins identified by two peptides were only considered to be statistically significant at less than 1% protein false discovery rate (FDR) using the ProValT algorithm as implemented in ProteoIQ [55,56]. From the results, the subcellular location of secreted proteins was manually determined for each protein from the Human Protein Reference Database (http://www.hprd.org/), Information Hyperlinked Over Proteins (iHOP, http://www.ihop-net.org/UniPub/iHOP/) and the UniProtKB/Swiss-Prot (http://www.ebi.ac.uk/uniprot) databases. The functional categories of secretome were annotated by the Ingenuity Pathways Analysis program (IPA, Ingenuity Systems, Inc.).

The N- and O-linked glycans released from the protein power of adipocytes were analyzed by full MS following permethylation and subsequent MS/MS fragmentations for specific glycan structures. We used GlycoWorkbench (http://glycomics.crrc.uga.edu/eurocarb/gwb/home.action) to facilitate manually interpretation of the glycan structures from the MS/MS spectra by TIM scan. The areas of each isotopic peak were calculated by their Gaussian distribution and then averaged to compare the relative abundance of each glycan between different samples. The ratio of peak areas from isotopic permethylation defined the relative abundance of each glycan.

Results
Insulin resistance and global O-GlcNAc levels in primary human adipocytes
Treatment with hyperglycemia and chronic insulin or normoglycemia and the pharmacological OGA inhibitor, PUGNAC, result in the global elevation of O-GlcNAc modified nucleocytoplasmic proteins and insulin resistance in rodent adipocytes [19,57]. We tested these conditions as well as treatment with a more specific OGA inhibitor, GlcNAcstatin, in primary human adipocytes [58]. Cryopreserved human preadipocytes were expanded and differentiated into mature adipocytes before treatment as described in Materials and Methods. Figure 1 shows that when compared to normoglycemia (LG), treatment with OGA inhibitors (LG + PUGNAC or LG + GlcNAcstatin) or treatment with hyperglycemia and hyperinsulinemia (HG + INS) raises global O-GlcNAc levels as measure by immunoblotting with an O-GlcNAc specific antibody. In a previous study using rodent adipocytes, we characterized and quantified the secretome and glycome during insulin sensitive and insulin resistant conditions induced by indirectly (HG + INS) or directly (LG + PUGNAC) modulating O-GlcNAc levels. We found that many secreted proteins were up or down-regulated upon the induction of insulin resistance. Here we took a parallel approach to ask the
same question using primary human adipocytes. As shown in Figure 2, after treatment both the cells and conditioned media were harvested. Before the final 16 hour serum-free treatment incubation, the cells were washed five times with serum-free media to remove any traces of serum. This greatly reduced the complexity of the conditioned media. The conditioned media was buffer exchanged and concentrated before being processed for analysis by LC-MS/MS. Cells were collected and delipidated as in Materials and Methods. Protein was digested and prepared for either N-glycan or O-glycan analysis (Figure 2).

**Characterization of the primary human adipose tissue secretome by LC-MS/MS**

The mass spectra from 24 mass spectrometric analyses were searched against a target nonredundant human database as well as a decoy human database using SEQUEST. Proteins identified by at least two non-redundant peptides with less than a 1% FDR were identified using ProteoIQ. Secreted proteins were manually determined by a reference search as described in Materials and Methods. Using these criteria, a total of 190 secreted proteins were identified. Table 1 shows the complete list of the 173 secreted proteins identified by at least 2 unique peptides. 17 of the secreted proteins identified based on a single peptide are presented in Supplemental Table 1 (Additional file 1: Table S1). Figure 3 shows the proportion of the secretome in each of the 9 main functional categories assigned by Ingenuity Pathways Analysis. The most highly represented categories are enzyme, peptidase, and other protein.

**Quantification of the primary human adipocyte secretome**

Several mass spectrometry techniques can be used for quantitative analysis. Both isotopic and non-isotopic
labeling are commonly used to provide quantitative analysis of proteins in complex protein samples [59-67]. For non-isotopic or label-free MS-based quantitative analysis, ion chromatogram intensity or observed peptide spectral counts are compared using quantitative software tools [68-76]. Peptide spectral counts quantification relies on the association between protein abundance and the number of MS/MS spectra identifying each protein. For this study, we used three biological replicates of conditioned media samples, which differed in complex proteome profiles and relative number of normalized spectral counts. For each of the biological replicates, prepared conditioned media from three treatment groups (LG, LG + PUG, HG + INS) was subjected to two technical replicates of reverse phase LC-MS/MS. All together, a total of 18 LC-MS/MS experiments were performed. SEQUEST was used to search MS data files from each LC-MS/MS run against a forward and reversed human database to calculate a FDR. ProteoIQ was used to cluster the peptides to their assigned proteins and to gather normalized spectral counts for proteins identified at less than a 1% FDR and assigned by at least two non-redundant peptides. Spectral counts were normalized for each technical replicate. For each biological replicate the normalized spectral counts were averaged followed and then the comparative ratios between the two insulin resistant conditions and the insulin responsive condition were calculated. Finally, the comparative ratios were averaged between all of the biological replicates. We set the threshold for reporting statistically significant differences at ≥150% (1.5-fold), an average of ≥5 total spectral counts, and an average of ≥3 normalized spectral counts for each treatment group. We identified 20 human adipocytokines whose secretion levels were upregulated and 4 proteins that were downregulated by at least 1.5-fold by the transition from the insulin sensitive condition to both insulin resistant conditions (Table 2). We also identified 28 and 8 proteins that were upregulated or downregulated, respectively, in the transition from insulin sensitive to one of the insulin resistant conditions (Additional file 1: Table S2).

To confirm the validity of the ratio that we used for statistical significance, we used the orthogonal method of immunoblotting. We confirmed that the regulation we observed in the proteomic experiment with immunoblotting for two proteins whose levels changed by only 1.5-fold in at least one of the insulin resistant conditions as determined by proteomics. In addition, we used cells where insulin resistance was induced using a more specific OGA inhibitor, GlcNAcstatin, to confirm the regulation we observed by using the less specific OGA inhibitor, PUGNAc [58,77]. Figure 4 shows that both SPARC and Chitinase-3-like protein 1 are upregulated in both insulin resistant conditions as determined by immunoblotting, which confirms our proteomic experiment.

**N-glycan site-mapping of the human adipocyte secretome**

Many secreted proteins are modified by N-glycans. During disease states, the glycoforms of these proteins may change and have the potential to be used as disease biomarkers. Since the glycome of adipocytes is not well-defined, we determine sites of N-linked glycosylation on secreted adipocytokines as an initial step in biomarker discovery. The tryptic peptides generated from conditioned media were...
| No. | Protein ID | Identified proteins                                      | Subcellular location |
|-----|------------|----------------------------------------------------------|----------------------|
| 1   | Q92484     | Acid sphingomyelinase-like phosphodiesterase 3a          | Extracellular        |
| 2   | P07108     | Acyl-CoA-binding protein                                  | Cytoplasm            |
| 3   | Q8IUX7     | Adipocyte enhancer-binding protein 1                      | Membrane             |
| 4   | P01023     | Alpha-2-macroglobulin                                     | Nucleus              |
| 5   | P06733     | Alpha-enolase                                            | Extracellular        |
| 6   | P15144     | Aminopeptidase N                                         | Cytoplasm            |
| 7   | P01019     | Angiotensinogen                                           | Membrane             |
| 8   | P07355     | Annexin A2                                               | Extracellular        |
| 9   | P08758     | Annexin A5                                               | Membrane             |
| 10  | Q8NCW5     | Apolipoprotein A-I binding protein                        | Extracellular        |
| 11  | P05090     | Apolipoprotein D                                          | Extracellular        |
| 12  | P02649     | Apolipoprotein E                                          | Extracellular        |
| 13  | P15289     | Arylsulfatase A                                           | Cytoplasm            |
| 14  | P61769     | Beta-2-microglobulin                                      | Membrane             |
| 15  | P21810     | Biglycan                                                 | Extracellular        |
| 16  | P43251     | Biotinidase                                              | Extracellular        |
| 17  | P55290     | Cadherin-13                                              | Membrane             |
| 18  | P27797     | Calreticulin                                              | Cytoplasm            |
| 19  | O43852     | Calumenin                                                 | Cytoplasm            |
| 20  | P16870     | Carboxypeptidase E                                        | Membrane             |
| 21  | P49747     | Cartilage oligomeric matrix protein                       | Extracellular        |
| 22  | P07858     | Cathepsin B                                               | Cytoplasm            |
| 23  | P07339     | Cathepsin D                                               | Cytoplasm            |
| 24  | P43235     | Cathepsin K                                               | Cytoplasm            |
| 25  | P07711     | Cathepsin L                                               | Cytoplasm            |
| 26  | Q9UBR2     | Cathepsin Z                                               | Cytoplasm            |
| 27  | P36222     | Chitinase-3-like protein 1                                | Extracellular        |
| 28  | Q15782     | Chitinase-3-like protein 2                                | Extracellular        |
| 29  | Q59FG9     | Chondroitin sulfate proteoglycan 2 (versican) variant     | Extracellular        |
| 30  | P10909     | Clusterin                                                 | Extracellular        |
| 31  | P02452     | Collagen alpha-1(I) chain                                 | Extracellular        |
| 32  | P02458     | Collagen alpha-1(II) chain                                | Extracellular        |
| 33  | P02461     | Collagen alpha-1(III) chain                               | Extracellular        |
| 34  | P02462     | Collagen alpha-1(IV) chain                                | Extracellular        |
| 35  | P20908     | Collagen alpha-1(V) chain                                 | Extracellular        |
| 36  | P12109     | Collagen alpha-1(V) chain                                 | Extracellular        |
| 37  | Q02388     | Collagen alpha-1(VII) chain                               | Extracellular        |
| 38  | P12107     | Collagen alpha-1(IX) chain                                | Extracellular        |
| 39  | Q09715     | Collagen alpha-1(XII) chain                               | Extracellular        |
| 40  | P39059     | Collagen alpha-1(XV) chain [Contains: Endostatin]         | Extracellular        |
| 41  | P39060     | Collagen alpha-1(XVII) chain [Contains: Endostatin]       | Extracellular        |
| 42  | P08123     | Collagen alpha-2(I) chain                                 | Extracellular        |
| 43  | P08572     | Collagen alpha-2(IV) chain [Contains: Canstatin]          | Extracellular        |
| 44  | P05997     | Collagen alpha-2(V) chain                                 | Extracellular        |
| No. | Accession No. | Protein Name                           | Location       |
|-----|---------------|----------------------------------------|----------------|
| 45  | P12110        | Collagen alpha-2(VI) chain             | Extracellular  |
| 46  | P25940        | Collagen alpha-3(V) chain              | Extracellular  |
| 47  | P12111        | Collagen alpha-3(VI) chain             | Extracellular  |
| 48  | P08253        | Collagenase (72 kDa type IV)           | Extracellular  |
| 49  | P00736        | Complement C1r subcomponent            | Extracellular  |
| 50  | P09871        | Complement C1s subcomponent            | Extracellular  |
| 51  | P01024        | Complement C3                          | Extracellular  |
| 52  | P29279        | Connective tissue growth factor        | Extracellular  |
| 53  | Q9Y240        | C-type lectin domain family 11 member A| Extracellular  |
| 54  | O75462        | Cytokine receptor-like factor 1        | Extracellular  |
| 55  | P07385        | Decorin                                | Extracellular  |
| 56  | Q07507        | Dermatopontin                          | Extracellular  |
| 57  | Q4WWZ6        | Diazepam binding inhibitor, splice form 1c| Cytoplasm |
| 58  | Q9UBP4        | Dickkopf-related protein 3             | Extracellular  |
| 59  | Q14118        | Dystroglycan                            | Membrane       |
| 60  | Q13822        | Ectonucleotide pyrophosphatase/phosphodiesterase 2 | Membrane |
| 61  | O12805        | EGF-containing fibulin-like extracellular matrix protein 1 | Extracellular |
| 62  | O95967        | EGF-containing fibulin-like extracellular matrix protein 2 | Extracellular |
| 63  | Q9Y6C2        | EMILIN-1                                | Extracellular  |
| 64  | Q9BXZ0        | EMILIN-2                                | Extracellular  |
| 65  | P61916        | Epididymal secretory protein E1        | Extracellular  |
| 66  | Q9Y2E5        | Epididymis-specific alpha-mannosidase  | Cytoplasm      |
| 67  | Q16610        | Extracellular matrix protein 1         | Extracellular  |
| 68  | P06294        | Extracellular superoxide dismutase [Cu-Zn] | Extracellular |
| 69  | P35555        | Fibrillin-1                             | Extracellular  |
| 70  | Q53TP5        | Fibroblast activation protein, alpha subunit | Cytoplasm |
| 71  | Q96828        | Fibromodulin                            | Extracellular  |
| 72  | P02751        | Fibronectin                             | Membrane       |
| 73  | P23142        | Fibulin-1                               | Extracellular  |
| 74  | P98095        | Fibulin-2                               | Extracellular  |
| 75  | Q9UBX5        | Fibulin-5                               | Extracellular  |
| 76  | Q12841        | Follistatin-related protein 1           | Extracellular  |
| 77  | P16930        | Fumarylacetoacetase                     | Cytoplasm      |
| 78  | P09382        | Galectin-1                              | Extracellular  |
| 79  | Q08380        | Galectin-3-binding protein              | Membrane       |
| 80  | Q92820        | Gamma-glutamyl hydrolase                | Cytoplasm      |
| 81  | P06396        | Gelsolin                                | Extracellular  |
| 82  | Q9UUJ9        | GlcNAc-1-phosphotransferase subunit gamma | Cytoplasm |
| 83  | P07093        | Glia-derived nexin                      | Extracellular  |
| 84  | P04406        | Glyceraldehyde-3-phosphate dehydrogenase| Cytoplasm      |
| 85  | P35052        | Glypican-1                              | Membrane       |
| 86  | P28799        | Granulins                               | Extracellular  |
| 87  | Q14393        | Growth-arrest-specific protein 6        | Extracellular  |
| 88  | P00738        | Haptoglobin                             | Extracellular  |
| 89  | P00739        | Haptoglobin-related protein             | Extracellular  |
| Entry | Accession | Description                                      | Location       |
|-------|-----------|--------------------------------------------------|----------------|
| 90    | O75629    | Human Protein CREG1                              | Nucleus        |
| 91    | P17936    | Insulin-like growth factor-binding protein 3     | Extracellular  |
| 92    | P22692    | Insulin-like growth factor-binding protein 4     | Extracellular  |
| 93    | P24592    | Insulin-like growth factor-binding protein 6     | Extracellular  |
| 94    | Q16270    | Insulin-like growth factor-binding protein 7     | Extracellular  |
| 95    | O95965    | Integrin beta-like protein 1                     | Unknown        |
| 96    | P19823    | Inter-alpha-trypsin inhibitor heavy chain H2     | Extracellular  |
| 97    | O14498    | ISLR                                             | Extracellular  |
| 98    | Q08431    | Lactadherin                                      | Extracellular  |
| 99    | Q8NHP8    | LAMA-like protein 2                              | Extracellular  |
| 100   | Q59H37    | Laminin alpha 2 subunit isofrom b                | Extracellular  |
| 101   | Q16363    | Laminin subunit alpha-4                          | Extracellular  |
| 102   | P07942    | Laminin subunit beta-1                           | Extracellular  |
| 103   | P55268    | Laminin subunit beta-2                           | Extracellular  |
| 104   | P11047    | Laminin subunit gamma-1                          | Extracellular  |
| 105   | Q14767    | Latent-transforming growth factor beta-binding protein 2 | Extracellular |
| 106   | Q99538    | Legumain                                         | Cytoplasm      |
| 107   | Q07954    | Low-density lipoprotein receptor-related protein 1 | Membrane      |
| 108   | P51884    | Lumican                                          | Extracellular  |
| 109   | P10619    | Lysosomal protective protein                      | Cytoplasm      |
| 110   | P13473    | Lysosome-associated membrane glycoprotein 2      | Membrane       |
| 111   | Q9Y4K0    | Lysyl oxidase homolog 2                          | Extracellular  |
| 112   | P09603    | Macrophage colony-stimulating factor 1           | Extracellular  |
| 113   | Q9UM22    | Mammalian ependymin-related protein 1            | Nucleus        |
| 114   | P48740    | Mannan-binding lectin serine protease 1           | Extracellular  |
| 115   | P50281    | Matrix metalloproteinase-14                      | Extracellular  |
| 116   | P01033    | Metalloproteinase inhibitor 1                    | Extracellular  |
| 117   | P16035    | Metalloproteinase inhibitor 2                    | Extracellular  |
| 118   | Q71SW6    | Muscle type neuropilin 1                         | Membrane       |
| 119   | P14543    | Nidogen-1                                        | Extracellular  |
| 120   | Q14112    | Nidogen-2                                        | Extracellular  |
| 121   | Q02818    | Nucleobindin-1                                   | Cytoplasm      |
| 122   | Q9NPN5    | Olfactomedin-like protein 3                      | Extracellular  |
| 123   | Q8667D1   | Out at first protein homolog                     | Unknown        |
| 124   | P26022    | Pentraxin-related protein PTX3                   | Extracellular  |
| 125   | P62937    | Peptidyl-prolyl cis-trans isomerase A            | Cytoplasm      |
| 126   | P23284    | Peptidylprolyl isomerase B                       | Cytoplasm      |
| 127   | Q15063    | Periostin                                        | Extracellular  |
| 128   | P98160    | Perlecan                                         | Membrane       |
| 129   | Q92626    | Peroxidasin homolog                              | Unknown        |
| 130   | P30086    | Phosphatidylethanolamine-binding protein 1       | Cytoplasm      |
| 131   | P55058    | Phospholipid transfer protein                    | Extracellular  |
| 132   | P36955    | Pigment epithelium-derived factor                | Extracellular  |
| 133   | Q9BTY2    | Plasma alpha-L-fucosidase                        | Extracellular  |
| 134   | Q9Y646    | Plasma glutamate carboxypeptidase                | Extracellular  |
Table 1 Total secreted proteins from human dispose tissue by LC-MS/MS (Continued)

| Protein ID | Protein Name                      | Location          |
|------------|-----------------------------------|-------------------|
| P05155     | Plasma protease C1 inhibitor      | Extracellular     |
| P05121     | Plasminogen activator inhibitor 1 | Extracellular     |
| Q6GZP0     | Platelet-derived growth factor D  | Extracellular     |
| P07602     | Proactin A polypeptide [Contains: Saposin-A] | Extracellular |
| Q15113     | Procollagen C-endopeptidase enhancer 1 | Extracellular |
| Q02809     | Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 | Extracellular |
| P07737     | Profilin-1                        | Cytoplasm         |
| P41222     | Prostaglandin-H2 D-isomerase      | Cytoplasm         |
| Q6UXB8     | Protease inhibitor 16             | Membrane          |
| Q07237     | Protein disulfide-isomerase       | Cytoplasm         |
| Q15084     | Protein disulfide-isomerase A6    | Cytoplasm         |
| P14618     | Pyruvate kinase isozymes M1/M2    | Cytoplasm         |
| Q6ED15     | Reticulocalbin-3                  | Cytoplasm         |
| Q99969     | Retinoic acid receptor responder protein 2 | Membrane |
| O75326     | Semaphorin-7A                     | Membrane          |
| Q12884     | Seprase                           | Cytoplasm         |
| Q92743     | Serine protease HTRA1             | Extracellular     |
| P02787     | Serotransferrin                   | Extracellular     |
| P09486     | SPARC                             | Extracellular     |
| Q98UD6     | Spondin-2                         | Extracellular     |
| Q9BRK5     | Stromal cell-derived factor 4     | Cytoplasm         |
| C00391     | Sulfhydryl oxidase 1              | Cytoplasm         |
| P00441     | Superoxide dismutase [Cu-Zn]      | Cytoplasm         |
| Q9Y490     | Talin-1                           | Membrane          |
| P24821     | Tenascin                          | Extracellular     |
| P22105     | Tenascin-X                        | Extracellular     |
| Q08629     | Testican-1                        | Extracellular     |
| P10599     | Thioredoxin                       | Cytoplasm         |
| Q16881     | Thioredoxin reductase 1           | Cytoplasm         |
| P07996     | Thrombospondin-1                  | Extracellular     |
| P35442     | Thrombospondin-2                  | Extracellular     |
| Q6FGX5     | TIMP1 protein                     | Extracellular     |
| Q15582     | Transforming growth factor-beta-induced protein ig-h3 | Extracellular |
| O14773     | Tripeptidyl-peptidase 1           | Cytoplasm         |
| Q6EMK4     | Vasorin                           | Membrane          |
| P13611     | Versican core protein             | Extracellular     |
| P08670     | Vimentin                          | Cytoplasm         |
| P04004     | Vitronectin                       | Extracellular     |
| O76076     | WNT1-inducible-signaling pathway protein 2 | Extracellular |

*The subcellular location was determined for each protein based on the Ingenuity Pathway Analysis software (Ingenuity Systems).*

digested with PNGase F in the presence of $^{18}$O water to convert any N-glycan-modified Asp to an $^{18}$O-Asp residue. The resulting peptides were analyzed by LC-MS/MS using the previously described method for ITMS and the parent mass list method for FTMS as described in *Materials and Methods*. To obtain the parent mass list, the protein sequences of the 190 identified secreted proteins from Table 1 and Additional file 1: Table S1 were extracted from the database. From these sequences, tryptic peptides, with allowance for two internal missed cleavage sites, that
Figure 3 The functional categories of the primary human adipocyte secretome. The biological function analysis was determined for each protein based on the Ingenuity Pathway Analysis software (Ingenuity Systems).

Table 2 Human adipocytokines regulated a minimum of 150% under both insulin resistant conditions

| No. | Protein ID | Identified proteins                            | LGPUG/LG | HGINS/LG | Ave. SC | SD   | Ave. peptides | SD   |
|-----|------------|-----------------------------------------------|----------|----------|---------|------|---------------|------|
| 1   | P61769     | Beta-2-microglobulin                          | 2.40     | 1.90     | 68.17   | 35.76| 3.17          | 0.98 |
| 2   | P00739     | Haptoglobin-related protein                   | 1.87     | 1.66     | 5.67    | 0.58 | 2.00          | 0.00 |
| 3   | Q16270     | Insulin-like growth factor-binding protein 7  | 1.75     | 1.59     | 19.00   | 9.76 | 4.33          | 1.03 |
| 4   | Q05965     | Integrin beta-like protein 1                  | 1.68     | 1.57     | 9.00    | 1.41 | 3.00          | 0.00 |

LG: low glucose; HG: high glucose. Catagories were determined using Ingenuity Pathway Analysis (Ingenuity Systems).
contained the consensus sequence, N-X-S/T, were obtained for theoretical mass calculations. Table 3 shows a total of 91 N-linked glycosylation sites that were identified on 52 proteins. These sites covered 10.3% of the theoretical 882 N-linked glycosylation site sequences generated from the secreted protein list.

Characterization of the human adipocyte glycome
To better define the glycome of human adipocytes, we performed N-linked and O-linked glycan analysis using mass spectrometry. Glycans were released from whole-cell human adipocyte extracts by PNGase F for N-linked glycans and by β-elimination for O-linked glycans as shown in Figure 2C. Figure 5A shows that the permethylated N-linked glycans were characterized by full FTMS spectrum using a LTQ Orbitrap XL (left panel, top) followed by MS/MS fragmentation by TIM analysis in the ion trap (left panel, bottom). The fragmentation of a biantennary N-linked glycan is shown as an example. We used the GlycoWorkbench to manually interpret a total of 155 N-linked glycan structures from MS/MS spectra at different charge states as shown in Additional file 1: Table S3. In Figure 5A (right panel), we show that 28 of the predominant N-linked glycans are assigned to a full FTMS and a MS/MS spectrum.

Figure 5B (left panel) shows a full FTMS spectrum from an O-linked glycan mixture. 10 of the predominant O-linked glycans were assigned from the full FTMS spectra (Figure 5B, bottom panel). A representative fragmentation spectrum is shown for one of the core 2 O-linked glycans (Figure 5B, right panel). A total of 29 O-linked glycans were characterized from MS/MS spectra at singly and doubly charged states as shown in Additional file 1: Table S4.

Relative quantification of the human adipocyte glycome
upon the induction of insulin resistance using 13C labeling and prevalence
For the comparative quantification of glycans, we performed isotopic labeling using heavy/light iodomethane (13CH3I and 12CH3I) [78,79]. The 13C/12C ratio from the sum of the peak areas and the prevalence ratio between the different treatment conditions were used for relative quantification. Permethylated glycans were mixed in a 1:1 protein ratio and analyzed in quadruplicate using an LTQ Orbitrap XL. Figure 6A (left panel) shows the isotopic pairs of N-linked glycans on a full FTMS spectrum. Figure 6A (right panel) shows an example of a calculated 13C/12C ratio from sum of peak area between LG + PUGNAc (LGPG) and LG conditions. Additional file 1: Table S5 shows a total of 48 N-linked glycans that were relatively quantified between the insulin resistant conditions (LGPG and HGINS) and insulin responsive condition (LG) by the average 13C/12C ratio and the average prevalence ratio. This approach simultaneously quantified a broad range of N-glycan structures in a complex mixture; however we did not observe highly significant changes in N-glycan 13C/12C ratios or prevalence ratios. Figure 6B (left panel) shows the isotopic pairs of O-linked glycans on a full FTMS spectrum.
| No. | Protein ID   | Identified proteins                  | N-linked peptides |
|-----|--------------|--------------------------------------|------------------|
| 1   | P15144       | Aminopeptidase N                     | KLN@TYLSQGHR     |
| 2   | P21810       | Biglycan                             | LLQVYVLHNSN@HTK  |
| 3   | P16870       | Carboxypeptidase E                   | GN@ETINLIHSTR    |
| 4   | P07339       | Cathepsin D                          | GSLSYLN@VTR      |
| 5   | P43235       | Cathepsin K                          | SN@DTLY1PEWEGR   |
| 6   | P07711       | Cathepsin L                          | YSVAN@DTGFVDIPK  |
| 7   | P07711       | Cathepsin L                          | YSVAN@DTGFVDIPKQEK|
| 8   | P10909       | Clusterin                            | LAN@LTQGECQYLYR  |
| 9   | P02461       | Collagen alpha-1(III) chain          | ASQN@ITYHCK      |
| 10  | P02461       | Collagen alpha-1(III) chain          | DGSRPGKGDRGERN@GSPGAPGAPGHPGGPMGGPK  |
| 11  | P20908       | Collagen alpha-1(V) chain            | VYCNQ@FTAGGSTDYVDPKK |
| 12  | P12109       | Collagen alpha-1(VI) chain           | ENYAELEDALNK@TAQJCIDKK |
| 13  | P12109       | Collagen alpha-1(VI) chain           | GEDPGAN@GTEFGPFPFGPFGP         |
| 14  | P12109       | Collagen alpha-1(VI) chain           | NQ@FTAADWQGSR     |
| 15  | P12109       | Collagen alpha-1(V) chain            | RN@FTAADWQGSR     |
| 16  | Q02388       | Collagen alpha-1(VII) chain          | TAPFEGVRVSRQLNL@ASDDVL     |
| 17  | Q99715       | Collagen alpha-1(XII) chain          | EAGN@ITTVDGELGK   |
| 18  | P08123       | Collagen alpha-2(I) chain            | LLLNYASQNP@ITYHCK  |
| 19  | P05997       | Collagen alpha-2(V) chain            | EASQNP@ITYHCK     |
| 20  | P12111       | Collagen alpha-3(V) chain            | GNPGEPLNL@GTTGPKGR |
| 21  | P12111       | Collagen alpha-3(VI) chain           | GPPGVL@GTOGFOPFGCPQR  |
| 22  | P12111       | Collagen alpha-3(V) chain            | GYPIPGEGRPGERPGPVN@GTOGFOPFGCPQR |
| 23  | P09871       | Complement C1 subcomponent           | NCGN@CSGDVFATALQGPNPKYPENSR  |
| 24  | O75462       | Cytokine receptor-like factor 1      | VLN@ASTLALANLNL@GSR     |
| 25  | O75462       | Cytokine receptor-like factor 1      | VDTDNSN@GTSR       |
| 26  | P07585       | Decorin                              | IADTNP@ISPGQPLLPSLTELHLDGKN  |
| 27  | P07585       | Decorin                              | LGLSFNSISAVDNP@GLSANPFLR     |
| 28  | Q098P4       | Dickkopf-related protein 3           | GSN@GTDQCDNR       |
| 29  | Q13822       | Ectonucleotide pyrophosphatase/phosphodiesterase 2 | AEIGVEEGPTVLS3S5PN@STSGCK |
| 30  | Q13822       | Ectonucleotide pyrophosphatase/phosphodiesterase 2 | AILAN@LTCK       |
| 31  | Q96C2        | EMILIN-1                             | LGALN@SSQLEDR    |
| 32  | P35555       | Fibrillin-1                          | TAIAF@ISHSVSNK    |
| 33  | P02751       | Fibronecin                           | DQCIVDDITVYNN@OTFHKK |
| 34  | P02751       | Fibronecin                           | DQCIVDDITVYNN@OTFHKK |
| 35  | P02751       | Fibronecin                           | LDAPNLQFVN@ETDSTVLVR |
| 36  | Q12841       | Follistatin-related protein 1        | GSN@YSYELDK     |
| 37  | Q12841       | Follistatin-related protein 1        | GSN@YSYELDKYFK   |
| 38  | P09382       | Galectin-1                           | FNAHGDANTVCNSK   |
| 39  | Q08380       | Galectin-3-binding protein           | ALCFEN@ATQALGR    |
| 40  | Q08380       | Galectin-3-binding protein           | DAGVUCN@ETR      |
| 41  | Q08380       | Galectin-3-binding protein           | TVRFPSYTN@SSGVD   |
| 42  | P00738       | Haptoglobin                          | WLLHP@YSQVXGLIK  |
| 43  | O75629       | Human Protein CREG1                  | LNA@ITNWLVDYFGPK  |
| Protein ID | Description | N-linked glycosylation site |
|-----------|-------------|----------------------------|
| P17936    | Insulin-like growth factor-binding protein 3 | GLCVN@ASAVSR |
| O14498    | ISLR        | SLSDLHNLISOFAWSLDLHNgLSALQLLK |
| Q68HP8    | LAMA-like protein 2 | SDLNPA@GYSYFKALR |
| Q16363    | Laminin subunit alpha-4 | DAVRNg@TEVPPQDQLR |
| Q16363    | Laminin subunit alpha-4 | FYFGGSPISAYAN@FTGCISNAYFTR |
| Q16363    | Laminin subunit alpha-4 | LITEAN@R |
| Q16363    | Laminin subunit alpha-4 | LTSLEDDIIKn@AGSIAEIDGAK |
| Q16363    | Laminin subunit alpha-4 | RPASNg@VASIQR |
| P07942    | Laminin subunit beta-1 | LSDTTSQNg@STAK |
| P11047    | Laminin subunit gamma-1 | VN@NTRLSSQSR |
| P11047    | Laminin subunit gamma-1 | KYTEQAN@SQDLEK |
| P11047    | Laminin subunit gamma-1 | LN@NRLTSIK |
| P11047    | Laminin subunit gamma-1 | TAN@DTSTEAYNLLL |
| P11047    | Laminin subunit gamma-1 | TLGEN@QPTAFEELNR |
| P11047    | Laminin subunit gamma-1 | VN@NKLTAEEAELR |
| Q14767    | Latent-transforming growth factor beta-binding protein 2 | DTGTQQAVPLEHPSWGLNgLTEK |
| P51884    | Lumican | AFE@N@TDLQQWILDHNLLENSK |
| P51884    | Lumican | LGSNFLigin@LTHIQLNHQ |
| P51884    | Lumican | LIH@NINNg@LTEVGPLPK |
| P13473    | Lysosome-associated membrane glycoprotein 2 | IA@QPGFPPSWIAN@FTK |
| P10033    | Metalloproteinase inhibitor 1 | AKFVTPT VN@Q@QTTLYQR |
| P10033    | Metalloproteinase inhibitor 1 | FV@PT@PNeQ@QTLYQR |
| P10033    | Metalloproteinase inhibitor 1 | SH@N@RSEFLIAQK |
| Q9NRNS    | Olfactomedin-like protein 3 | IY@VDGTQNg@DTAFVPFR |
| P26022    | Pentraxin-related protein PTX3 | ADTV@NgK |
| Q15063    | Periostin | EV@N@DTLVLNLK |
| Q15063    | Periostin | IFL@Evn@DLTVNLNLK |
| P98160    | Perlecain | SLTQGSLVLGLAPVN@GSQGK |
| P55058    | Phospholipid transfer protein | VSN@VSCOASVSR |
| P36955    | Pigment epithelium-derived factor | VTN@NLTLIEESLTSEFHDIDR |
| P36955    | Pigment epithelium-derived factor | VTN@NLTLIEESLTSEFHDIDRELK |
| P05155    | Plasma protease C1 inhibitor | VGQLQ@SHNg@LSLVLVPQNLK |
| P05155    | Plasma protease C1 inhibitor | VLSNg@NSDANELINTWVAK |
| P07602    | Proactivator polypeptide [Contains: Saposin-A] | TN@STFVOALVEHK |
| P07602    | Proactivator polypeptide [Contains: Saposin-A] | LIDNN@KTEK |
| P07602    | Proactivator polypeptide [Contains: Saposin-A] | LIDNN@KTEKEILDADFK |
| P07602    | Proactivator polypeptide [Contains: Saposin-A] | NLEKN@GSTK |
| P07602    | Proactivator polypeptide [Contains: Saposin-A] | NLEKN@SKQELIALEK |
| P07602    | Proactivator polypeptide [Contains: Saposin-A] | TN@STFVOALVEHVK@EEDCR |
| P09486    | SPARC | VCS@NDNgK |
| P09486    | SPARC | VCS@NDNgKTDFSSCHFATK |
| P24821    | Tenascin | Ng@TTSYLR |
| P24821    | Tenascin | LN@YSLPTGQWGVQLPR |
| P07996    | Thrombospondin-1 | VWN@STTPGGEHLR |
Table 3 Identification of N-linked glycosylation sites using PNGase F with the incorporation of 18O water in human adipocytokines (Continued)

| No. | Accession | Protein | Amino acid sequence | Calculated 13C/12C ratio |
|-----|-----------|---------|---------------------|-------------------------|
| 88  | P35442    | Thrombospondin-2 | WN@STITGTGEHLR | 2.05 (2.03-2.07) |
| 89  | Q6FGXS    | TIMP1 protein | FVGTPV@NQQTLYQR  | 2.06 (2.04-2.07) |
| 90  | Q6FGXS    | TIMP1 protein | SH@RSEELJAGK    | 2.07 (2.04-2.08) |
| 91  | Q6EMK4    | Vasorin  | LHEITN@ETFR       | 2.08 (2.04-2.09) |

*An @ indicates the site of N-linked glycosylation.

Discussion

Several groups have reported the rodent adipocyte secretome during insulin resistance, but there have been fewer such studies performed with human adipocytes [25,80-82]. It is advantageous to investigate the secretome of human adipose tissue because there are bound to be differences between the rodent and human secretome [8]. The differences in the secretome of human adipose tissue have been investigated during adipogenesis and between different fat pad depots [83-86]. Other studies have characterized the secretome without quantification [87,88]. Another approach has been to study a limited list of known adipocytokines during insulin sensitivity and insulin resistance [89,90]. Therefore, our study fills an important gap in the literature by taking a discovery approach to characterizing and quantifying the human adipocyte secretome in both insulin sensitive and two different insulin resistant conditions. In addition, we characterize and quantify the glycome of human adipocytes, which has not been described to our knowledge.

We report a total of 193 secreted proteins from human adipocytes (Table 1 and Additional file 1: Table S1). Using Ingenuity Pathways Analysis we determined that many of the secreted proteins were enzymes, peptidases, or other functions (Figure 3). The largest pool of identified proteins from the other category corresponds to extracellular matrix proteins. A common theme among the adipose tissue secretome studies is that extracellular matrix (ECM) proteins, ECM remodelers, inflammatory, and angiogenesis proteins are highly represented [7,81,87,91]. The accumulation of fat mass during obesity requires extensive tissue remodeling that is accomplished by ECM remodelers. The remodeling can generate an inflammatory response during obesity and insulin resistance due to limited angiogenesis, localized hypoxia, adipocyte necrosis, increased fibrosis, altered adipocytokine secretion, and M1-stage macrophage and other immune cell infiltration [4,92-96]. Many of the adipocytokines we identified, such as SPARC and Chitinase-3-like protein 1, have been implicated in inflammation [97,98]. In addition, tissue remodeling requires extensive crosstalk between the different cell types that comprise adipose tissue. Several of the adipocytokines we identified, such as Gelsolin, Calreticulin, and Cathepsin D, may be expressed in cell types other than adipocytes [87,99]. By using primary adipocytes instead of a cell line, the secretome we identified better represents the physiological status of adipose tissue. In the future, these identified adipocytokines may potentially be used as prognostic/diagnostic biomarkers for metabolic syndrome, T2DM, and other complications.

We generated insulin resistance in the human adipocytes by both directly and indirectly modulating O-GlcNAc levels. The elevation of O-GlcNAc levels has been shown to be sufficient to induce insulin resistance in many systems [19-24]. We have previously demonstrated that the induction of insulin resistance in this way in rodent adipocytes alters the secretion of several adipocytokines [25]. Here, we showed that 20 and 4 adipocytokines were upregulated or downregulated, respectively, upon the transition to insulin resistance by both directly and indirectly modulating O-GlcNAc levels (Table 2). We also identified 28 and 8 adipocytokines that were upregulated or downregulated, respectively, upon the induction of insulin resistance by one of the two methods (Additional file 1: Table S2). In this study, we find that Sulphydryl oxidase 1 (Quiescin Q6), laminin B1, and Chitinase-3-like protein 1 are upregulated during both insulin resistant conditions, which was also observed in the rodent study. SPARC was upregulated in both insulin resistant conditions in human adipocytes and was found to be upregulated in HG + INS in rodent adipocytes by proteomics. Four other proteins were observed to be regulated by insulin resistance in both rodent and human adipocytes; however, the regulation was slightly different between species. Thioredoxin was downregulated in both insulin resistant conditions in rodent adipocytes and during HG + INS in human adipocytes but was upregulated during LG + PUG in human adipocytes. Gelsolin was upregulated in both insulin resistant conditions in rodent adipocytes and during HG + INS in human adipocytes but was not regulated by LG + PUG. Fibulin 2 was downregulated in both insulin resistant conditions in rodent adipocytes but was not...
Figure 5 (See legend on next page.)
regulated or upregulated in LG + PUG and HG + INS, respectively, in human adipocytes. From these examples, it is clear that there are differences in adipocytokine secretion between rodents and humans. Because the proteome sample is extremely complex, we may not have achieved sufficient resolution to quantify some of the proteins that were observed in both rodent and human adipocyte secretomes, such as Angiotensinogen, Cathespin B, and Spordin 1 [25,100]. Because we assigned significance to a relatively small fold change (1.5 fold) for quantification, we verified the regulation of two adipocytokines that changed by 1.5 fold by an independent, orthogonal method. Both SPARC and Chitinase-3-like protein 1 protein levels were altered in a similar manner as measured by both quantitative proteomics and immunoblotting (Figure 4). In addition, the regulation of these proteins by O-GlcNAc was verified using a more OGA-specific N-acetylgalactosaminidase inhibitor, GlcNAcstatin.

The inclusion of an insulin resistance condition generated by directly modulating O-GlcNAc levels in our proteomic study helped us to better define the proteins regulated by O-GlcNAc in human adipocytes. The O-GlcNAc modification is found on hundreds in nucleocytoplasmic proteins and can affect protein function in diverse ways, such as protein-protein interactions, protein degradation, interplay with phosphorylation, localization, and transcriptional activation [101]. Compiling a list of proteins modulated by O-GlcNAc is the first step in understanding the mechanism of how O-GlcNAc affects adipocytokine expression. We have used the data generated by our previous rodent proteomic study to investigate the transcriptional regulation of adipocytokines by global O-GlcNAc levels (unpublished data). Although several studies have associated elevated O-GlcNAc levels with adipocytokine expression, no specific molecular mechanism has been reported [13-16,21,102,103].

We also characterize the N-linked and O-linked glycome for human adipocytes. Glycosylation and other PTMs provide an additional layer of regulatory complexity to expressed proteins. To understand the full functionality of a protein, one must know the state of the PTMs. Since secretory proteins, such as adipocytokines, are exposed to the glycosylation machinery that reside in the ER and Golgi apparatus as they make their way through the secretory pathway, they often have a high degree of complex glycosylation. The subset of glycans expressed by cells depends on multiple factors, such as developmental stage, tissue type, and the genetic and physiological state of the cell. It is not known whether adipocytes change glycan expression patterns in response to insulin resistance. Since glycosylation can affect diverse processes that are relevant to adipose tissue remodeling in obesity, such as cell-cell interactions, growth factor sequestration, cytokine activation, and cell migration, there is a need to define the adipocyte glycome [34,104,105].

Characterizing the glycome poses many challenges because there is no template for modification, as in DNA, and there is an incredible diversity of glycoforms. Mass spectrometry can provide both structural and abundance information using a small amount of material from a relatively complex mixture [106]. However, it is still difficult to assign specific glycoforms to specific protein residues in a complex mixture. To determine sites of N-glycosylation on the secreted proteins, we used PNGase F and 18O water to convert the glycan-modified Asn to an 18O-Asp residue, thereby leaving a chemical marker of the glycosylation site. We identified 91 sites on 51 proteins of N-linked glycosylation by Orbitrap mass spectrometer using the parent mass list obtained from theoretical consensus sequence in the secretome list (Table 3). For relative glycan quantification, we permethylated using heavy or light iodomethane (13CH3I and 12CH3I) and used prevalence data. The use of isotopic labels allows for mixing of the samples to be compared before analysis, thus providing an internal standard and preventing run to run technical variation [49,78]. These techniques allow us to determine the full diversity and changes in major and minor adipocyte glycans during insulin sensitive and resistant conditions. We characterized a total of 155 N-linked glycans and 29 of O-linked glycans (Figure 5, Additional file 1: Table S3, Table S4). The results presented here demonstrate that the human adipocytes are expressing extended hybrid and complex N-linked glycans, in addition to high mannose glycans. Adipose tissues possess the biosynthetic ability to generate diverse O-linked glycans such as core structure, O-glucose glycans, and O-fucose glycans.

Following characterization, we determined the change in glycan prevalence during insulin resistance using isotopic and prevalence ratios. We quantified 48 N-linked glycans and 12 O-linked glycans (Figure 6, Additional file 1: Tables S5-S6). Our results showed that changes of glycan prevalence were not statistically different between
Figure 6 (See legend on next page.)
the insulin sensitive and insulin resistant conditions. It has been suggested that altering the intracellular UDP-GlcNAc pool, such as during increased glucose flux, changes the degree of N-glycan branching in an ultrasensitive manner [37]. While it is clear that the induction of insulin resistance in adipocytes massively upregulates intracellular glycosylation (Figure 1), we did not observe significant changes in complex glycosylation. This suggests that changes in complex glycosylation are not necessary for the induction of insulin resistance; although we cannot rule out that they play a role in insulin resistance that occurs over a long period of time. Since insulin resistance was generated by treatment for 40 hours, it is possible that the changes in glycan structure had not had time to significantly change.

In conclusion, we have characterized the secretome and glycome of primary human adipocytes during insulin resistance using a proteomic approach. We generated insulin resistance by either directly or indirectly modulating O-GlcNAc levels, which provides a list of adipocytokines that are modulated by O-GlcNAc levels. Adipocytokine and glycogen levels were quantified between insulin sensitive and resistant conditions and insulin responsive condition by 13C/12C ratio and prevalence ratio.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
JML participated in the design and execution of experiments and in the writing of the manuscript. EEB-H participated in the execution of experiments as in the writing of the manuscript. CFT and DH participated in the execution of experiments and editing of the manuscript. LW participated in the design of experiments and the writing and final editing of the manuscript. All authors read and approved the final manuscript.

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Author details
1Complex Carbohydrate Research Center, The University of Georgia, 315 Riverbend Road, 30602-4712 Athens, Georgia. 2Department of Chemistry, The University of Georgia, 30602 Athens, Georgia. 3Department of Biochemistry and Molecular Biology, The University of Georgia, 30602 Athens, Georgia. 4Department of Foods and Nutrition, The University of Georgia, 30602 Athens, Georgia. 5Department of Chemistry, Changwon National University, Changwon, Gyeongnam 641-773, South Korea.

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