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Proteomic study of endothelial dysfunction induced by AGEs and its possible role in diabetic cardiovascular complications

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ABSTRACT

Endothelial dysfunction is one of the primary steps in the development of diabetes associated cardiovascular diseases. Hyperglycemic condition in diabetes promotes accumulation of advanced glycation end products (AGEs) in the plasma, that interact with the receptor for AGEs (RAGE) present on the endothelial cells and negatively affect their function. Using Human umbilical vascular endothelial cells (HUVECs) in culture, the effect of glycated human serum albumin on global proteomic changes was studied by SWATH-MS, a label free quantitative proteomic approach. Out of the 1860 proteins identified, 161 showed higher abundance while 123 showed lesser abundance in cells treated with glycated HSA. Bioinformatic analysis revealed that the differentially regulated proteins were involved in various processes such as apoptosis, oxidative stress *etc.* that are associated with endothelial dysfunction. Furthermore, the iRegulon analysis and immunofuorescence studies indicated that several of the differentially regulated proteins were transcriptionally regulated by NF-κB, that is downstream to AGE-RAGE axis. Some of the important differentially regulated proteins include ICAM1, vWF, PAI-1 that affect important endothelial functions like cell adhesion and blood coagulation. qPCR analysis showed an increase in expression of the AGE receptor RAGE along with other genes involved in endothelial function. AGE treatment to HUVEC cells led to increased oxidative stress and apoptosis. This is the first proteomics study that provides insight into proteomic changes downstream to AGE-RAGE axis leading to endothelial dysfunction and predisposing to cardiovascular complications.

Significance: Cardiovascular disease (CVD) is a major pathological outcome in diabetic patients and it is important to address ways that target its development before the onset. Elevated plasma AGEs in diabetes can affect endothelial function and can continue to show their effects even after blood glucose levels are back to normal. Since endothelial dysfunction acts as one of the initiating factors for the development of CVD, understanding how AGEs affect the endothelial cell proteome to cause dysfunction will provide insight into the mechanisms involved and aid designing new therapeutic approaches.

1. Introduction

Diabetes is a metabolic disorder that has currently become a global pandemic affecting about 8.5% of the world's population [1]. It is mainly characterized by increased blood glucose levels, either due to insufficient insulin (Type I) or ineffective insulin action (Type II). The morbidity and mortality associated with diabetes is mainly because of the complications that arise as its result and which include cardiovascular diseases, nephropathy and retinopathy among others [2].Cardiovascular diseases, comprising of atherosclerosis leading to myocardial infarction or stroke, and diabetic cardiac myopathy, are one of the most predominant complications of diabetes [3]. Diabetic patients are at a greater risk of mortality associated heart disease as compared to non-diabetics, with > 65% of Type 2 diabetes patients dying of cardiovascular disease [4]. Current treatments in diabetes only slow down the progression of these complications, and therefore, any new therapy that can inhibit their development can be a useful approach to improve quality of life of patients.

Endothelial dysfunction involves a transition from its quiescent phenotype to a pro-inflammatory one and acts as initial step for the development of cardiovascular diseases, including diabetic cardiovascular complications [5–8]. For example, increased adhesion molecule expression in endothelial dysfunction and consequent leukocyte migration is one of the steps that lead to the formation of atherosclerotic plaques [9]. Even other complications of diabetes like nephropathy and retinopathy involve abnormal functioning of vascular endothelial cells.

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For example, hyperproliferation of retinal capillary endothelial cells is the hallmark of proliferative diabetic retinopathy [10]. A number of mechanisms have been shown to affect endothelial function in diabetes such as protein kinase C activation, increased flux through polyol pathway and so on, one of them being increased protein glycation [11]. Glycation is a non-enzymatic reaction of sugar molecules with free amino groups of proteins to form Amadori products, which further undergo rearrangement to form Advanced Glycation End products (AGEs) [12]. Increase in plasma protein glycation occurs in diabetes due to the elevated levels of glucose and glucose derived intermediates such as 3-deoxyglucosone, glyoxal and methylglyoxal in blood [13, 14]. It is also reported that diabetic patients with CVD have even higher levels of plasma AGEs as compared to other diabetic patients [15, 16]. This indicates that plasma AGEs could play a role in the development of cardiovascular complications in such patients.

AGEs interact with the Receptor for Advanced Glycation End products (RAGE) and activate numerous signaling cascades which induce oxidative stress and inflammatory response [17, 18]. In vascular endothelial cells, RAGE is expressed at basal levels but is highly induced in presence of AGEs [19, 20]. AGE-RAGE interaction in diabetes can negatively affect endothelial cell physiology predisposing to cardiovascular disease [21, 22] and this has been shown in clinical studies wherein diabetics show higher RAGE expression [23] and AGE localization near atherosclerotic plaques [24]. Animal models of diabetic atherosclerosis have also shown improved regression of atherosclerotic plaques after RAGE knockout [25]. Therefore, understanding the AGE RAGE axis in development of endothelial dysfunction and its inhibition can be helpful in designing new therapies that target endothelial dysfunction and impair development of cardiovascular diseases. In our study, we have used glycated human serum albumin to treat Human Umbilical Vein Endothelial Cells (HUVEC) in culture, followed by label-free quantitative proteomics using SWATH to understand how AGEs affect the endothelial cell proteome and predisposes to the development of cardiovascular complications.

2. Materials and methods

2.1. Chemicals used

All chemicals were procured from Merck (formerly Sigma-Aldrich) unless otherwise stated. Antibodies used were anti-CML from Abcam, anti-p65 Nf-κB from Santa Cruz Biotechnology, anti-rabbit IgG tagged with HRP from Merck (formerly Bangalore Genei) and anti-rabbit IgG tagged with Alexafluor 594 from Thermo Fisher Scientific. All reagents used for RNA extraction and cDNA synthesis and the TaqMan assays used for qPCR (vWF: Hs01012930-m1, endothelin-1: Hs00174961-m1, ICAM-1: Hs00164932-m1, NOS3: Hs01574659_m1, VEGFR-2: Hs00911708_m1 and β-Actin: Hs99999903_m1) were from Thermo Fisher Scientific, USA.

2.2. Synthesis and characterization of glycated human serum albumin

Human serum albumin (50 mg/ml) was incubated with 0.5 M glucose in 200 mM phosphate buffer containing 0.05% sodium azide at 37 °C for 90 days. After completion of the reaction, it was dialyzed against phosphate buffer overnight to remove unreacted glucose and further concentrated using centrifugal filters of 30 kDa molecular weight cut-off. Modification of albumin over time was monitored on a 5800 MALDI TOF/TOF™ mass spectrometer (SCIEX, USA) in linear mode using sinapic acid as matrix. Since many AGEs are known to exhibit characteristic fluorescence at excitation wavelength 370 nm and emission 440 nm, fluorescence was measured to confirm formation of AGEs [26]. Also, since CML is one of the most prevalent AGEs, western blot using anti carboxymethyl-lysine (CML) antibody was performed for validation.

2.3. Western blotting

5 µg unmodified and modified HSA were separated on an SDS-PAGE gel followed by semi-dry transfer of the proteins on to a PVDF membrane. The membrane was then stained with Ponceau S to check equal transfer of protein from gel. Blocking was done using 5% skimmed milk for 1 h at 37 °C, followed by incubation with 1:500 *v*/v dilution anti-CML antibody (Abcam, UK) in blocking buffer at room temperature for 1 h. Post incubation washes were given with phosphate buffer saline (PBS) containing 0.1% Tween-20, followed by incubation with 1:5000 *v*/v dilution of anti-rabbit secondary antibody in PBS for 1 h at room temperature. Chemiluminescent detection was performed using Clarity™ Western Chemiluminescent Substrate (Bio-Rad, USA) on a Syngene Dyversity Gel Documentation system.

2.4. Cell culture

Human Umbilical Vein Endothelial Cells (HUVEC)were obtained from Thermo Fisher Scientific (Cat.No. C0035C) and grown as per supplier's instructions in Medium 200 supplemented with 2% Large Vessel Endothelial Supplement (LVES) at 37 °C in a humidified chamber with 5% CO_2. All plasticware used for growing cells were coated with 2% gelatin overnight prior to seeding of cells. Cells between passages 3–8 were used for all experiments.

2.5. Apoptosis assay

Annexin V and PI staining was performed to detect apoptotic cells. An early indicator of apoptosis is translocation of phosphatidylserine (PS) to the external cell membrane and since annexins have high affinity for PS, Annexin V conjugated to fluorescent tags can be used for detection of apoptotic cells. Propidium iodide (PI) is a fluorescent DNA binding dye that does not enter live cells and hence, is used to detect dead cells. Thus, simultaneous staining with Annexin V-FITC and PI can be used to differentiate early and late apoptotic cells from necrotic dead cells. HUVECs were seeded on 6-well plates and allowed to grow up to 80% confluence followed by 3 h serum starvation. Cells were then treated with 3 mg/ml unmodified HSA (Con-HSA) or glycated HSA (Gly-HSA) and harvested using trypsin after 24 h. Percentage of apoptotic cells was determined using Annexin V-FITC Apoptosis Detection Kit (Sigma) as per manufacturer's instructions. Briefly, the harvested cells were resuspended in Annexin Binding Buffer and incubated with Annexin V-FITC and PI followed by flow cytometry acquisition on BD Accuri C6 Cytometer using 488 nm excitation laser. 10,000 cells were analyzed for each treatment and Annexin V-FITC and PI staining were monitored on green channel (530/30 nm) and red channel (585/40 nm) respectively. Appropriate quadrants were set to detect viable cells (negative for both dyes), early apoptotic cells (positive for Annexin V), late apoptotic cells (positive for both dyes) and necrotic cells (positive for PI). Carboplatin and Methylglyoxal were used as compensation controls for Annexin V and PI staining respectively.

2.6. Reactive oxygen species detection

ROS production in cells was monitored by DCFH-DA staining. DCFH-DA is a cell permeant dye that gets deacetylated by cellular esterases and oxidized by ROS to form fluorescent DCF that can be detected by flow cytometry. Serum starved HUVECs were treated with3mg/ml Con-HSA and Gly-HSA for 24 h. After treatment, cells were washed with PBS and incubated with 5 µM 2',7'-dichlorofluorescein diacetate (DCFH-DA) in phenol red free medium for 15 min at 37 °C. Cells were washed with PBS to remove unreacted DCFH-DA and trypsinized. Harvested cells were then resuspended in PBS and reactive oxygen species (ROS) were quantified by flow cytometry using BD AccurieC6 flow cytometer (BD Biosciences, USA) using 488 nm excitation laser and detection on green channel (530/30 nm). Fluorescence