CD44, a Predominant Protein in Methylglyoxal-Induced Secretome of Muscle Cells, is Elevated in Diabetic Plasma

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ABSTRACT: Methylglyoxal (MG), a glycolytic intermediate and reactive dicarbonyl, is responsible for exacerbation of insulin resistance and diabetic complication. In this study, MG-induced secretome of rat muscle cells was identified and relatively quantified by SWATH-MS. A total of 643 proteins were identified in MG-induced secretome, of which 82 proteins were upregulated and 99 proteins were downregulated by more than 1.3-fold in SWATH analysis. Further, secretory proteins from the classical secretory pathway and nonclassical secretory pathway were identified using SignalP and SecretomeP, respectively. A total of 180 proteins were identified with SignalP, and 113 proteins were identified with SecretomeP. The differentially expressed proteins were functionally annotated by KEGG pathway analysis using Cytoscape software with plugin clusterMaker. The differentially expressed proteins were found to be involved in various pathways like extracellular matrix (ECM)–receptor interaction, leukocyte transendothelial migration, fluid shear stress and atherosclerosis, complement and coagulation cascades, and lysosomal pathway. Since the MG levels are high in diabetic conditions, the presence of MG-induced secreted proteins was inspected by profiling human plasma of healthy and diabetic subjects (n = 10 each). CD44, a predominant MG-induced secreted protein, was found to be elevated in the diabetic plasma and to have a role in the development of insulin resistance.

INTRODUCTION

Methylglyoxal (MG) is produced as a minor byproduct of various metabolic processes. However, in insulin-resistant states like obesity and type 2 diabetes, MG levels are known to increase due to altered glucose metabolism. The concentration of MG in the plasma of diabetic patients is associated with the level of blood glucose. The intracellularly produced MG can readily diffuse into the extracellular matrix. Since MG is highly reactive, it readily reacts with and modifies proteins, lipids, and DNA and can affect their normal structure and function. It is also a major precursor of advanced glycation end products, which are reported to be involved in the development of diabetes and diabetic complications and whose detrimental effects have been widely reported. Therefore, various detoxification mechanisms are present in the body, one of them being the glyoxalase system. This system comprises glutathione and two enzymes, viz., glyoxalase I and glyoxalase II. MG first reacts with glutathione to form a hemithioacetal, which is metabolized by glyoxalase I into S-D-lactoylglutathione. This intermediate is further metabolized into D-lactate by glyoxalase II, and glutathione is regenerated. However, in diabetes, because of higher levels of MG, it can modify proteins from several tissues, including vascular endothelium and smooth muscles. MG-induced modification in proteins may have adverse effects on cellular processes, and therefore, such proteins are removed by exocytosis or secretion into the extracellular matrix.

Secreted proteins comprise an important class of biomolecules, that include a wide array of proteins such as serum proteins and matrix proteins as well as hormones and growth factors. It is believed that approximately 10% of the human genome encodes for secreted proteins. Quintessential examples of secreted proteins include serum albumin, immunoglobulins, and enzymes of the digestive tract. Even in the regulation of cell renewal and differentiation, proteins secreted in low abundance but that are highly bioactive, such as cytokines, and growth factors contribute significantly.

Recent studies have indicated that apart from being the largest organ, skeletal muscles also have an endocrine function,
producing a large number of proteins called myokines. These proteins have been reported to have widespread action on different organs and tissues. A number of tissues are reported to be regulated by myokines, and the synthesis and secretion of myokines are controlled by several factors including differentiation, exercise, and in vitro electrical stimulation. Apart from these factors, insulin resistance affects the secretion of myokines. Various factors including oxidative stress, palmitic acid, and TNF-α are known to induce insulin resistance and affect the myokine secretion in muscle cells. Similarly, it has been shown that MG induces insulin resistance in muscle cells, however, MG-induced changes in myokine secretion are not yet studied. Characterization of skeletal muscle secretome is still not complete even after the identification of numerous myokines in recent profiling studies, which is evident from the amount of overlap seen among these studies. The pathogenesis of type 2 diabetes affects the functioning of all major organs that govern metabolic control including skeletal muscles. Since myokines influence a number of different organs, any alteration of the skeletal muscle secretome can have wide and detrimental effects. Herein, we sought to identify the MG-induced secretome of rat muscle cells and its validation in clinical plasma samples to identify potential biomarkers for diabetes.

**METHODOLOGY**

**Chemicals.** All fine chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM) for cell culture was procured from HiMedia. Fetal bovine serum (FBS) (US origin) and trypsin were purchased from Invitrogen (Carlsbad, CAA). Plasticware for tissue culture was procured from Nunc (Rochester, NY). The Bradford protein estimation kit was purchased from Bio-Rad Laboratories. Centrifugal filters (3 kDa cutoff) and C18 Zip-Tip desalting columns were procured from Millipore (Millipore, Billerica, MA). Liquid chromatography columns, Eskigent C18-RP HPLC column (100 × 0.3 mm, 3 μm, 120 Å), were procured from Sciex (Framingham, MA). The RapiGest SF surfactant was purchased from Waters (#186001860, Waters Corporation, MA). All solvents for liquid chromatography—mass spectrometry (LC—MS) were procured from J.T. Baker (J T. Baker, PA). The ELISA kit sCD44std Instant ELISA was procured from Thermo (#BMS209INST, ebioscience, Vienna, Austria).

**Research Design.** The schematic of the complete experimental design adopted in this study is depicted in Figure 1. The study was conducted to characterize methylglyoxal-induced secretome of muscle cells and identify the presence of methylglyoxal-induced secreted proteins in the diabetic plasma. The conditioned media from three individual experiments, each of control cells and methylglyoxal-treated cells were used for the identification of secretome by SWATH-MS. The mass spectrometric acquisition was performed for three different experiments in technical triplicates. The presence of methylglyoxal-induced secreted proteins was evaluated by performing proteomics analysis of 10 plasma samples each of healthy control and diabetic subjects by SWATH-MS in technical triplicates. CD44, one of the predominant methylglyoxal-induced secreted proteins, was evaluated for its presence in the same set of clinical samples in technical duplicates. Since this was a pilot study, the sample size was limited to a maximum of 20 samples.

![Figure 1](https://dx.doi.org/10.1021/acsomega.0c01318)  
**Figure 1.** Overview of the complete experimental design.
cell secretome. Clinical plasma samples were collected from the Chellaragam Diabetes Institute, Pune, India, as per the guidelines of the Indian Council of Medical Research. The approval of the Institutional Ethics Committee was taken for the study, and informed consent was obtained from all of the subjects before blood collection. Only subjects with no history of other chronic clinical disorders, such as liver/kidney disease, thyroid disorders, or pregnancy, were included in the study. The proteomics study and enzyme-linked immunosorbent assay (ELISA) study both comprised healthy subjects \((n = 10)\) and diabetic subjects \((n = 10)\). Plasma preparation was done by collecting peripheral venous blood in ethylenediaminetetraacetic acid (EDTA) vacutainers (BD Biosciences). After the sample collection, biochemical parameters such as fasting blood glucose and glycated hemoglobin \((\text{HbA}_{1c})\) were analyzed immediately. Details of patients are given in Table S1. Erythrocytes were separated from plasma by centrifugation at 200g for 5 min. Next, the supernatant was centrifuged at 9300g for 15 min and plasma was carefully separated and stored at \(-80^\circ\text{C}\) until use.

**In-Solution Trypsin Digestion.** Total protein \((100 \mu\text{g})\) in 0.1% Rapigest for each plasma sample was used for digestion. Before reduction and alkylation, proteins were denatured by incubating at 80 °C for 15 min. Further, proteins were reduced using 100 mM dithiothreitol for 15 min at 60 °C and then alkylated by treating with 200 mM iodoacetamide for 30 min in the dark at room temperature. Trypsin was added at 1:25 (w/w) enzyme-to-protein ratio and incubated at 37 °C for 18 h; 2 \(\mu\text{L}\) of formic acid was added to inactivate trypsin and stop the digestion. For desalting of the digested peptides, C18 ZipTips were used, and the desalted peptides were concentrated using Speed Vac. For LC–MS analysis, peptides were reconstituted in 3% acetonitrile with 0.1% formic acid.

**Liquid Chromatography–Mass Spectrometry Analysis.** SWATH-MS Analysis for Secretome. SWATH analysis was performed on a Triple-TOF 6600 (AB Sciex; Concord, Canada) mass spectrometer coupled with Micro LC 200 (Eksigent; Dublin, CA) in high-sensitivity mode. A spectral library was generated by pooling equal amounts of peptide samples from each treatment and analyzing by information-dependent acquisition (IDA). Briefly, peptides were separated on a Eksigent C18-RP HPLC column \((100 \times 0.3 \text{ mm}, 3 \mu\text{m}, 120 \AA)\) using a 95 min gradient of 3% to 35% mobile phase B at a flow rate of 8 \(\mu\text{L}\)/min (mobile phase A: water with 0.1% formic acid; mobile phase B: acetonitrile with 0.1% formic acid). Accumulation time for MS was 250 ms and for MS/MS was 100 ms. Fragmentation was done using rolling collision energy. For SWATH-MS acquisition, the precursor mass range of 400–1250 \(m/z\) was divided into a set of 34 overlapping windows of 25 \(m/z\) each. For MS/MS, collision energy was optimized for each window and spectra were collected from 100 to 2000 \(m/z\). Fragment ion scans were performed over an accumulation time of 70 ms, while for the precursor survey scan, it was 100 ms. All samples were acquired in biological and technical triplicates.

To get spectral library from IDA run, data was analyzed by ProteinPilot software version 5.0 using UniProt *Rattus norvegicus* database updated with Uniprot release 2019_02; 8,060 reviewed protein entries. The enzyme used for digestion was set to trypsin, and carbamidomethylation of cysteine residues was set as fixed modification. Search was performed using a false discovery rate (FDR) of 1%. The resultant spectral library was imported into PeakView v2.2 software, and SWATH runs for all samples were processed using 50 ppm mass error, 5 min RT window, 99% confidence, and 1% FDR. The peak areas thus generated in PeakView were imported into MarkerView v 1.2.1, wherein statistical analysis using the t-test was performed. Normalization across the runs was performed using total area sum. All of the raw mass spectrometric data have been deposited at the public repository PeptideAtlas (PASS01477).

**SWATH-MS Analysis for Clinical Plasma.** LC–MS Conditions. The trypsin-digested samples (healthy and diabetic) were desalted offline by Zip-Tip (Millipore) and loaded on to an EksigentTrap (ChromXP C-18-CL: 0.35 × 0.5 mm, 3 \(\mu\text{m}\), 120 Å) and analytical Nano column (ChromXP3C-18-CL: 0.075 × 15 mm, 3 \(\mu\text{m}\), 120 Å) fitted with an Eksigent nanoLC-Ultra 2D system, which was connected to a Triple-TOF 6600 mass spectrometer (Sciex, Ontario, Canada). Peptides were separated on nanoLC using the following mobile phase composition: water/acetonitrile/formic acid \((\text{A}, 98/2/0.2%; \text{B}, 2/98/0.2%).\) The flow rate was kept at 300 nL/min, and 2000 ng of each sample was injected on the column. An overall 3.5 h gradient run was used for separation, which started with 5 min of 5% B, followed by a linear increase of B to 35% in 80 min and further increase to 50% B in 90 min. In the end, mobile phase B was increased to 90% in 1 min and maintained for 13 min, after which the gradient was brought back to the initial 5% B in 1 min and held for 6 min.

MS/MS Conditions. The sample data was acquired in information-dependent acquisition (IDA) mode using an ESI ion source. The ESI source was maintained at a voltage of 2300 V and a temperature of 130 °C, with curtain gas of 25 psi, nebulizer gas of 20 psi, and heater gas of 10 psi. For MS survey scan, mass range \(m/z\) 350–1250 was scanned with a resolution \(\geq 30\,000\) full width at half-maxima (FWHM) with an accumulation time of 250 ms. For MS/MS, 35 product ion scans over the mass range \(m/z\) 100–1600 were collected in high-sensitivity mode. The criteria for precursor selection for MS/MS were set at a threshold of more than 120 counts per second and a charge state of 2+ to 5+. Also, the dynamic exclusion for precursor selection was set for 3 s. Collision energy (CE) for fragmentation was optimized for each precursor using an IDA CE parameter script, and collision energy spread (CES) was set at 5 eV. The total cycle time for the survey scan and product ion scan was set to 2.35 s.

**Label-Free Quantitation.** Label-free quantification was performed using SWATH-MS, wherein the mass range of 350–1250 Da was divided into a set of 75 overlapping windows of 12 Da each with 1 Da overlap between each window. The CE for each window was optimized, and a CES of 5 eV was set. MS/MS scans were acquired in high-sensitivity mode with a mass resolution of 15,000. The total duty cycle comprised one MS survey scan of 50 ms and 75 MS/MS scans of 50 ms each. Each sample was acquired in triplicate runs for quantification. The raw mass spectrometric data for all IDA and SWATH runs is deposited at the public repository PeptideAtlas (PASS01478).

**SWATH Data Processing.** The IDA runs were searched against the UniProt protein database \(\text{release 2018_04; 20,341}\) reviewed protein entries) limited to *Homo sapiens* taxonomy with ProteinPilot v5.0 software. For protein identification, the PEAK score was set to 0.05. The enzyme used for digestion was set to trypsin, and carbamidomethylation of cysteine residues was set as fixed modification. Search was performed using a false discovery rate (FDR) of 1%. The resultant spectral library was imported into PeakView v2.2 software, and SWATH runs for all samples were processed using 50 ppm mass error, 5 min RT window, 99% confidence, and 1% FDR. The peak areas thus generated in PeakView were imported into MarkerView v 1.2.1, wherein statistical analysis using the t-test was performed. Normalization across the runs was performed using total area sum. All of the raw mass spectrometric data have been deposited at the public repository PeptideAtlas (PASS01477).
unique peptides with a minimum 95% confidence and detected with less than 1% FDR were considered for relative quantitation analysis.

The result of the IDA analysis was used as a reference spectral library for SWATH processing using PeakView v2.2 software. The spectral library was imported and filtered by setting the mass tolerance to 50 ppm, peptide confidence to 99%, number of peptides to 5, and number of transitions to 6. Peptides shared between two or more protein families were excluded from quantitation. Further, the proteins whose peptides passed the 1% FDR criteria set in the SWATH 2.0 plugin of PeakView were exported to MarkerView v 1.2.1. Statistical analysis was performed in MarkerView using a t-test, and those proteins with a p-value ≤ 0.05 were considered for quantitation. The proteins having fold change ≥1.3 or ≤1.3 were considered as differentially expressed in our experiment.

**Bioinformatic Analysis.** The differentially secreted proteins were analyzed for protein–protein interactions and pathways using the STRING 11.0 database (Search Tool for the Retrieval of Interacting Genes), and the protein–protein interaction (PPI) network was imported into Cytoscape 3.2, an open-source network visualization software. The clustering of proteins was performed using the clusterMaker plugin in Cytoscape. The identification of secretory proteins was done using both classical and nonclassical secretion prediction tools. The web-based SignalP 5.0 server (http://www.cbs.dtu.dk/services/SignalP/) was used for predicting classically secreted proteins, and the data was processed using the “eukarya” option. For the prediction of nonclassically secreted proteins, SecretomeP 2.0 (http://www.cbs.dtu.dk/services/SecretomeP/) was used.

**Data Analysis for Quantification of Methylglyoxal-Modified Peptides.** Skyline (version 4.1.0, MacCoss Lab) was used for the quantification of peptides using SWATH.wiff files. The FASTA file of human serum albumin was used for theoretical mass spectral library generation. Unmodified peptides and corresponding carboxyethyl-modified peptides of albumin were specified for the targeted quantification of peptides. Retention times of precursors were manually corrected wherever required. Only intense and co-eluting fragment ions of a particular precursor were considered for quantification. The sum of the area under the curve (AUC) of these selected fragment ions was used for the quantification of the precursor. The digestion enzyme was specified as trypsin. The maximum missed cleavage was set to 1. Carbamidomethylation at cysteine (57.021464 Da), MG-H1 at arginine (54.01565 Da), and carboxyethyl modification at lysine and arginine (72.021126 Da) were specified as methylglyoxal-associated modifications. Precursor ion charge states were specified as +2 and +3, whereas fragment ion charge states were specified as +1 and +2. Fragment ion mass tolerance was set to 0.5 Da. The acquisition method was selected as DIA, and the product mass analyzer was set as TOF. The isolation scheme was selected as SWATH (15 m/z) at a resolving power of 15 000. The peak area of the modified peptide was normalized with the peak area of its corresponding unmodified peptide. The CEL-modified peptide content in diabetic plasma was expressed as a fold change ratio of the normalized peak area of the CEL-modified peptide of diabetic plasma to the
The prediluted human plasma samples (20 μL; 1:60) were added to each well, and the volume was made up with 130 μL of distilled water. The plate was covered and incubated for 3 h at room temperature with intermittent agitation on a microplate shaker. The plate was washed thrice with (400 μL per well) wash buffer. After the washes, the excess wash buffer was removed by tapping the plate on an absorbent pad. Then, 100 μL of TMB substrate solution was added to all wells, including the blank wells, and incubated at room temperature for about 10 min. The enzyme reaction was stopped by adding 100 μL of stop solution into each well, and the absorbance of each microwell was recorded at 450 nm. The standard curve of CD44 was plotted in the concentration range of 62.5–4000 pg/mL, and CD44 concentration in the plasma was calculated by elisaanalysis.com online software using the 4-parameter logistic regression algorithm. The box plot of CD44 concentration was plotted for healthy and diabetic subjects using the web app PlotsOfData.23

**Statistical Analysis.** Mass spectrometry acquisition for cell secretome experiments was performed for three individual cell culture experiments in technical triplicates. The clinical plasma proteomic analysis was performed for 10 plasma samples each of healthy control and diabetic subjects in technical triplicates for each sample, and ELISA was performed in duplicates for the same set of plasma samples. Statistical analysis was performed by Student’s t-test for quantitation of both proteomic data, peptide quantification in clinical plasma and for ELISA, and one way analysis of variance (ANOVA) was performed for the cell viability assay. Data were expressed as mean ± SEM. A p-value ≤ 0.05 was considered as statistically significant. For proteomic analysis, the proteins with more than two matching peptides and fold change difference of ≥1.3 in protein expression were considered.

Figure 3. Bioinformatic analysis: PPI network clusters showing differentially abundant proteins involved in (A) ECM–receptor interactions, (B) leukocyte transendothelial migration, (C) fluid shear stress and atherosclerosis, (D) complement and coagulation cascades, and (E) lysosomal pathway; green indicates >1.3-fold higher abundance and red indicates >1.3-fold lesser abundance in MG-treated cells.
180 proteins were identified with SecretomeP (Table S3). Proteins were identified with SecretomeP (Table S3). Proteins were identified with SecretomeP (Table S3). Proteins were identified with SecretomeP (Table S3). Proteins were identified with SecretomeP (Table S3).

**RESULTS AND DISCUSSION**

**Methylglyoxal Does Not Affect Cell Viability up to 3 mM Concentration.** Methylglyoxal, a highly reactive dicarbonyl compound, is reported to induce oxidative stress in cells. Therefore, we have evaluated its cytotoxicity on L6 rat skeletal muscle cells. MG concentration above 3 mM reduced the cell viability, as observed by trypan blue staining (Figure S1) and, therefore, 3 mM MG treatment was used for all secretome experiments. Methylglyoxal-induced secretome was tryptically digested, and SWATH-MS analysis was carried out for three individual experiments in technical triplicates for identification and expression analysis. The volcano plot indicates the proteins with differential abundance >1.3-fold and p-value < 0.05 in pink (Figure 2A). The IDA spectral library had 643 proteins (Table S2), and 82 of those were upregulated while 99 were downregulated by more than 1.3-fold in MG-induced secretome in SWATH analysis (Figure 2B). All of the differentially expressed proteins (Table S3) were significant at p-value < 0.05. Secretory proteins from the classical secretory pathway and nonclassical secretory pathway were determined using SignalP and SecretomeP, respectively. Of total identified proteins, 180 proteins were identified with SignalP and 113 proteins were identified with SecretomeP (Figure 2C). The KEGG pathway analysis was done using Cytoscape software with plugin clusterMaker. Differentially expressed proteins were found to be involved in various pathways like extracellular matrix (ECM)—receptor interaction (Figure 3A), leukocyte transendothelial migration (Figure 3B), fluid shear stress and atherosclerosis (Figure 3C), complement and coagulation cascades (Figure 3D), and lysosomal pathway (Figure 3E). ECM—receptor interactions are known to contribute to histopathological lesions in diabetic nephropathy. Previously, methylglyoxal was found to inhibit endothelial cell adhesion to type IV collagen of renal glomerular cells, podocytes, and mesangial cells, leading to the development of diabetic nephropathy. In this study, the extracellular matrix (ECM) proteins such as integrin β1, fibronectin, and CD44 antigen were upregulated in methylglyoxal-induced secretome and may induce insulin resistance. Aberrant regulation of ECM components is observed in obesity associated with insulin resistance. MG-induced upregulation of key proteins such as ezrin, integrin β1, Ras-related protein Rap-1b, transforming protein RhoA, Thy-1 membrane glycoprotein, and vascular cell adhesion protein 1 and downregulation of 72 kDa type IV collagenase, which are involved in leukocyte transendothelial migration, form an important step in eliciting inflammatory...
immune response. MT1-MMP-dependent shedding of CD44 plays a key role in regulation of leukocyte adhesion to the pancreatic blood vessels and the transendothelial migration of diabetogenic, cytotoxic T cells into the islet cells. This was also associated with enrichment of proteins involved in complement and coagulation cascades such as complement C3, α-2-macroglobulin, and urokinase-type plasminogen activator that were upregulated and proteins such as complement C1s, clusterin, vitamin K-dependent protein S, and plasminogen activator inhibitor 1 were downregulated in MG-induced secretome. A complement and coagulation system has been implicated in the pathogenesis of metabolic disorders, including insulin resistance, non-alcoholic fatty liver disease, and type 2 diabetes. In obesity and type 2 diabetes, adipocytes are actively involved in production of complement components such as C3 factor. Higher levels of serum C3 are correlated with insulin resistance, endothelial dysfunction, and progression of diabetic nephropathy. C3 is also associated with cardiovascular diseases, as it interacts with the coagulation system. Similarly, proteins involved in fluid shear stress and atherosclerosis such as endoplasm, heat shock protein HSP 90-β, heat shock protein HSP 90-α, transforming protein RhoA, vascular cell adhesion protein 1, NAD(P)H dehydrogenase [quinone] 1, and glutathione S-transferase Mu 2 were upregulated, and proteins such as platelet-derived growth factor subunit A, vascular endothelial growth factor A, 72 kDa type IV collagenase, and C–C motif chemokine 2 were downregulated in response to MG treatment. People with diabetes tend to develop accelerated atherosclerosis. Glycation of extracellular matrix proteins such as glycated collagen alters the endothelial cell function and could contribute to the development of atherosclerotic plaques. In this study, ECM proteins were differentially regulated in response to MG treatment. Proteins such as vascular cell adhesion protein 1 and transforming protein RhoA were upregulated. NAD(P)H dehydrogenase [quinone] 1 and its homologue NADPH oxidase act downstream to the AGE-RAGE axis and are involved in the production of ROS and inflammatory response. Another important pathway that was enriched in the MG-induced secretome was the lysosomal pathway. MG is a highly reactive dicarbonyl responsible for the formation of diverse AGEs, which are implicated in the pathogenesis of diabetes. Cells have evolved combat mechanisms to degrade AGEs via lysosome-mediated autophagy pathways. In a recent study, it has been demonstrated that modulation of lysosome biogenesis leading to autophagy was responsible for the degradation of AGEs in the diabetic kidney. In the case of autophagy-deficient mutant cells, lysosomal biogenesis was not observed, resulting in the accumulation of AGEs in the

Figure 4. Quantification of CEL-modified peptides. Representative extracted ion chromatograms showing co-elution of y fragment ions of CEL-modified peptides (A) FK(CEL)DLGEENFK and (B) K(CEL)VPQYSTPTLVEVSR of human serum albumin in diabetic plasma. (C) Fold change in expression of CEL-modified albumin peptides FK(CEL)DLGEENFK and K(CEL)VPQYSTPTLVEVSR in plasma sample of diabetic subjects compared to healthy subjects.
glomeruli and renal vasculature, which was associated with enhanced inflammation and fibrosis. However, MG-induced secretome showed downregulation of various proteins such as N(4)-(β-N-acetylglucosaminy1)-l-asparaginase, acid ceramidase, V-type proton ATPase subunit S1, cathepsin D, pro-cathepsin H, cathepsin L1, deoxyribonuclease-2-α, tissue α-1-fucosidase, N-acetylgalactosamine-6-sulfatase, β-glucuronidase, β-hexosaminidase subunit α, β-hexosaminidase subunit β, hyaluronidase-1, legumain, α-N-acetylgalactosaminidase, palmitoyl-protein thioesterase 1, prosaposin, and tripeptidyl-peptidase 1, which are involved in the lysosomal degradation pathway. The impaired lysosomal degradation system is associated with aging and diabetes. Accumulated AGEs exacerbate diabetes and diabetic complications.

Apart from the above pathways, MG-induced secretome had several proteins related to insulin resistance, obesity, and metabolic syndrome. Table 1 provides information on these important proteins. Proteins such as vascular cell adhesion protein 1, cell adhesion molecule 1, CD44 antigen, atrial natriuretic peptide receptor 3, α-2-macroglobulin, monocyte differentiation antigen CD14, annexin A2, complement C3 and C4 proteins, calreticulin, urotensin-2B, protein S100-A4, and galectin-3 were upregulated, and similarly, some of the downregulated proteins included were lanosterol synthase, acid ceramidase, cathepsin D, cathepsin L1, carboxypeptidase E, metalloproteinase inhibitor 2, meteorin-like protein, etc. In our previous study, we have shown that MG downregulates enzymes such as lanosterol synthase involved in cholesterol biosynthesis.

**Elevated Levels of Methylglyoxal-Modified Serum Albumin Peptides in Diabetic Plasma.** The hyperglycemic condition in diabetes promotes glycation and formation of reactive carbonyls such as glyoxal and methylglyoxal, which can, in turn, modify proteins leading to the formation of carboxymethyllysine (CEL) and argpyrimidines (ARGPYR). In severe diabetes, due to elevated levels of MG, it is expected that MG-associated modifications such as CEL and ARGPYR can be observed in plasma proteins. Human serum albumin has been considered as a primary target for glycation due to its abundance, many lysine and arginine residues, and relatively long half-life. We, therefore, studied MG-associated modification of HSA, which could reflect elevated levels of MG. A label-free SWATH-MS approach was used for the quantification of MG-modified peptides. The peptides and their fragments that are consistently observed in all of the samples were considered for analysis (Figure 4A,B). The y ions were considered for peptide quantification as they were more intense than b ions. The sum of the area under the curve (AUC) of selected y ions was used for peptide quantification. Two peptides, FKDLGEEFNFK and KVPQVSTPTLVEVSR, showed CEL modification of lysine residues, and their intensities were found to be high in diabetic plasma. The peak area of CEL-modified peptides in healthy and diabetic plasma samples was normalized with the peak area of their corresponding unmodified peptide. The increase in CEL modification in diabetes was expressed as fold change over healthy control (Figure 4C). The level of CEL-modified peptide FK(CEL)DLGEEFNFK in diabetic subjects was 1.31-fold higher than in healthy subjects. Similarly, the level of CEL-modified albumin peptide K(CEL)VPQVSTPTLVEVSR in diabetic subjects was 1.47-fold higher than in healthy subjects. Details of precursor and fragment ions used for quantification are summarized in Table 2. The retention time of quantified peptides has been shown in Figure S2. Normalized CEL-modified areas for both peptides are shown in Figure S3.

**Proteomic Analysis of Clinical Plasma.** The usefulness of MG-induced secretome was studied in clinical plasma. Since MG levels increase in diabetes, it is expected that some of the secreted proteins observed in cell culture may also be found in clinical plasma. Therefore, to identify such proteins, SWATH-MS was performed to identify and quantify MG-associated secreted proteins in the plasma. Tryptic digest of clinical plasma was subjected to expression analysis using SWATH-MS workflow. A total of 238 proteins were identified in the spectral

| sr. no. | modification site | peptide sequence | charge state | modification | monoisotopic m/z | fragment ion (m/z) used for quantification |
|---------|------------------|------------------|-------------|--------------|-----------------|---------------------------------------|
| 1       | K36              | FKDLGEEFNFK      | +3          | unmodified   | 409.5399        | y²⁺ (294.1812)                         |
|         |                  |                  |             |              |                 | y⁴⁺ (537.2667)                        |
|         |                  |                  |             |              |                 | y⁵⁺ (666.3093)                        |
|         |                  |                  |             |              |                 | y⁶⁺ (723.3308)                        |
|         |                  |                  |             |              |                 | y⁷⁺ (836.4149)                        |
|         |                  |                  |             |              |                 | y⁸⁺ (951.4418)                        |
|         |                  |                  |             |              |                 | y²⁺ (294.1812)                         |
|         |                  |                  |             |              |                 | y⁴⁺ (537.2667)                        |
|         |                  |                  |             |              |                 | y⁵⁺ (666.3093)                        |
|         |                  |                  |             |              |                 | y⁶⁺ (723.3308)                        |
|         |                  |                  |             |              |                 | y⁷⁺ (836.4149)                        |
|         |                  |                  |             |              |                 | y⁸⁺ (951.4418)                        |
| 2       | K36              | FKDLGEEFNFK      | +3          | CEL          | 433.347         |                                        |
| 3       | K438             | KVPQVSTPTLVEVSR  | +3          | unmodified   | 547.3174        |                                        |
| 4       | K438             | KVPQVSTPTLVEVSR  | +3          | CEL          | 571.3245        |                                        |
library obtained from IDA (Table S4), of which 37 proteins were upregulated and 13 proteins were downregulated by more than 1.3-fold (Figure 5A). All of the differentially expressed proteins (Table S5) were significant at p-value < 0.05. Of the total identified proteins, 189 proteins were identified with SignalP and 24 proteins with SecretomeP (Figure 5B). The KEGG pathway analysis was done using Cytoscape software with a plugin clusterMaker. The differentially expressed proteins were found to be involved in pathway complement and coagulation cascades (Figure 5C). Among differentially expressed proteins, upregulated proteins were complement C1r subcomponent, complement C1s subcomponent, vitronectin, complement C5, complement component C7, fibrinogen α chain, coagulation factor XIII A chain, vitamin K-dependent protein S, plasma kallikrein, and plasma serine protease inhibitor, while downregulated proteins included complement C3, antithrombin-III, plasma protease C1 inhibitor, and complement C4-A. Interestingly, two pathways, mainly the complement system and ECM–receptor interaction, were shared between MG-induced secretome and diabetic plasma.

**CD44 Antigen Protein Was Found to be Common among Secretome and Clinical Data.** To identify MG-induced secreted proteins in the clinical plasma, the differentially expressed proteins from secretome and clinical plasma were analyzed for the presence of common proteins. There was a scarce overlap between MG-induced secretome and plasma proteins since plasma is a heterogeneous matrix that represents proteins secreted from various tissues including muscle cells, liver, adipose, and nervous tissue. Among 189 proteins identified, only four proteins were observed to be common between MG-induced secretome and clinical plasma (Figure 5D); however, only one protein, particularly CD44 antigen protein, showed a common trend in both the groups (Table 3). CD44 antigen protein was found to be upregulated with a 6-fold change in MG-induced secretome and 1.88-fold in diabetic plasma.

**Table 3. Common Proteins between Cell Secretome and Clinical Plasma Proteome**

| sr. no. | protein name       | fold change (secretome) | fold change (clinical plasma proteomics) |
|--------|--------------------|-------------------------|-----------------------------------------|
| 1      | complement C1s subcomponent | 3.02 down | 2.48 up |
| 2      | vitamin K-dependent protein S | 2.99 down | 2.09 up |
| 3      | CD44 antigen       | 6 up                    | 1.88 up                                 |
| 4      | complement C3      | 2.13 up                 | 1.71 down                               |

**Validation of CD44 Antigen Protein by ELISA in Clinical Subjects.** To validate CD44, an MG-induced secreted protein upregulated in diabetes, ELISA was performed in duplicate in the plasma from healthy and diabetic subjects (n = 10 from each group). The CD44 median for healthy and diabetic subjects was 1321 and 1840 pg/mL, respectively (Figure 6A). The fold change was calculated, which showed a similar trend as that of proteomics data, and the protein was found to be 1.6-fold upregulated in diabetic plasma (Figure 6B).

CD44 is a multifunctional cell surface receptor molecule. It interacts with hyaluronan and osteopontin and regulates inflammatory and proinflammatory responses. It exists in several isoforms due to alternate splicing mechanisms. The protein is expressed in several cell types and is involved in various disease conditions, including insulin resistance, obesity,

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**Figure 5.** Proteomic analysis for clinical plasma: differential proteomics analysis done by SWATH-MS. (A) Number of differentially expressed proteins in diabetic healthy control. (B) Number of secretory proteins obtained through SignalP and SecretomeP. (C) PPI network cluster showing proteins involved in complement and coagulation cascades; green indicates >1.3-fold higher abundance and red indicates >1.3-fold lesser abundance in diabetic subjects. (D) Venn diagram showing the common proteins in secretome and clinical data.
metabolic syndrome, diabetes, and cancer.\textsuperscript{45–47} The levels of CD44 were found to be significantly increased in insulin-resistance conditions (IR) in a study involving 58 healthy, overweight, and moderately obese white adult participants. The elevated level of the CD44 gene was accompanied with increased expression of OPN, CD68, and IL6.\textsuperscript{48} In another study, CD44 protein levels were positively correlated with insulin resistance as measured by HbA1c (\textit{p}-value < 0.0002). Similarly, genome-wide association studies, as well as computational biology studies, involving analysis of microarray data suggested that CD44 plays an important role in the pathogenesis of adipose tissue inflammation and insulin resistance.\textsuperscript{49} Further studies are required to develop CD44 as a molecular marker for dysregulated methylglyoxal metabolism.

\section*{CONCLUSIONS}

This is the first study that describes the secretome of muscle cells in response to methylglyoxal treatment in muscle cells. About 180 proteins were identified to be secretory proteins either with SignalP or SecretomeP. The differentially expressed secretory proteins were involved in various pathways like ECM–receptor interaction, leukocyte transendothelial migration, fluid shear stress and atherosclerosis, complement and coagulation cascades, and lysosomal pathway. To identify the MG-induced secreted proteins in the clinical plasma, the proteins identified in the plasma were compared with the MG-induced secreted proteins in the muscle cells. CD44 was found to be a common protein between MG-induced secretome and clinical plasma proteome. It was found to be upregulated in the diabetic plasma as measured by ELISA and to have a role in the development of insulin resistance. The elevated levels of CD44 were accompanied by an increase in MG-induced CEL modifications of two HSA peptides, FKDLGEENFK and KVPQVSTPTLVEVSR, suggesting that the high levels of CD44 could be associated with an increase in the levels of MG in diabetes.

\section*{ASSOCIATED CONTENT}

\section*{Supporting Information}

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01318.

Effect of methylglyoxal (MG) on cell viability (Figure S1), retention time of quantified peptides (Figure S2), normalized CEL-modified peptide area (Figure S3) (PDF)

Clinical characteristics of diabetic and healthy subjects (Table S1), list of identified proteins in cell secretome (Table S2); secretory proteins showing differential abundance in SWATH-MS analysis (cell secretome) (Table S3); list of identified proteins in clinical plasma (Table S4); secretory proteins showing differential abundance in SWATH-MS analysis (clinical plasma) (Table S5) (XLSX)

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\section*{Notes}

The authors declare no competing financial interest.

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\section*{REFERENCES}

(1) Thornalley, P. J. The glyoxalase system in health and disease. \textit{Mol. Aspects Med.} 1993, \textbf{14}, 287–371.

(2) McLellan, A. C.; Thornalley, P. J.; Benn, J.; Sonksen, P. H. Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. \textit{Clin. Sci.} 1994, \textbf{87}, 21–29.

(3) Maessen, D. E. M.; Stehouwer, C. D. A.; Schalkwijk, C. G. The role of methylglyoxal and the glyoxalase system in diabetes and other age-related diseases. \textit{Clin. Sci.} 2015, \textbf{128}, 839–861.
(4) Rabbani, N.; Thornalley, P. J. Dicarbonyl proteome and genome damage in metabolic and vascular disease. Biochem. Soc. Trans. 2014, 42, 425–432.

(5) Jang, J. H.; Kim, E. A.; Park, H. J.; Sung, E. G.; Song, I. H.; Kim, J. Y.; Woo, C. H.; Doh, K. O.; Kim, K. H.; Lee, T. J. Methylglyoxal-induced apoptosis is dependent on the suppression of c-FLIPL expression via down-regulation of p65 in endothelial cells. J. Cell. Mol. Med. 2017, 21, 2720–2731.

(6) Miyazawa, N.; Abe, M.; Souma, T.; Tanemoto, M.; Abe, T.; Nakayama, M.; Ito, S. Methylglyoxal augments intracellular oxidative stress in human aortic endothelial cells. Free Radical Res. 2010, 44, 101–107.

(7) Schalkwijk, C. G. Vascular AGE-ing by methylglyoxal: the past, the present and the future. Diabetologia 2015, 58, 1715–1719.

(8) Yamawaki, H.; Saito, K.; Okada, M.; Hara, Y. Methylglyoxal mediates vascular inflammation via JNK and p38 in human endothelial cells. Am. J. Physiol. Cell Physiol. 2008, 295, C1510–C1517.

(9) Thornalley, P. J. The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life. Biochem. J. 1990, 269, 1.

(10) Dhar, A.; Dhar, I.; Desai, K. M.; Wu, L. Methylglyoxal scavengers attenuate endothelial dysfunction induced by methylglyoxal and high concentrations of glucose. Br. J. Pharmacol. 2010, 161, 1843–1856.

(11) Chang, T.; Wang, R.; Wu, L. Methylglyoxal-induced nitric oxide and peroxynitrite production in vascular smooth muscle cells. Free Radical Biol. Med. 2005, 38, 286–293.

(12) Thornalley, P. J. Cell activation by glycated proteins. AGE receptors, receptor recognition factors and functional classification of AGES. Cell. Mol. Biol. 1998, 44, 1013–1023.

(13) Pavlou, M. P.; Diamandis, E. P. The cancer cell secretome: a good source for discovering biomarkers? J. Proteomics 2010, 73, 1896–1906.

(14) Lodish, H.; Berk, A.; Zipursky, S. L.; Matsuda, P.; Baltimore, D.; Darnell, J. Molecular Cell Biology, 4th ed.; National Center for Biotechnology Information: Bookshelf, 2000.

(15) Skalnikova, H.; Motlik, J.; Gadher, S. J.; Kovarova, H. Mapping of the secretome of primary isolates of mammalian cells, stem cells and derived cell lines. Proteomics 2011, 11, 691–708.

(16) Eckardt, K.; Gögens, S. W.; Raschke, S.; Eckel, J. Myokinase in insulin resistance and type 2 diabetes. Diabetologia 2014, 57, 1087–1099.

(17) Norheim, F.; Raastad, T.; Thiede, B.; Rastan, A. C.; Drevon, C. A.; Haugen, F. Proteomic identification of secreted proteins from human skeletal muscle cells and expression in response to strength training. Am. J. Physiol. Endocrinol. Metab. 2011, 301, E1013–E1021.

(18) Nikolic, N.; Bakke, S. S.; Kase, E. T.; Rudberg, I.; Halle, I. F.; Rastan, A. C.; Thoresen, G. H.; Aas, V. Electrical pulse stimulation of cultured human skeletal muscle cells as an in vitro model of exercise. PLoS One 2012, 7, No. e33023.

(19) Giacco, F.; Brownlee, M. Oxidative stress and diabetic complications. Circ. Res. 2010, 107, 1058–1070.

(20) Deshmukh, A. S.; Cox, J.; Jensen, L. J.; Meissner, F.; Mann, M. Secretome analysis of lipid-induced insulin resistance in skeletal muscle cells by a combined experimental and bioinformatics workflow. J. Proteome Res. 2015, 14, 4885–4895.

(21) Yoon, J. H.; Song, P.; Jang, J.-H.; Kim, D.-K.; Choi, S.; Kim, J.; Ghim, J.; Kim, D.; Park, S.; Lee, H. Proteomic analysis of tumor necrosis factor-alpha (TNF-α)-induced L6 myotube secretome reveals novel TNF-α-dependent myokinases in diabetic skeletal muscle. J. Proteome Res. 2011, 10, 5315–5325.

(22) Ahmad, K.; Shaikh, S.; Lee, E. J.; Lee, Y. H.; Choi, I. Consequences of dicarbonyl stress on skeletal muscle proteins and in Type 2 diabetes. Curr. Protein Pept. Sci. 2019, DOI: 10.2174/13892072066619119100759.

(23) Postma, M.; Goedhart, J. PlotsOfData—A web app for visualizing data together with their summaries. PLoS Biol. 2019, 17, No. e3000202.
(42) Jagadeeshwarasad, M. G.; Venkatasubramani, V.; Unnikrishnan, A. G.; Kulkarni, M. J. Albumin Abundance and Its Glycation Status Determine Hemoglobin Glycation. ACS Omega 2018, 3, 12999–13008.

(43) Rathore, R.; Somwane, B. P.; Jagadeeshwarasad, M. G.; Kahar, S.; Santhakumari, B.; Unnikrishnan, A. G.; Kulkarni, M. J. Glycation of glucose sensitive lysine residues K36, K438 and K549 of albumin is associated with prediabetes. J. Proteomics 2019, 208, No. 103481.

(44) Nagano, O.; Saya, H. Mechanism and biological significance of CD44 cleavage. Cancer Sci. 2004, 95, 930–935.

(45) Hasibi, A.; Henayake, C. K.; Bracy, D. P.; Bugler-Lamb, A. e. R.; Lantier, L.; Khan, F.; Ashford, M. L. J.; McCrimmon, R. J.; Wasserman, D. H.; Kang, L. CD44 contributes to hyaluronan-mediated insulin resistance in skeletal muscle of high-fat-fed C57BL/6 mice. Am. J. Physiol. Endocrinol. Metab. 2019, 317, E973–E983.

(46) Nomiyama, T.; Perez-Tilve, D.; Ogawa, D.; Gizard, F.; Zhao, Y.; Heywood, E. B.; Jones, K. L.; Kawamori, R.; Lantier, L.; Khan, F.; Ashford, M. L. J.; McCrimmon, R. J.; Wasserman, D. H.; Kang, L. CD44 contributes to systemic insulin resistance and proinflammatory macrophages in human adipose tissue. Diabetesologia 2015, 58, 1579–1586.

(47) Mattheolabakis, G.; Milane, L.; Singh, A.; Amiji, M. M. Hyaluronic acid targeting of CD44 for cancer therapy: from receptor biology to nanomedicine. J. Drug Targeting 2015, 23, 605–618.

(48) Liu, L. F.; Kodama, K.; Wei, K.; Tolentino, L. L.; Choi, O.; Engleman, E. G.; Butte, A. J.; McLaughlin, T. The receptor CD44 is associated with systemic insulin resistance and proinflammatory macrophages in human adipose tissue. Diabetes Technol. 2011, 5, 982–988.

(50) Indulekha, K.; Surendar, J.; Mohan, V. High sensitivity C-reactive protein, tumor necrosis factor-α, interleukin-6, and vascular cell adhesion molecule-1 levels in Asian Indians with metabolic syndrome and insulin resistance (CURES-105). J. Diabetes Sci. Technol. 2011, 5, 982–988.

(51) Wang, T.-T.; Wang, X.-M.; Zhang, X.-L. Circulating Vascular Cell Adhesion Molecule-1 (VCAM-1) and Intercellular Adhesion Molecule-1 (ICAM-1): Relationship with carotid artery elasticity in patients with impaired glucose regulation (IGR). Ann. Endocrinol. 2019, 72, 76–82.

(52) Pivovarova, O.; Gögebakan, Ö.; Klötting, N.; Sparwasser, A.; Weikert, M. O.; Haddad, I.; Nikiforova, V. J.; Bergmann, A.; Kruse, M.; Seltmann, A.-C. Insulin up-regulates natriuretic peptide clearance receptor expression in the subcutaneous fat depot in obese subjects: a missing link between CVD risk and obesity? J. Clin. Endocrinol. 2012, 97, E731–E739.

(53) Zhang, R.; Barker, L.; Pinchev, D.; Marshall, J.; Rasamoeilisolo, M. I.; Smith, C.; Kupchak, P.; Kireeva, I.; Ingratta, L.; Jackowski, G. Mining biomarkers in human sera using proteomic tools. Proteomics 2004, 4, 244–256.

(54) Lim, P. S.; Chang, Y.-K.; Wu, T.-K. Serum Lipopolysaccharide-Binding Protein is Associated with Chronic Inflammation and Metabolic Syndrome in Hemodialysis Patients. Blood Purif. 2019, 47, 28–36.

(55) Cura-Esquível, I.; Cordero-Pérez, P.; Torres-González, L.; Muñoz-Espinosa, L. E. Acute phase markers in obese children and adolescents with metabolic disorders. Arch. Argent. Pediatr. 2018, 116, 275–282.

(56) Liu, B.; Xu, Y.; Voss, C.; Qiu, F.-h.; Zhao, M.-z.; Liu, Y.-d.; Nie, J.; Wang, W.-l. Altered protein expression in gestational diabetes mellitus placentas provides insight into insulin resistance and coagulation/fibrinolysis pathways. PLoS One 2012, 7, No. e44701.

(57) Wang, Y.; Koh, W.-p.; Jensen, M. K.; Yuan, J.-M.; Pan, A. Plasma Fetuin-A Levels and Risk of Type 2 Diabetes Mellitus in A Chinese Population: A Nested Case-Control Study. Diabetes Metab. J. 2019, 43, No. 474.
PPARδ-dependent pathways in skeletal muscle of mice. Exp. Mol. Med. 2018, 50, 122.

(75) Nagareddy, P. R.; Rajput, P. S.; Vasudevan, H.; McClure, B.; Kumar, U.; Macleod, K. M.; McNeill, J. H. Inhibition of matrix metalloproteinase-2 improves endothelial function and prevents hypertension in insulin-resistant rats. Br. J. Pharmacol. 2012, 165, 705–715.

(76) Kang, L.; Ayala, J. E.; Lee-Young, R. S.; Zhang, Z.; James, F. D.; Neufeld, P. D.; Pozzi, A.; Zutter, M. M.; Wasserman, D. H. Diet-induced muscle insulin resistance is associated with extracellular matrix remodeling and interaction with integrin α2β1 in mice. Diabetes 2011, 60, 416–426.

(77) Liew, C. W.; Assmann, A.; Templin, A. T.; Raum, J. C.; Lipson, K. L.; Rajan, S.; Qiang, G.; Hu, J.; Kawamori, D.; Lindberg, I. Insulin regulates carboxypeptidase E by modulating translation initiation scaffolding protein eIF4G1 in pancreatic β cells. Proc. Natl. Acad. Sci. U.S.A. 2014, 111, E2319–E2328.

(78) Huang, X.; Vaag, A.; Carlsson, E.; Hansson, M.; Ahren, B.; Groop, L. Impaired cathepsin L gene expression in skeletal muscle is associated with type 2 diabetes. Diabetes 2003, 52, 2411–2418.

(79) Liu, L.; Chen, B.; Zhang, X.; Tan, L.; Wang, D. W. Increased cathepsin D correlates with clinical parameters in newly diagnosed type 2 diabetes. Dis. Markers 2017, 2017, No. S286408.

(80) Chavez, J. A.; Holland, W. L.; Bär, J.; Sandhoff, K.; Summers, S. A. Acid ceramidase overexpression prevents the inhibitory effects of saturated fatty acids on insulin signaling. J. Biol. Chem. 2005, 280, 20148–20153.