MODULATION OF ENDOTHELIAL CELL SIGNALLING BY CHEMICALLY MODIFIED PROTEINS

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CERTIFICATE

This is to certify that the work incorporated in this Ph.D. thesis entitled '**MODULATION OF ENDOTHELIAL CELL SIGNALLING BY CHEMICALLY MODIFIED PROTEINS**' submitted by **Ms. Reema Mohan Banarjee** to the Academy of Scientific and Innovative Research (AcSIR) in fulfilment of the requirement for the award of the degree of **Doctor of Philosophy**, embodies original research work under my guidance. I further certify that this work has not been submitted to any other University or institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources have been duly cited and acknowledged.

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Date: June 29, 2018

Place: Pune

"It has been said that one is as old as one's arteries. In view of the supreme importance of endothelium in arterial function, I should like to modify. . . this statement by saying that

one is as old as one's endothelium."

- Rudolf Altschul, 1954

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Declaration by Research Scholar

I, Reema Mohan Banarjee, hereby declare that the work incorporated in the thesis entitled '**MODULATION OF ENDOTHELIAL CELL SIGNALLING BY CHEMICALLY MODIFIED PROTEINS**' submitted by me to the **Academy of Scientific and Innovative Research (AcSIR)**, for the degree of **Doctor of Philosophy in Biological Sciences** has been carried out by myself. All the work, except that in Chapter 5, have been carried out at CSIR-National Chemical Laboratory, Pune under the guidance of Dr. Mahesh J. Kulkarni. The work reported in Chapter 5 was conducted at The Faculty of Biological Sciences, University of Leeds, United Kingdom as a visiting scholar as a part of the Newton Bhabha PhD placement program under the supervision of Dr. Sreenivasan Ponnambalam. The reported work is original and has not been submitted to any other university or institution for the award of any degree or diploma. Such material, as has been obtained from other sources, has been duly acknowledged.

Date: 29-06-2018

Place: Pune

Reema Banarjee

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Abbreviations

AGE	Advanced Glycation End-products
BCA	Bicinchoninic Acid
BHT	Butylated Hydroxytoluene
BSA	Bovine Serum Albumin
Cbs	cystathionine β synthase
CML	Carboxymethyl lysine
CEL	Carboxyethyl lysine
CVD	Cardiovascular Diseases
DAVID	Database for Annotation, Visualization and Integrated Discovery
DCFH-DA	2',7'- dichlorodihydrofluorescein diacetate
DCF	Dichlorofluorescein
DIA	Data Independant Acquisition
ED	Endothelial Dysfunction
eNOS	Endothelial Nitric oxide Synthase
FITC	Fluorescein Isothiocyanate
Hcy	Homocysteine
Hct	Homocysteine thiolactone
HEK cells	Human Embryonic Kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHcy	Hyperhomocystenemia

HSA	Human Serum Albumin
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Intercellular Adhesion Molecule
IDA	Information Dependant Acquisition
LDL	Low Density Lipoprotein
LOX-1	Lectin-like Oxidised LDL Receptor 1
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS^E	MS Exponential
Mthfr	Methionine tetrahydrofolate reductase
Nf-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NRF1	Nuclear Respiratory Factor 1
oxLDL	Oxidized LDL
OPLS-DA	Orthogonal Projections to Latent Structures Discriminant Analysis
PCA	Principal Component Analysis
PBS	Phosphate Buffer Saline
PPI	Protein-protein Interaction
PVDF	Polyvinylidene fluoride
RAGE	Receptor for Advanced Glycation End-products
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SWATH	Sequential Window Acquisition of Theoretical Masses
TNF α	Tumour Necrosis Factor alpha
vWF	von Willebrand's Factor
XIC	Extracted Ion Chromatogram

Preface

Chapter 1: Introduction

Plasma proteins *in vivo* can react with number of endogenous metabolites as well as external entities like drugs which can lead to their chemical modification and affect their structure and function. Many such modified proteins are recognized by pattern recognition receptors like RAGE and LOX1 that function to remove the altered proteins from the system. However, under situations where there is excess of modifying agents, there can be over-accumulation of modified proteins. For example, hyperglycemia or elevated blood glucose levels in diabetes has been associated with elevated levels of glycated plasma proteins formed as a result of their reaction with glucose. Endothelial cells that line the vasculature are one of the first cells to interact with such modified plasma proteins. Accumulation of modified proteins in plasma can have deleterious effects on these cells and lead to endothelial dysfunction or ED. ED is characterized by a pro-inflammatory condition wherein there is vasoconstriction, thrombogenesis, leukocyte adhesion and vascular permeability which can act as an initial step in the development of vascular diseases. A number of risk factors of cardiovascular diseases are also associated with increased accumulation of modified proteins. For instance, higher level of glycated proteins in diabetic plasma can be an important contributing factor in the pathogenesis of diabetic cardiovascular complications. Understanding the mechanism of development of endothelial dysfunction in response to modified proteins can be useful in designing strategies for management of cardiovascular diseases. Therefore, in the present study, plasma proteins with different modifications were prepared and used to study the response of endothelial cells to treatment with such modified proteins.

Chapter 2: Synthesis and Characterization of Different Chemically Modified Proteins

Different metabolites and drugs have been reported to be capable of modifying proteins in plasma. Reducing sugars like glucose, and reactive metabolites formed during oxidative stress and lipid peroxidation, such as 4-hydroxynonenal and peroxynitrite can react with protein side chains and lead to the formation of adducts. Excess accumulation of modified proteins in plasma can have deleterious effects on the endothelial cells lining the vasculature culminating in vascular disorders. Therefore it is important to study the effects of different modifications on the protein structure as well as the effect of modified proteins on

endothelial cells. For this reason, serum albumin, the most abundant plasma protein, was modified at number of residues by different modifying agents followed by characterization of the modified proteins by MALDI mass spectrometry, fluorescence spectroscopy, CD spectroscopy and western blot. The extent of modification and modification sites on albumin were identified by high resolution accurate mass spectrometry. The modifications also induced a change in the helical structure of albumin which was seen by CD spectroscopy. Treatment with modified albumin reduced the viability of endothelial cells. Thus modified plasma proteins can negatively affect endothelial cells and can predispose to endothelial dysfunction.

Chapter 3: Study of Endothelial Dysfunction in Response to Glycated Albumin via RAGE and its Involvement in Diabetic Vascular Complications

Diabetics have higher risk of developing cardiovascular diseases even with controlled blood glucose levels as compared to non-diabetics. Since ED acts as an initial step in the development of cardiovascular diseases, negative effects of glycated proteins on expression of proteins involved in endothelial functions such as vasodilation and blood coagulation can play a role in the development of diabetic cardiovascular complications. The interaction of glycated proteins with their receptor RAGE has already been implicated in the development of a number of conditions such as neurodegeneration and insulin resistance. Therefore, the effect of glycated albumin on endothelial cell function and global protein expression was studied. Glycated albumin induced oxidative stress and apoptosis in HUVECs. Further, proteomic analysis by SWATH MS identified 1860 proteins, of which 161 proteins showed higher abundance while 123 proteins showed lower abundance after treatment with glycated albumin. Among the proteins showing altered abundance were number of proteins involved in endothelial functions such as cell adhesion, platelet aggregation and angiogenesis, including ICAM-1 and vWF, which were also validated by quantitative PCR. Increased expression of the receptor RAGE was also observed by qPCR, along with nuclear translocation of Nf-κB which was seen by immunofluorescence. Thus, in this chapter, we could demonstrate the role of glycated proteins in inducing endothelial dysfunction and subsequent cardiovascular complications in diabetes.

Chapter 4: Study of Endothelial Response to Homocysteinylated Albumin

Folate deficiency is associated with hyperhomocystenemia, i.e. elevated levels of homocysteine, and an increased risk of cardiovascular diseases. Homocysteine thiolactone, a cyclic analogue of homocyteine formed as a result of its interaction with methionyl tRNA synthase, is reported to modify lysine side chains of proteins. Since homocysteine is present in blood in its protein bound form and not in free form, homocysteine modified proteins can play a role in the pathogenesis of cardiovascular diseases in patients with hyperhomocystenemia. Therefore, the effect of homocysteinylated proteins on endothelial cell function and its total cell proteome was studied. Homocysteinylated albumin induced oxidative stress and apoptosis in HUVECs. SWATH MS identified 1410 proteins out of which 53 proteins showed altered abundance including those involved in protein degradation pathways such as ubiquitin ligases and peptidases along with those involved in endothelial function. The differentially abundant proteins also included upstream regulators of Nf-κB which controls the expression of numerous mediators of endothelial dysfunction. Thus, prolonged presence of homocysteinylated proteins can lead to endothelial dysfunction.

Chapter 5: Study of Modulation of Insulin Signal Transduction by Oxidized LDL via LOX-1

Interaction of oxidized LDL with its receptor LOX-1 plays an important role in the development of atherosclerosis. In endothelial cells, insulin signaling regulates a number of functions including expression and activation of eNOS, which is an important vasodilator and anti-inflammatory molecule and alteration of insulin signaling by LOX-1 can negatively affect eNOS functions. Therefore, the cross-talk between LOX1 and insulin signaling was studied using HEK293 cells over-expressing human LOX1 receptor. LOX-1 expressed in HEK293 cells could bind and internalize oxidized LDL as seen by immunofluorescence studies. Activation of signaling pathways in response to oxLDL binding to LOX1 was studied using western blot and a reduction in the phosphorylation of the serine/threonine kinase Akt could be observed. Additionally, insulin dependent pAkt activation was inhibited in the presence of oxidized LDL. Thus, interaction of oxLDL with LOX1 can negatively affect insulin mediated endothelial functions and lead to insulin resistance.

Dedicated to my Parents

&

to everyone who inspired me to do a little better each time...

Chapter 1: Introduction

1.1. Protein Chemical Modifications

Biomolecules interact with a number of metabolites, drugs and other chemicals that are capable of reacting and modifying them. Chemical modifications occur routinely under physiological conditions and many are integral part of biological processes. For example, methylation of DNA is a well-known epigenetic modification which serves to regulate gene expression in cells[1]. A number of such reactions have been reported for proteins, lipids as well as nucleic acids; oxidation[2], nitration[3], glycation[4], homocysteinylation[5], citrullination[6], carbonylation[7], acetylation[8] are to name a few. These reactions may involve formation of an adduct or loss of proton or water molecule[9].

Proteins, *in vivo* undergo various chemical modifications both as a part of various cellular processes as well as due to changes in the external environment. Protein phosphorylation[10] and acetylation[11] which help signal transduction in the cell are major examples of physiologically important chemical modifications. Likewise, ubiquitination, which is the conjugation of a 76-residue protein named ubiquitin to proteins, tags the protein for recognition by the proteosomal system and is one the important pathways for cell protein degradation[12]. Chemical modifications enhance the size of the proteome and thereby its complexity by 1-2 orders of magnitude. These modifications can be spontaneous as in case of glycation and nitration or enzymatically mediated as in case of phosphorylation and acetylation. Most physiologically important modifications are mediated by a complex enzymatic machinery which regulates both addition as well as removal of the modification. For example, acetylation of histones for transcriptional control involves both acetyltransferases and deacetylases which perform antagonistic functions and therefore, their levels and activities are tightly regulated so as to maintain a balance in the level of acetylation as per the physiological requirement of the cell[13].

Non-enzymatic protein modifications, on the other hand, are caused by exogenous compounds or certain metabolites. For example, drugs such as penicillin[14] and cocaine[15] can covalently bind proteins. Similarly, dopamine quinone, an oxidation product of the

neurotransmitter dopamine can undergo nucleophillic addition reaction with free sulfhydryl groups of cysteine residues[16]. These reactions are spontaneous and the levels of such modified proteins vary greatly depending on the external environment and the levels of the modifying agent. Some of these modifications can also negatively affect protein structure and function. Few important ones are discussed below.

1.1.1. Protein Oxidation

Redox reactions are an integral part of biological functions as *in vivo* oxidation reactions of carbohydrates and fatty acids provide energy. But under certain conditions, biomolecules can undergo aberrant oxidation reactions producing toxic moieties that can harm normal physiological functions. Oxidative stress, wherein there is an imbalance between oxidant formation and removal mechanisms, leads to protein oxidation[17], a reaction also termed as protein carbonylation since there is an increase in levels of protein carbonyl groups[18]. These reactions can occur with reactive oxygen species[19] or reactive nitrogen species [20, 21] formed during oxidative stress and are termed as 'primary protein carbonylation' reactions. They can also occur with other by-products of oxidative stress such as glycation by-products like glyoxal and methyl glyoxal or lipid peroxidation by-products like HNE and malondialdehyde[22], which then are termed as 'secondary protein carbonylation' reactions. Oxidation can also occur in the presence of metal ions like iron (Fe) or copper (Cu) at arginine, lysine, threonine and proline residues and can lead to cleavage of polypeptide backbone and cross-linking within the peptide chains [23].

1.1.2. Glycation

Glycation is a non-enzymatic reaction of reducing sugars like glucose and its auto-oxidation products with free amino groups of proteins, lipids and nucleic acids. Reducing sugars react with free amino groups of proteins to form Schiff bases which rearrange to form Amadori products. These further undergo a series of reactions to form Advanced Glycation End products (AGEs) (Figure 1.1). This reaction is also known as Maillard reaction since is it was first described by Louis Camille Maillard, who noticed the browning effect when a mixture of amino acids and reducing sugars was heated[24]. Several other non-sugar compounds have also been reported to lead to glycation reaction such as ascorbic acid (via its oxidative degradation product threose)[25], glyceraldehyde[26], and so on.

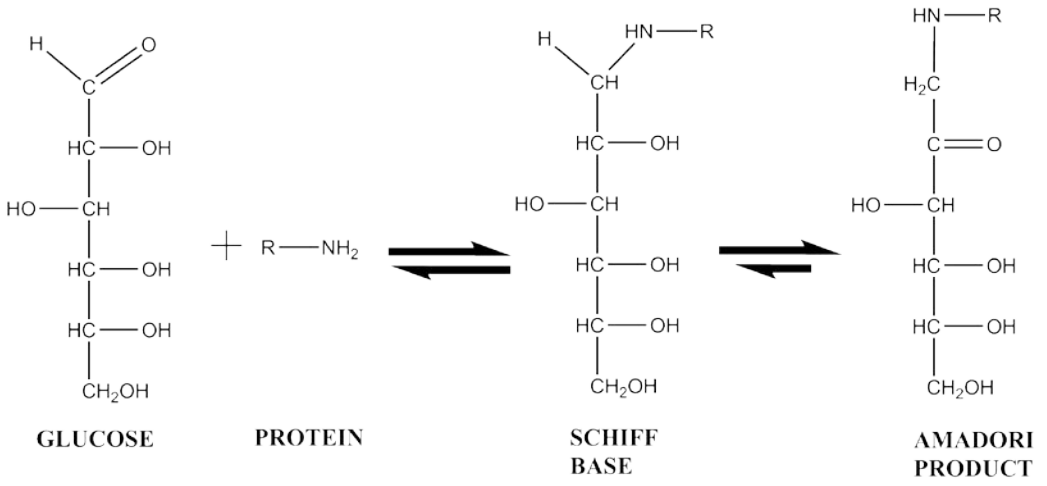


Figure 1. 1: Glycation reaction
Reaction of reducing sugars like glucose leads to glycation at free amino groups of proteins

The reaction also leads to formation of highly reactive α-dicarbonyls and oxoaldehydes, such as like 3-deoxyglucosone, glyoxal and methylglyoxal from glucose, which themselves can lead to formation of AGEs[4] (Figure 1.2).

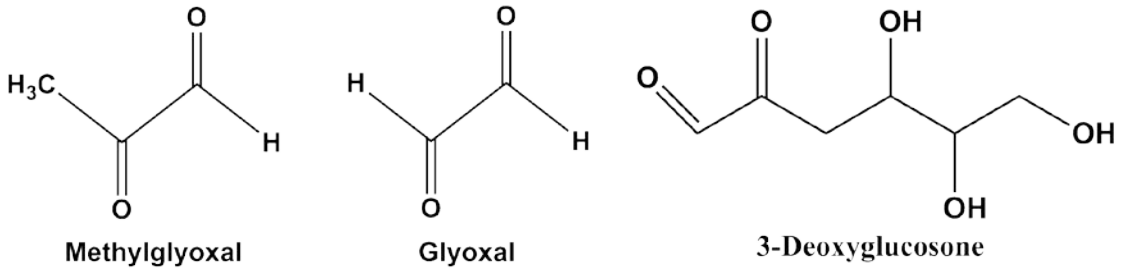


Figure 1. 2: Reactive Dicarbonyls in Glycation
α-dicarbonyl compounds that are potent glycating agents

The formation of AGEs occurs over a period of weeks. It can be accompanied by oxidation leading to a process called glycoxidation. Different AGEs have been identified and characterized[27] and some their names and structures are given in Figure 1.3. Protein glycation has been reported to lead to aggregation and cross-linking[28] and also reduce the enzymatic activity of a number of proteins such as aminotransferases[29], HDL paraoxonase[30] and so on[31].

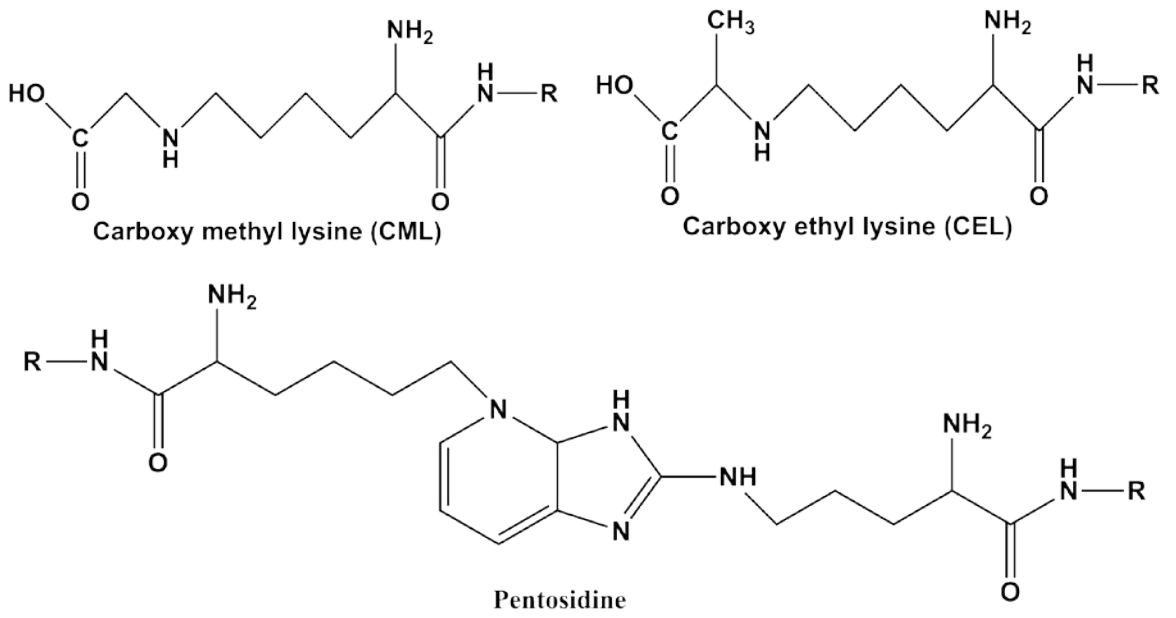


Figure 1. 3: Advanced Glycation End-products
Chemical structures of some of the major AGEs present in plasma (R= polypeptide chain)

1.1.3. Homocysteinylation

Homocysteine (Hcy) is a non-protein sulphur containing amino acid produced in all human tissues. It is formed via transmethylation of methionine, wherein methionine is first converted to S-adenosyl-L-methionine (SAM) by SAM synthase. SAM is then converted by methyltransferase to S-adenosyl-L-homocysteine (SAH), which is further converted by SAH hydrolase to adenosine and homocysteine. Hcy can get remethylated to form methionine by a reaction that requires 5-methyltetrahydrofolate and vitamin B12 as cofactors. Remethylation can also be mediated by betaine homocysteine S-transferase in a B12 independant reaction. Alternatively, it can get converted to cysteine via the transulfuration pathway which is catalyzed by cystathionine-β-synthase using pyridoxal-5-phosphate, a vitamin B6 derivative, as cofactor[32]. Due to its structural similarity to methionine, Hcy gets activated by methionyl tRNA synthetase but instead of being transferred to the tRNA, it gets cyclized to form a thioester, homocysteine thiolactone (Hct)[33] (Figure 1.3).

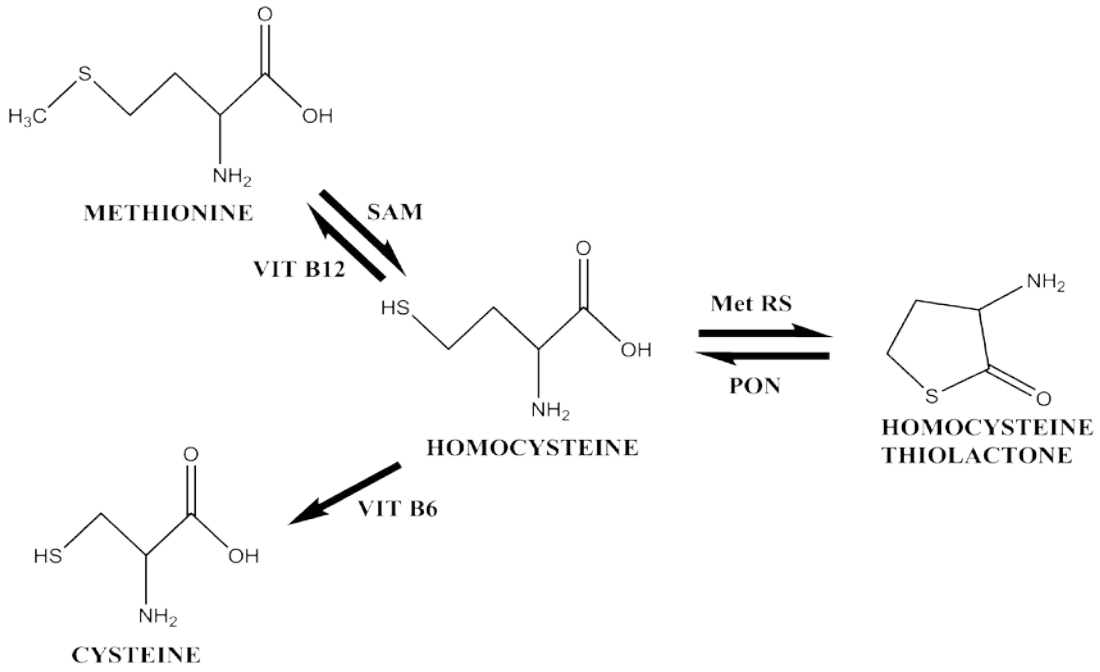
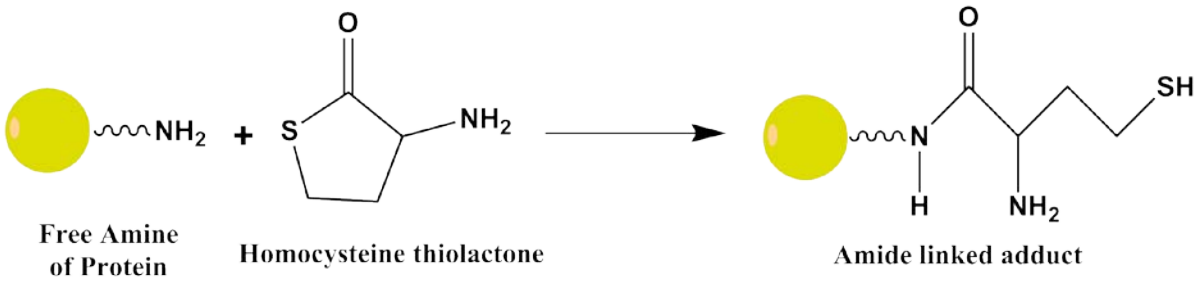


Figure 1. 4: Biosynthesis of Homocysteine
Homocysteine is formed as an intermediate during methionine cycle and can get reconverted to methionine or converted to cysteine or to homocysteine thiolactone by various different enzymes

Homocysteine thiolactone is a highly reactive compound with a carboxyl group that readily reacts with free amino (-NH_2) groups of protein lysine residues to form an amide linked adduct, homocystamide (Figure 1.4a). It has been shown to react with a number of plasma proteins such as serum albumin, fibrinogen, transferrin and so on, unde r physiological conditions by a reaction termed as N-homocysteinylation[34]. Hcy itself can also bind to free thiol groups of proteins via a reversible reaction termed as S-homocysteinylation (Figure 1.5).



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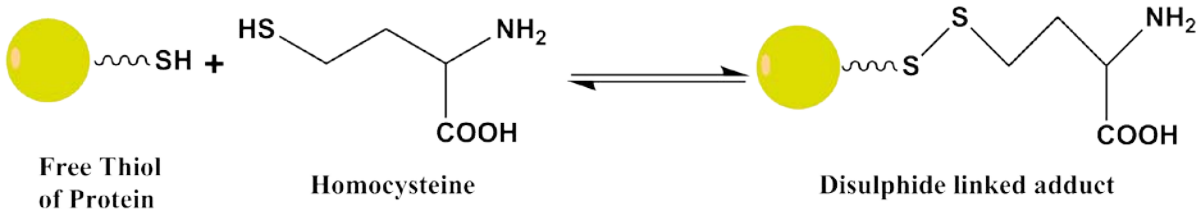


(b)

Figure 1. 5: Protein Homocysteinylation
Homocysteine can modify proteins in two ways (a) N-homocysteinylation, i.e. reaction with free amino groups via Hct, or (b) S-homocysteinylation, i.e. reaction with free thiol groups

Protein N-homocysteinylation can affect protein secondary structure since it results in incorporation of free thiol groups. This can lead to protein aggregation and precipitation which is reported for a number of proteins after reaction with Hct *in vitro*[35]. It also leads to the formation of toxic amyloid like protofibrils in BSA[36]. Homocysteinylation can also lead to loss of protein activity as reported in case of homocysteinylated NO synthase [37]. In contrast, homocysteinylation of angiotensin convertase enzyme or ACE has been reported to increase its activity [38].

1.1.4. Acetylation

Acetylation is one of the well known chemical modifications of protein which involves transfer of acetyl group to the amino acid side chains of proteins. Acetylation can occur enzymatically, via N-terminal acetyltransferases (NATs) and lysine acetyltransferases (KAT) which is important for stabilization and activation of proteins[39, 40] or non-enzymatically, by transfer of acetyl group from chemical molecules such as acetic anhydride[41] and acetyl salicylic acid or aspirin[42] (Figure 1.6).

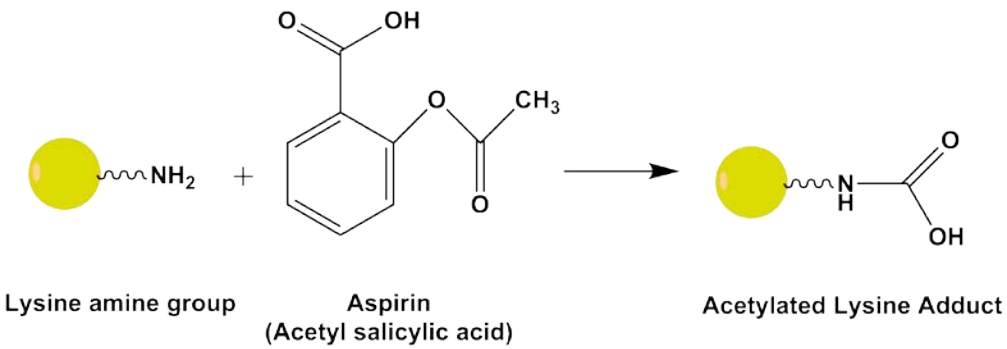
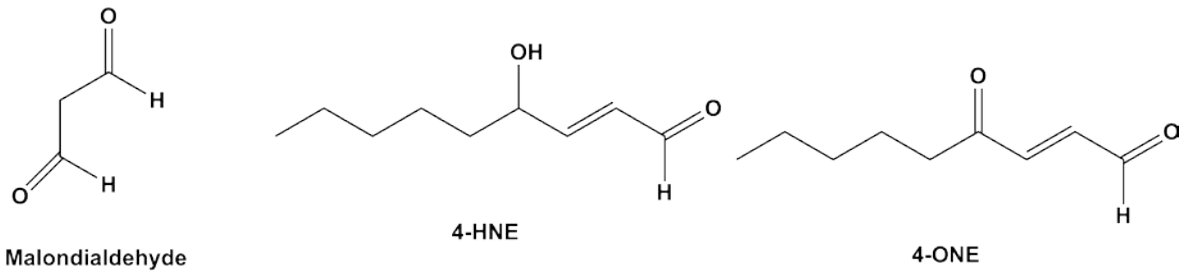


Figure 1. 6: Protein Acetylation
Aspirin leads to acetylation of free amino groups of proteins

Aspirin has been reported to acetylate a number of plasma proteins *in vitro*[42, 43] as well as many cellular proteins[44]. Since acetylation also occurs at epsilon amino group of lysines similar to glycation and homocysteinylation, it has been shown to inhibit these modifications and thus prevent some of their negative effects on p rotein structure and function[45, 46, 47].

1.1.5. Carbonylation by Lipid Peroxidation Products

Lipid peroxidation under oxidative stress leads to the formation of highly reactive α, β-unsaturated aldehydes or reactive carbonyl species such as 4-hydroxynonenal (4-HNE), 4-oxononal (4-ONE), malondialdehyde and acrolein (Figure 1.7a). These, in turn, can undergo Michael's addition reaction with side chains of amino acids like lysine, histidine and cysteine leading to addition of reactive carbonyl species to the protein side chains, forming Advanced Lipoxidation End-products or ALEs[48] (Figure 1.7b). The reaction is irreversible and irreparable, and serves as an indicator of oxidative stress[7].



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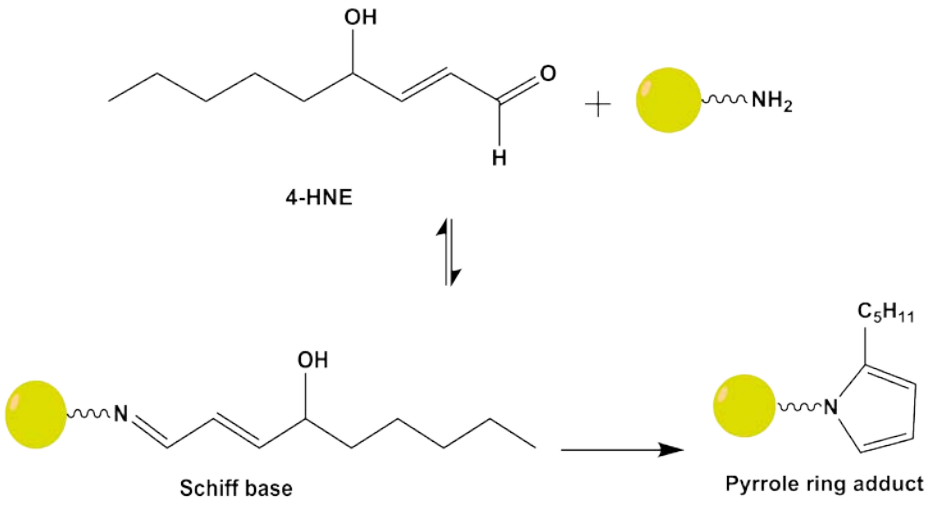


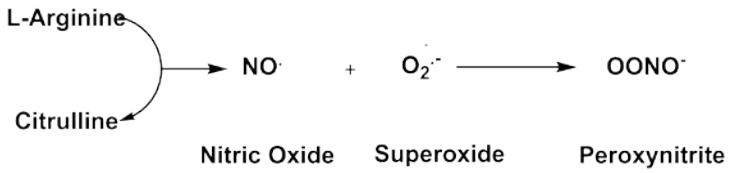
(b)

Figure 1. 7: Lipid Peroxidation Products and Protein Carbonylation
(a) Reactive aldehydes formed due to lipid peroxidation (b) 4-HNE reacts with free amino groups of proteins to form Schiff base which further undergoes rearrangement to form pyrrole ring adduct

Carbonylation of skin keratin by acrolein affects its fibrous structure and thus, the appearance of skin with advancing age[49]. In similar manner, carbonylation of cellular actin has been reported to lead to its aggregation and reduced cell proliferation in T-lymphocytes and consequent reduction in immune function with aging[50]. 4-HNE is highly toxic and most abundant of all lipid aldehydes and numerous plasma proteins[51] as well as cellular proteins[52] have been reported to be modified by HNE.

1.1.6. Protein nitration

Protein nitration occurs by addition of nitro group (NO_2) to the *ortho* position of phenolic hydroxyl group of tyrosine residue by *in vivo* nitrating agents such as peroxynitrite ($OONO^-$) and its derivatives. These reactive nitrogen species are formed by reaction between superoxide ($O_2^{\cdot -}$) and nitric oxide (NO), a relaxing factor synthesized from arginine by NO synthase and released into blood by epithelial cells[53] (Figure 1.8a).



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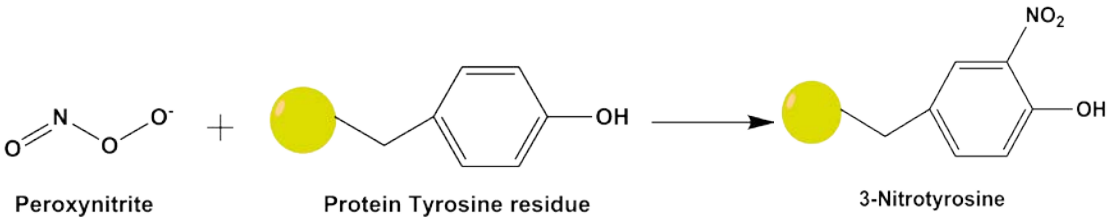

(b)

Figure 1. 8: Peroxynitrite and Protein Nitration
(a) Formation of peroxynitrite from reaction between superoxide and nitric oxide **(b)** Protein nitration at tyrosine by peroxynitrite

Nitration of protein is a selective reaction depending on protein structure and folding, and can lead to change in protein activity, degradation and immunogenicity. Many cellular and plasma proteins have been reported to lose their activity after nitration *in vitro*[54, 55]. Tyrosine nitration also increases aggregation of tau proteins and can influence the progression of Alzheimer's disease[56].

1.2. Regulation of Protein Modifications

Although many protein modifications are prerequisite for the normal functioning of mature proteins, in instances where there is excess accumulation of the modifying molecule, these chemical modifications of proteins can occur at higher rates and affect normal physiology. This is illustrated by the increased accumulation of glycated proteins in diabetic patients due to elevated plasma glucose levels[57]. Consequently, there exist mechanisms that function for their removal, either via enzymes that remove the modifying agent or receptors that recognize and remove the modified protein.

1.2.1 Enzymatic Removal Mechanisms

Cellular defense systems against protein modifications involve a number of enzymes that scavenge modifying agents and maintain them at non-toxic levels. Major detoxifying enzymes for the removal of reactive oxygen species involved in protein oxidation include SOD, catalase and glutathione peroxidase. SOD or superoxide dismutase is a metalloenzyme which converts superoxide (O_2^-) to molecular oxygen and hydrogen peroxide. This hydrogen peroxide is further detoxified into water and molecular oxygen by catalase in peroxisomes and glutathione peroxidase (a selenocysteine peroxidase) in mitochondria[58]. A similar system

which removes the highly reactive dicarbonyls responsible for protein glycation involves the enzyme glyoxalase[59]. In this cytoplasmic enzymatic pathway, glutathione (GSH) reacts spontaneously with MG to form hemiacetal adducts. These adducts are metabolized by glyoxalse-1 enzyme (Glo-1) to S-lactoylglutathione (SLH)[60] which is further metabolized by glyoxalase-2 (Glo-2) to regenerate GSH and form lactate[61]. Other enzymes like aldoketoreductases and aldehyde dehydrogenases also play a minor role in detoxifying reactive dicarbonyls[62]. Paraoxonase-1 (PON1) or homocysteine thiolactonase-1[63] is an enzyme which detoxifies Hct and protects against protein homocysteinylation[64, 65]. It is a calcium dependant hydrolyzing enzyme present in serum and endothelial cells[66], [67]. PON1 is associated with HDL in serum and has also been shown to prevent copper induced LDL oxidation[68]. Apart from these, numerous proteolytic enzymes in the lysosomes[69] and the proteosomal complex[70], [71] in conjunction with the ubiquitination system function to remove the modified proteins from the cells. For example, lysosomal endopeptidases cathepsin D and L have been shown to be involved in the degradation of AGEs[72].

1.2.2 Pattern Recognition receptors

Extracellular modified proteins are recognized by a specific class of receptors called as scavenger receptors, which internalize and present them to proteolytic degradation. Scavenger receptors are a family of membrane bound receptors that were first identified in macrophages to bind and internalize oxidized LDL[73]. These receptors can be classified based on their structure and biological function into 10 classes A-J[74]. Currently known to bind a number of other ligands like microbial pathogens, apoptotic cells, phospholipids and so on, these receptors have been implicated in a number of biological functions such as apoptosis and angiogenesis as well as in development of diseases such as atherosclerosis[75]. Different forms of these receptors have been identified which interact with particular modified proteins; for example, LOX-1 recognizes and binds oxidatively modified LDL or oxLDL whereas RAGE/AGE-R1 recognizes glycated proteins[76].

1.2.2.1. LOX1

Lectin-like receptor for oxidized LDL (LOX-1), also known as SR-E1, is a 50kDa type II transmembrane receptor with 273 amino acids[77] that has been found to be expressed on endothelial cells, macrophages[78], platelets[79] and smooth muscle cells[80]. A lthough it was

initially identified as the primary receptor for binding oxidized LDL, it has been later shown to bind a variety of ligands such as activated platelets, apoptotic bodies, advanced glycation end products and other forms of modified LDL[81]. Its expression has been shown to be induced by numerous factors such as oxLDL[82], proinflammatory cytokines[83], lysophosphatidylcholine[84], glucose[85] as well as by fluid shear stress[86]. LOX-1 expression has also been found to be induced by angiotensin in endothelial cells[87] and consequently, angiotensin receptor blockers like losartan inhibit its expression[88].

LOX1 plays an essential role in removal of oxLDL from blood stream and its proteolytic degradation in the cells[89]. 6 cysteine residues along with three clusters of basic amino acids in the C-terminal lectin-like extra-cellular domain have been shown to be essential for binding of oxLDL to LOX-1[90]. After oxLDL binding, LOX1 gets internalized via a dyanamin dependent mechanism followed by its dissociation from the bound oxLDL in the endocytic vesicles and recycling back to the cell membrane[91].

1.2.2.2 RAGE

Receptor for Advanced Glycation End-products or RAGE is a 42kDa multi-ligand pattern recognition receptor[92] that interacts with many endogenous ligands such as high mobility group protein 1 (HMGB1 or Amphoterin), S100 or Calgranulin, β-amyloid and advanced oxidation protein products (AOPPs), apart from AGEs[93]. It was first identified in bovine endothelium as a 35kDa receptor that binds glycated proteins[94], [95] and is expressed in a number of cells such as macrophages and monocytes[96], smooth muscle cells[97], endothelial cells[98], neuronal cells[99] and so on. Although its physiological expression levels are low, but it can get up-regulated in response to ligand binding and inflammation[92, 100]. It belongs to the immunoglobulin superfamily and the structure of full length RAGE contains a V-type (variable) domain for ligand binding, two C-type (constant) domains, a transmembrane spanning helix and a C -terminal cytosolic domain ctRAGE for signal transduction[101]. Binding of ligands to full-length RAGE leads to their internalization but does not lead to their clearance or degradation, instead leads to pro-inflammatory response via different signaling cascades[102].

RAGE has several isoforms in humans, one of which without cytosolic tail and transmembrane helix, also termed as variant 1 does not activate signaling cascade. It can be

formed as a s plice variant of RAGE[103] and is termed as endogenous secretory RAGE (esRAGE). Additionally, it can also be cleaved proteolytically from full-length RAGE expressed on cell surface by metalloproteases such as ADAM10[104], [105] in which case it is called as soluble RAGE (sRAGE). A number of studies indicate the beneficial role of sRAGE in blocking the pro-inflammatory response of RAGE[106], [107], [108] and therefore, it may function as a decoy receptor for the removal of modified proteins [109].

1.3. Protein modifications in disease

Although the body is equipped with mechanisms for removal of modified proteins, an excess exposure to modifying agent can lead to accumulation of these modified proteins in plasma. This can have deleterious effects and has been implicated in the etiology of a number of diseases. Increased accumulation of AGEs has been associated with aging[110] as well as a number of aging associated diseases like Alzheimer's[111], cardiovascular disease[112], chronic kidney disease[113], non-alcoholic fatty liver disease or NAFLD[114] and so on. Other modifications have also been associated with the development of these diseases, for example, increased HNE derived ALEs are observed in brains of Alzheimer's patients[115] and nitrated proteins have been shown to mediate development of liver diseases[116]. Moreover, increased modification of specific functional proteins has also been associated with some diseases. For instance, glycation and hyperphosphorylation of the microtubule associated Tau protein in neurons mediates its aggregation and is implicated in progression of Alzheimer's disease[117], [118]. Similarly, HNE mediated carbonylation of fatty acid binding protein or FABP is reported to reduce its affinity for fatty acids and is thereby involved in obesity related insulin resistance[119]. In some cases, the modification itself can also serve as biomarker for disease diagnosis, as seen in HbA1c measurement which quantifies the extent of glycation at valine residue at A-chain of haemoglobin, and is the routinely used evaluation test for diabetes[120], [121].

A different mechanism by which modified proteins are involved in disease pathology is via autoimmune response. Since protein modification affects its protein charge distribution and consequently its secondary structure, the modified protein can be recognized as non-self and induce inflammatory or immune response in the body[122]. Accordingly, autoantibodies against various modified proteins like glycated[123, 124] and homocysteinylated[125, 126] have been

observed in different diseases. Such autoantibodies have also been used for diagnosis of autoimmune diseases; for instance, detection of the antibody for cyclic citrullinated peptide (anti-CCP) is the basis of a diagnosis test for rheumatoid arthritis[127].

Increased protein modification can occur due to increase in levels of modifying agent or reduction in capacity of defensive mechanisms or both. A decline in the protein degradation machinery occurs with age[128] which is why the accumulation of modified proteins is observed in diseases associated with aging. At the same time, genetic variations in removal mechanisms can be also be associated with increase in protein modification as seen in case of PON1 wherein allelic variations have been associated with increased protein homocysteinylation and increased cardiovascular risk[129]. There has been a lot of focus on scavenger receptors and their role in the development of diseases as well. Since the binding of modified proteins to these receptors also initiates an inflammatory signaling cascade, excessive ligand interaction can play a causative role in the development of various complications. For example, interaction of AGEs with RAGE has been associated with diabetic cardiovascular complications[130], Alzheimer's disease[131] as well as cancer[132]. Similarly, interaction of oxidized LDL with LOX-1 receptors has been associated with increased atherosclerotic risk[133].

1.4. Plasma Protein Modifications and Cardiovascular Diseases

Cardiovascular diseases (CVD) comprise numerous disorders such as coronary heart disease, cerebrovascular disease, rheumatic heart disease and so on. They are one of the leading causes of morbidity and mortality worldwide, being responsible for 31% of all global deaths[134]. Atherosclerosis, a vascular pathology wherein there is build up of a plaque or occlusion in the vascular wall accompanied by stiffening or hardening of the arteries, is a characteristic feature seen in a number of CVDs[135]. Rupture of the atherosclerotic plaque leads to further thombogenesis and the resultant decrease in blood flow may lead to heart attack or stroke[136], which together are responsible for 80% of deaths due to CVD.

A disruption of the functions of the vascular endothelium usually acts as the initiating step of CVD. They are commonly associated with a number of factors such as smoking, hypercholesterolemia, hypertension, diabetes and obesity, many of which are also associated with increased protein modifications. For example, aldehydes in cigarette smoke and alcohol

are reported to increase plasma protein carbonylation[137, 138]. Plasma proteins being continuously exposed to these modifying agents are more likely to get modified and may contribute to development of vascular diseases in a n umber of ways. For example, homocysteinylated LDL exerts cytotoxic effects on endothelial cells[139] whereas homocysteinylation of fibronectin attenuates its interaction with fibrin leading to delay in blood coagulation[140]. Elevated levels of modified plasma proteins have also been associated with the development of atherosclerosis. One of the underlying processes for atherogenesis is the uptake of oxidized LDL via LOX-1 by macrophages which leads to formation of foam cells that comprise the core of the atherosclerotic plaque[141]. Other modified plasma proteins, such as nitrated apolipoprotein B[142], citrullinated fibrinogen[143] and so on, have also been associated with atherosclerotic lesions and can serve as potential biomarkers for atherosclerosis[144].

1.5. The Vascular Endothelium

Vascular endothelial cells line the entire circulatory system and form the boundary between circulating blood cells and vascular smooth muscle cells. They are the main mediators of vascular homeostasis as they control various functions such as vasomotion, angiogenesis, hemostasis and inflammation. The main function of the endothelium is to form a barrier allowing only selective molecules to travel across. Vascular permeability is maintained by different cell adhesion molecules (CAMs) such as cadherins, integrins or immunoglobulin-like CAMs[145]. The endothelium also has specialized adhesion molecules termed as selectins, which mediate adhesion and transmigration of leukocytes during inflammation[146, 147].

For the maintenance of normal vascular tone, the endothelium releases both vasodilators, like NO (nitric oxide), prostacyclins and C type natriuretic peptide, as well as vasoconstrictors, like endothelin and angiotensin II[148, 149]. Nitric oxide or endothelium derived relaxing factor (EDRF) is produced by endothelial nitric oxide synthase (eNOS) and released in response to shear stress and other mediators like bradykinin, adenosine and serotonin. It acts on vascular smooth muscle cells and causes vessel relaxation[148, 150, 151]. Endothelin-1 is the most potent vasoconstrictor produced by the vascular endothelial cells which acts via endothelin A receptors[152, 153]. Angiogenesis or the formation of new blood vessels from the existing ones, occurs in response to pro-angiogenic signals secreted by

endothelial cells as well as those secreted by other cells, such as fibroblast growth factors (FGF) and platelet derived growth factors (PDGF). The most important mediators of angiogenesis include vascular endothelial growth factors (VEGF), angiopoeitins (Ang) and some members of the ephrin family. VEGF is required for the initiation of angiogenesis whereas angiopoietins and ephrins are required for the maturation & remodelling of newly formed blood vessels[154]. The endothelium also regulates blood fluidity by producing a number of pro-coagulant as well as anticoagulant mediators. NO and prostaglandin I2 (PGI-2) themselves also act as antithrombotic molecules wherein they inhibit platelet activation and aggregation[155]. Thrombomodulin, expressed on t he endothelial cell surface, binds to thrombin and inhibits its protease activity which is required for converting fibrinogen to fibrin during clot formation[156]. Plasminogen activators cleave plasminogen to plasmin in the presence of fibrin and aid dissolution of the clot[157]. Endothelial cells also continuously produce and release tissue plasminogen activator (t-PA), which is rapidly degraded in the liver, unless it binds to fibrin[156]. Among the prothrombotic molecules produced by the endothelium are Von Willebrand factor (vWF) and plasminogen activator inhibitor-1 (PAI-1). vWF is stored in Weibel-Palade bodies in the endothelial cells and when released forms large multimers which serve as a ligand for platelet adhesion and aggregation during formation of a clot[158]. The endothelium also secretes the endothelial cell type plasminogen activator inhibitor i.e. PAI-1 which acts as protease inhibitor for the plasminogen activators[159].

1.5.1. Endothelial Dysfunction

Endothelial dysfunction or ED involves transition of the vascular endothelium from its quiescent phenotype to a pro-inflammatory one (Figure 1.9). The term was coined by Furchgott and Zawadzki after they discovered that acetylcholine requires the presence of the endothelial cells to relax the underlying vascular smooth muscle[148].

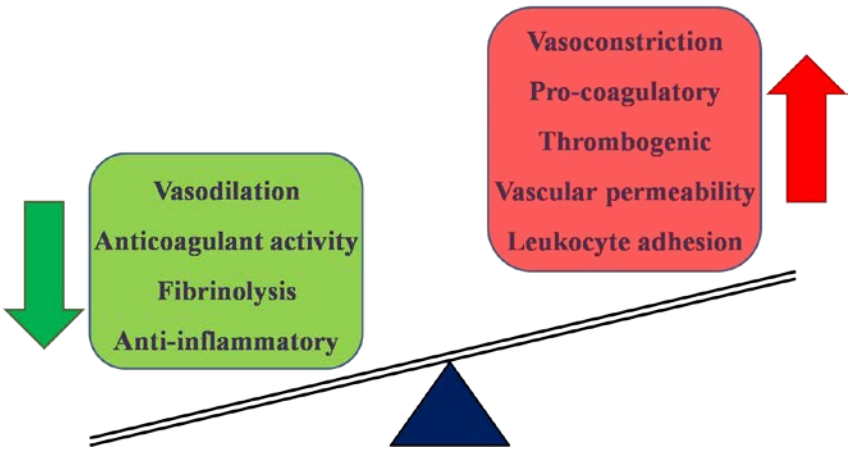

Figure 1. 9: Endothelial Dysfunction
Imbalance between the repair and damage mechanisms of the endothelium

ED is mainly characterized by reduced endothelium-mediated vasorelaxation and increased pro-coagulant tendency[160]. It is initiated by increased production of ROS[161], which themselves act as vasoconstrictors. ROS also cause degradation of tetrahydrobiopterin, the co-factor required by eNOS as a result of which eNOS switches towards synthesizing superoxide ions instead of NO, a process termed as eNOS uncoupling[162], [163] (Figure 1.10). The formed superoxide can react with NO to produce the reactive nitrogen species peroxynitrite[53]. Thus reduced NO synthesis and rapid destruction of formed NO by ROS leads to reduced NO bioavailability, which is the hallmark of endothelial dysfunction[164]. This is accompanied by increased expression of endothelin-1 and prostaglandins leading to vasoconstriction.

The ROS also regulate the expression of a number of many pro-apoptotic genes, via activation of NF-κB transcription factor leading to apoptosis of endothelial cells and destruction of endothelial layer releasing circulatory markers such as endothelial microparticles and circulating endothelial cells[165]. Under normal conditions, the endothelial surface does not allow adhesion of platelets and thrombus formation. However, endothelial injury allows platelets to adhere to its surface (Figure 1.10b) and release of thromboxane A2 and serotonin from the activated platelets add to vascular constriction[166]. Functional impairment of NO also affects fibrinolysis leading to prothrombotic phenotype[167], [168]. NF-κB also signals the production of inflammatory mediators like interleukin β and monocyte chemo-attractant protein-1[169], [170] and increased expression of adhesion molecules (VCAM-1,

ICAM-1) on t he endothelial cell membrane. This leads to the consequent migration of leukocytes into the vascular wall (Figure 1.10c) which is one of the steps that leads to the formation of atherosclerotic plaque.

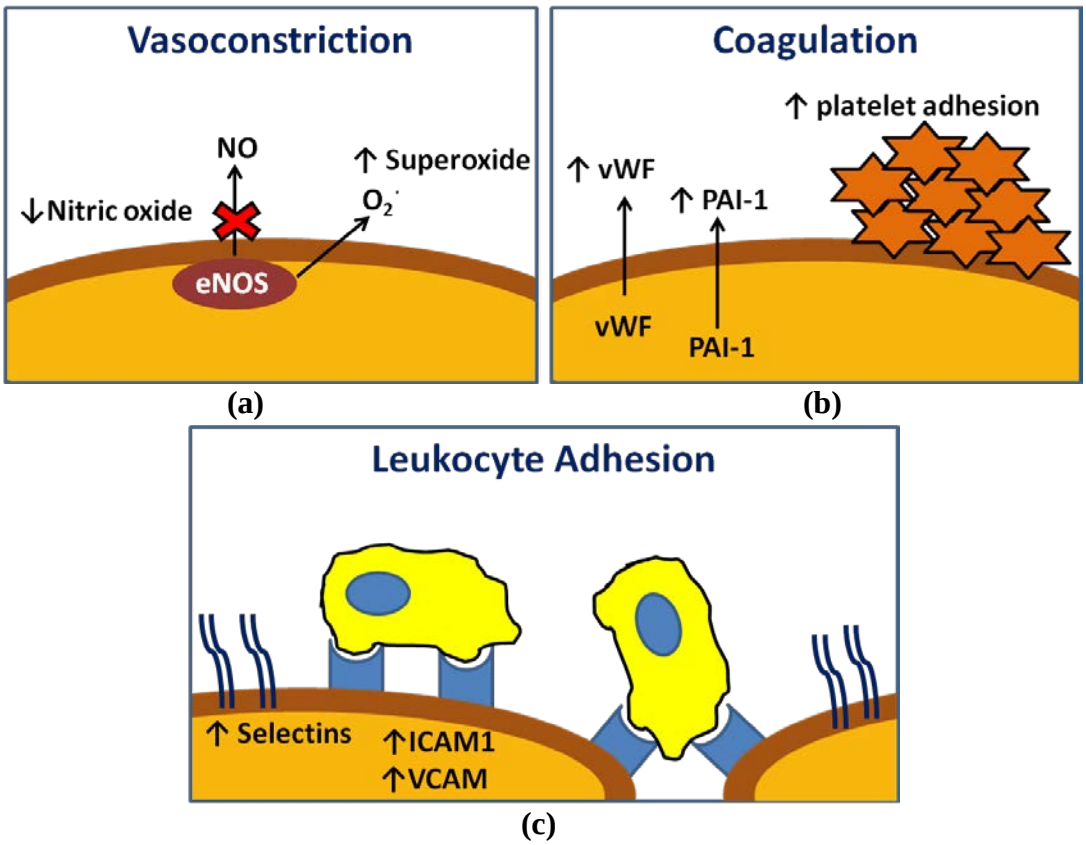

Figure 1. 10: Characteristics of Endothelial Dysfunction
ED is characterized by increased vasoconstriction, blood coagulation or thrombogenesis and leukocyte adhesion and transmigration into the sub-endothelial region

The accumulated lymphocytes and macrophages in the vascular wall secrete more chemokines and further aggravate the pro-inflammatory condition[171] leading to a destructive cyclic process [172] that acts as initial step for the development of cardiovascular diseases like atherosclerosis and hypertension. The cellular mechanism leading to ED is depicted in Figure 1.11.

Endothelial injury not only affects the vascular system but also other systems of the body and thus ED is also associated with various other processes such as aging, pre-eclampsia, microalbuminuria, insulin resistance, obesity and so on[173]. For instance,

dysfunction of the endothelial cells in blood brain barrier is speculated to be involved in Alzheimer's disease pathology[174]. Likewise, endothelial dysfunction is believed to play a role in the development of diabetic nephropathy[175] and macular edema[176].

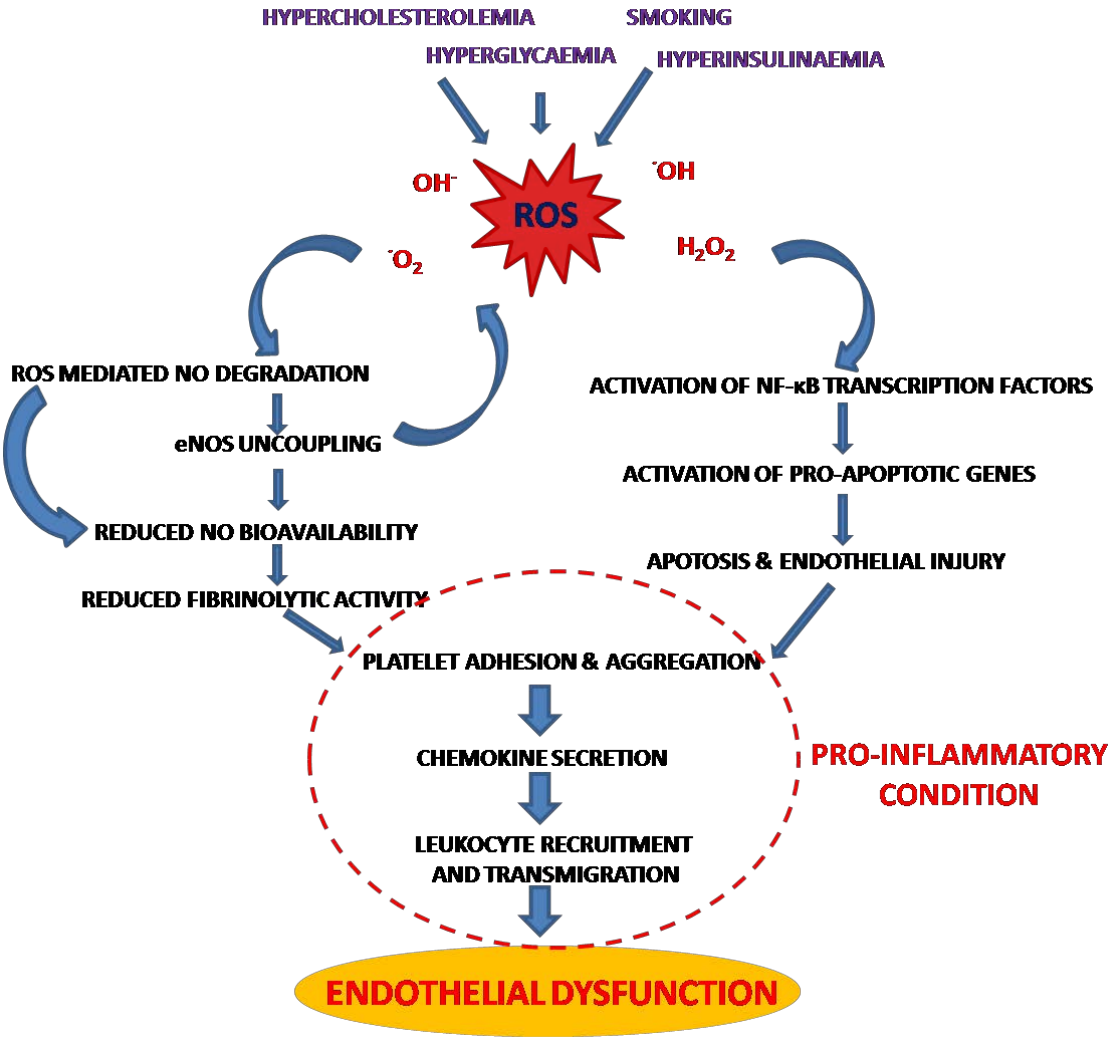

Figure 1. 11: Mechanism of Endothelial Dysfunction

1.5.2. Endothelial Dysfunction and Chemical Modifications

The vascular endothelial cells being in direct contact with blood and its components, are the first to interact with modified plasma proteins and can play an important role in the development of cardiovascular pathologies in response to modified proteins. Therefore, it is important to understand their function as well as their response to different modified proteins.

Few of the modified proteins have been shown to affect endothelial function via their interaction with the scavenger receptors present on endothelial surface. For example, the interaction of oxLDL with LOX-1 has been reported to lead to oxidative stress and reduced NO production in bovine aortic endothelial cells (BAECs)[177]. It has also been reported to increase the endothelial expression of adhesion molecules[178] and chemokines[179] like MCP-1[180] and consequently, increase monocyte adhesion to endothelial cells. Therefore, LOX-1 mediates a majority of the negative effects of oxLDL on endothelial function[181], [182]. Similarly, AGE-RAGE interaction has been reported to increase expression of adhesion molecules VCAM-1, ICAM-1 and E-selectin and induce endothelial cell activation[183]. However, for most of the other plasma protein modifications, limited information is available regarding their interaction with endothelial cells.

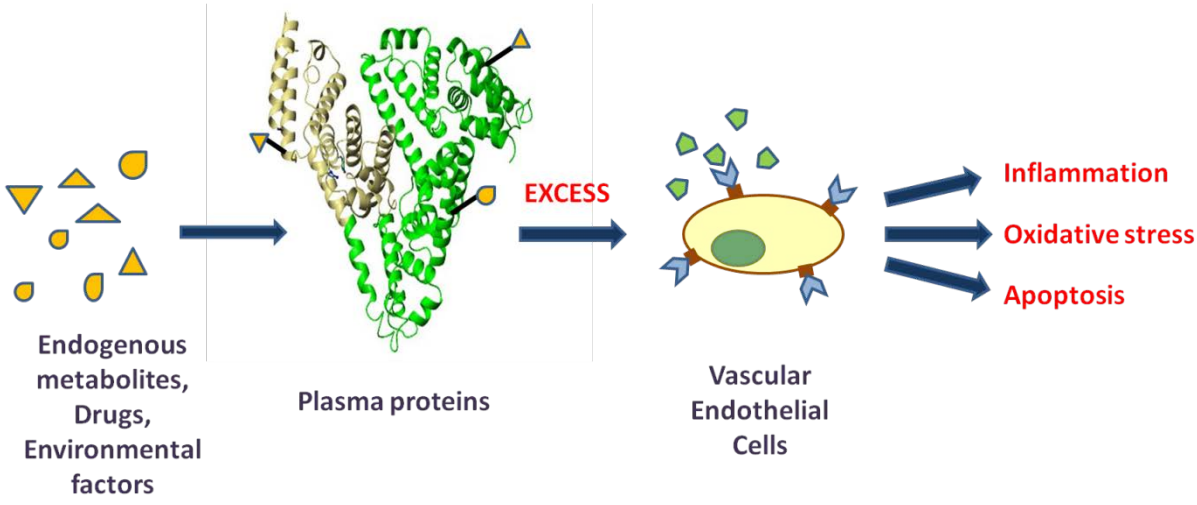

Figure 1. 12: Plasma protein modification and Endothelial Dysfunction
Excessive accumulation of modified plasma proteins negatively affects endothelial cell physiology leading to dysfunction

1.6. Scope of Work

Current treatments for cardiovascular disease do not aim at improvement of endothelial function. Since ED serves as an initial step for the development of CVD, targeting endothelial dysfunction as a therapeutic strategy can prove to be useful for better prognosis as seen by few clinical trials[184]. Also, since chemical modifications have been reported to be involved in development of endothelial dysfunction and CVD, it is necessary to understand

the effect of chemically modified proteins on endothelial cells and the mechanism involved in development of ED in response to such proteins. This will further aid development of novel therapeutic interventions based on inhibiting the interaction of chemically modified proteins with endothelial cells. This PhD study was designed with the aim to address these aspects.

Objectives of Thesis:

- Synthesize and characterize different chemically modified proteins
- Study the effect of chemically modified proteins on endothelial cells
- Identify receptors and signaling pathways associated with different modifications

1.7. References

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Chapter 2: Synthesis and Characterization of Different Chemically Modified Proteins

2.1. Background

Proteins routinely undergo chemical modifications, termed as post-translational modifications or PTMs, which confer them with new function or activity, allow molecular interactions and compartmentalization, and also determine their lifespan. Most of the physiologically important chemical modifications are mediated by cellular enzymatic systems. However, proteins can also undergo aberrant modifications under abnormal conditions wherein there is increased accumulation of a modifying agent or imbalance of the removal mechanisms. Such modifications are physiologically unfavourable since they can lead to loss or gain of protein function, increase their immunogenicity, or induce an inflammatory response via specific by pattern recognition receptors that recognize such modified proteins. Particularly plasma proteins are prone to such modifications since they are continuously exposed to an number of exogenous moieties as well as a number of metabolites which are capable of modifying them.

Serum albumin is a large plasma protein of molecular weight 64kDa that performs various functions such as maintaining intravascular oncotic or osmotic pressure, acts as a carrier of substances like vitamins and drugs, and a free-radical scavenger and so on. It is present in high concentration in serum (about 50-60mg/ml) and is characterized by several nucleophilic and accessible residues (35 cysteines, 21 tyrosines, 16 histidines and 59 lysines), therefore, making it a favorable target protein for modifications[1]. It also has a long circulating half life of about 21 days, which allows even slow modification reactions to proceed. Consequently, it is reported to be covalently modified by various modifiers such as metabolites, drugs such as aspirin, penicillin, billirubin, as well as environmental agents.

In this chapter, we report different *in vitro* modifications using serum albumin as a model protein and their effect on protein secondary structure as well as the effect of such modified proteins on endothelial cells.

2.2. Materials and Methods

2.2.1. Reagents

All chemicals were procured from Merck (formerly Sigma-Aldrich) unless otherwise stated. Antibodies used were anti-CML (Abcam), anti-acetyl lysine (Merck, formerly Millipore), anti 4-HNE (Abcam), anti-nitrotyrosine (Abcam), and secondary antibodies tagged to HRP from Merck (formerly Bangalore Genei). All solvents for mass spectrometry were obtained from J.T.Baker®.

2.2.2. Synthesis of Modified Albumin

2.2.2.1. Glycation

Human serum albumin (50mg/ml) was incubated with 0.5M glucose in 200mM 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 30kDa molecular weight cut-off.

2.2.2.2. Homocysteinylation

HSA (100μM) was incubated with 5mM homocysteine thiolactone hydrochloride in 100mM phosphate buffer containing 2mM EDTA at 37°C for 24h. After completion of the reaction, it was dialyzed against phosphate buffer overnight to remove unreacted thiolactone and concentrated using molecular weight cut-off filters.

2.2.2.3. Acetylation

50mg/ml HSA was incubated with 100mM aspirin in 200mM phosphate buffer containing 0.05% sodium azide at 37°C for 6 days followed by dialysis.

2.2.2.4. Carbonylation

5mg HNE-DMA (4-hydroxynonenal dimethylacetal) was dissolved in 1mM ice-cold HCl to make 32mM stock solution. For carbonylation reaction, 100μM or 6.66 mg/ml HSA was incubated with 5mM 4-HNE in 200mM phosphate buffer at 37°C for 1 day.

2.2.2.5. Nitration

2mM working stock of peroxynitrite was prepared by diluting it in 1M NaOH. Further, 100μM HSA (i.e. 6.66 mg/ml) was incubated with 1mM peroxynitrite in 100mM phosphate buffer containing 20mM sodium bicarbonate and vortexed to allow nitration reaction.

2.2.3. MALDI mass spectrometry

Modification of albumin over time was monitored on a 4800 MALDI TOF/TOF^{TM} mass spectrometer (SCIEX, USA) in Linear mode using Sinapic acid as matrix. Spectra were processed using Data Explorer software.

2.2.4. Western blotting

5µg unmodified and modified HSA was separated on an 12% SDS-PAGE gel followed by semi-dry transfer of the proteins on to a PVDF membrane using a TE 70 PWR Semidry transfer unit (GE Healthcare, Amersham, UK). The membrane was then stained with Ponceau S solution (0.5% w/v in 1% v/v glacial acetic acid) to check equal transfer of protein from gel. Blocking of the membrane was done using 5% skimmed milk for 1h at 37°C, followed by incubation with primary antibody in blocking buffer at room temperature for 1h or at 4°C overnight on a platform rocker. Post incubation washes were given with phosphate buffered saline or PBS (150mM NaCl, 3mM KCl, 8mM Na_2HPO_4, 2mM KH_2PO_4) containing 0.1% Tween-20, followed by incubation with 1:5000 v/v dilution of secondary antibody in PBS for 1h at room temperature. Chemiluminescent detection was performed using Clarity^{TM} Western Chemiluminescent Substrate (Bio-Rad, USA) on a Syngene Dyversity Gel Documentation system.

2.2.5. LC-MS/MS Analysis

50µg control and modified HSA in 50mM ammonium bicarbonate were denatured at 80°C for 20 minutes followed by reduction with 150mM dithiothreitol at 60°C for 15 minutes and alkylation with 200mM iodoacetamide at ambient temperature in dark for 30 minutes. Further, 1µg trypsin (1:50 w/w ratio of trypsin to protein) was used for digestion at 37°C for 16h, after which digestion was quenched using formic acid. 1.5ug digest was loaded on to an Hypersil Gold C18 reverse phase HPLC column (150 X 2.1 mm, 1.9 mm, Thermo Scientific) of a Accela UPLC system connected to a Q-exactive Quadrapole-Orbitrap mass spectrometer from Thermo Fisher Scientific, USA. Peptides were separated over a 45 minute gradient of 3-4% mobile phase A (Mobile Phase A: 100% ACN, Mobile Phase B: 100% Water + 0.1% formic acid) at a flow rate of 350µl per minute. MS survey scan was performed over the mass range 350-1800 m/z with a resolution of 17,500. Fragmentation for MS/MS was done using high energy collision dissociation (HCD) for the top five peptides.

To identify modified peptides, raw data was searched against database restricted to Human serum albumin protein from Uniprot using SEQUEST HT algorithm in the Proteome Discoverer^{TM} software version 1.4 from Thermo Fisher Scientific by specifying full tryptic digestion and 2 missed cleavages. Carbamidomethylation at cysteine was specified as fixed modification while variable modifications searched for were Amadori (m/z 162.053), carboxymethyl or CML (m/z 58.005) and carboxyethyl or CEL (m/z 72.021) at lysine and arginine for glycated albumin, acetyl-lysine (m/z 42.048) for acetylated, homocysteine (m/z 133) and homocysteine thiolactone (m/z 117.024) at lysine for homocysteinylated, HNE (m/z 156.115) at histidine and nitro-tyrosine (m/z 44.985) for nitrated albumin respectively.

2.2.6. Fluorescence Spectrometry

Fluorescence scan of glycated and control albumin was carried out using 370nm excitation wavelength and emission over 380nm to 600nm wavelengths using a Varian Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, USA).

2.2.7. CD Spectrometry

The effect of modification of albumin secondary structure was studied using far UV-CD spectroscopy. Control and modified albumin were analyzed on JASCO J-815 CD spectrophotometer using 1mm spacers at 190-250nm wavelength range.

2.2.8. 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.

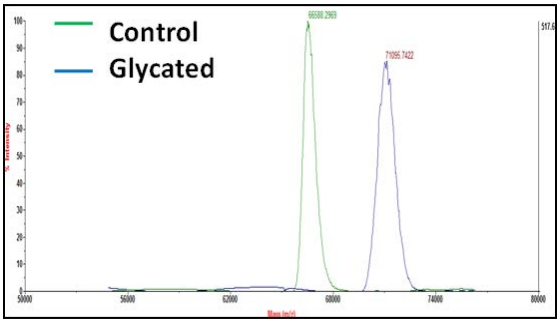
2.2.9. Cell viability assay

For viability assay, cells were grown in 24-well plates up to 80% confluence followed by 3h serum starvation and treatment with varying concentrations of unmodified and Gly-HSA. Viability was assessed after 24h using trypan blue dye exclusion assay by counting live and dead cells on a hemocytometer.

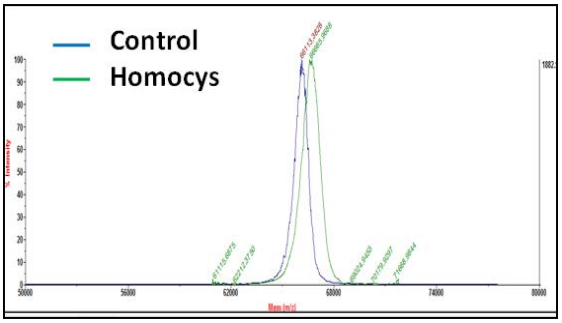
2.3. Results

2.3.1. MALDI

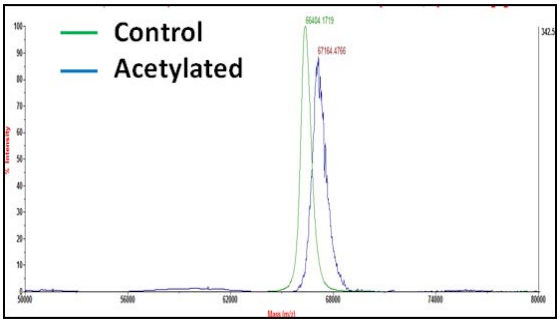
Modification of proteins by different modifying agents leads to formation of adducts that can be seen in MALDI spectrometry as an increase in molecular weight, denoted by Δm. For example, glycation of albumin at one lysine residue leads to an increase in molecular weight by 162Da. Similarly, the Δm for homocysteinylation is 117Da, acetylation is 42Da, carbonylation by HNE is 155Da and nitration is 46Da. Total increase in molecular weight of the protein observed corresponds to number of modifications and can be monitored over different concentrations of modifying agent and incubation times. An increase in molecular weight of serum albumin was observed with different modifying agents indicating increase in number of modifications or modified residues (Figure.2.1).



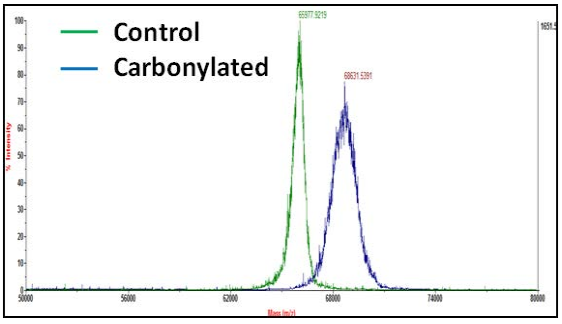
(a)



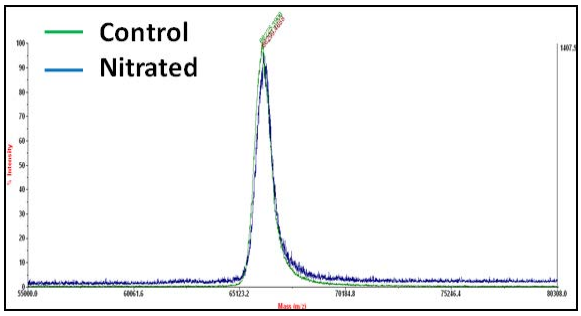
(b)



(c)



(d)



(e)

Figure 2. 1: MALDI spectra of modified albumin
Increase in molecular mass of albumin can be seen after modification as compared to unmodified control albumin by linear mode MALDI MS

2.3.2. Fluorescence Spectroscopy

Many crosslinking AGEs such as pentosidine and argpyrimidine are known to be fluorescent[2], [3]. Therefore, increase in fluorescence at λ_{ex} 370nm and λ_{em} 440nm was measured to confirm formation of AGEs[4]. Increased fluorescence shown by Gly-HSA in the fluorescence scan confirmed the formation of AGEs (Figure.2.2).

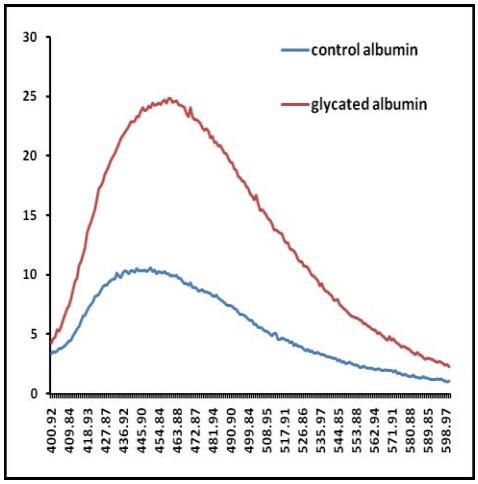


Figure 2. 2: Fluorescence scan spectrum of Con and Gly-HSA
Characteristic increase in fluorescence around 440nm is observed after glycation of HSA

2.3.3. Western Blot

Specific modifications were confirmed by western blot using modification specific antibodies. Since CML is one of the most prevalent AGEs, western blotting with anti-CML antibody validated the presence of AGEs (Figure.2.3a). Similarly, western blots with anti-acetyl lysine, anti-HNE and anti-nitrotyrosine antibodies validated the formation of respective modifications (Figure.2.3). Many modifications are reported to lead to the

formation of aggregates which can also be seen in the western blot as a higher molecular weight band in modified albumin.

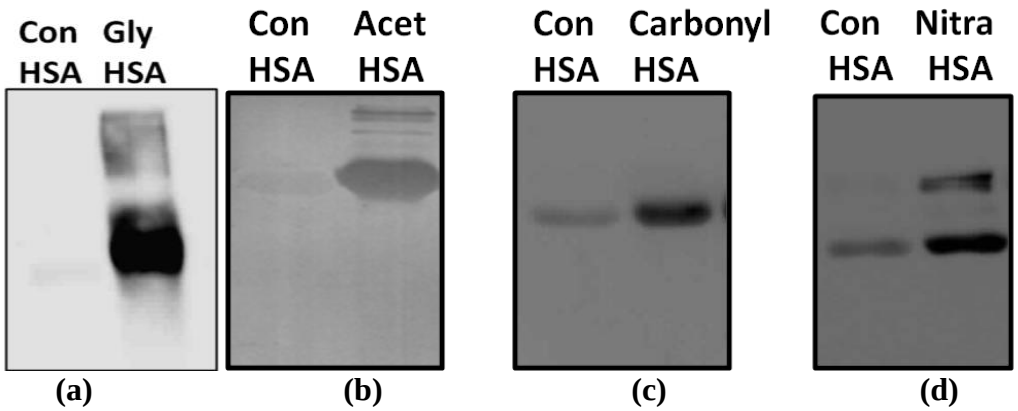


Figure 2. 3: Western blot for modified albumin
Western blots using modification specific antibodies (a) anti-CML (b) anti acetyl lysine (c) anti-HNE (d) anti-nitrotyrosine

2.3.4. Identification of modified sites by LC-MS/MS

Mass spectrometry is widely used to identify and characterize protein modifications. The characteristic mass shift or Δm observed in the MS/MS fragmentation spectra allows exact localization of the modification to the specific amino acid and therefore, the determination of extent of modification and number of modification sites. Modified peptides were identified from our *in vitro* modification reactions using high resolution accurate mass spectrometry and analyzed by filtering for high peptide identification confidence and minimum XCorr score of 1. Those peptides not showing good fragmentation were excluded. Modified peptides were also manually validated by checking for the presence of minimum 3 MS/MS fragments retaining the modification (Figure 2.4).

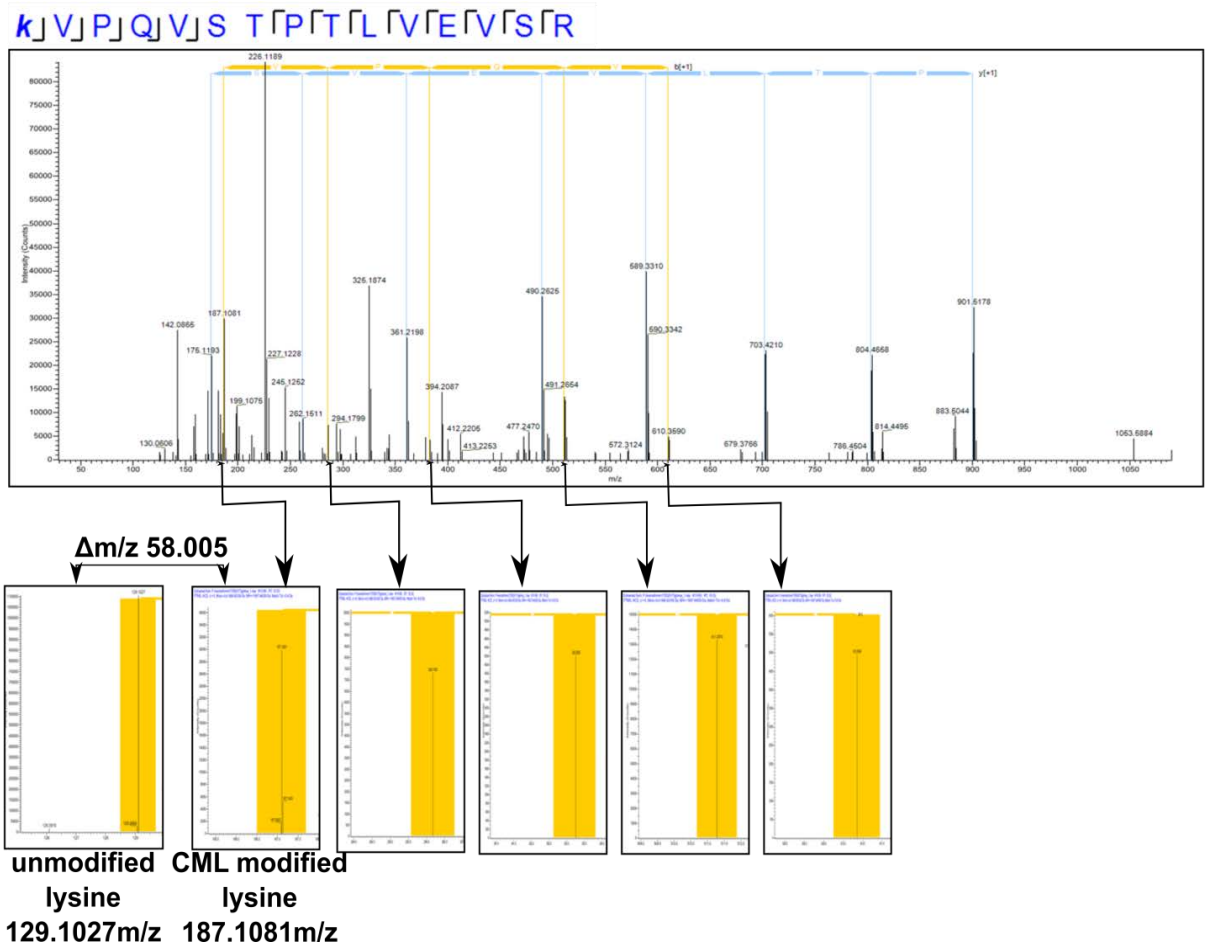


Figure 2. 4: MS/MS spectra for CML modified peptide of albumin
CML modified peptide of albumin shows modified lysine fragment at m/z of 129.1027 while unmodified lysine fragment is observed at m/z of 187.1081. The difference in the two m/z, or Δ m/z=58.005, corresponds to mass of CML adduct. Similar manual annotation was performed for at least 3 peptide fragments to confirm the presence of modification.

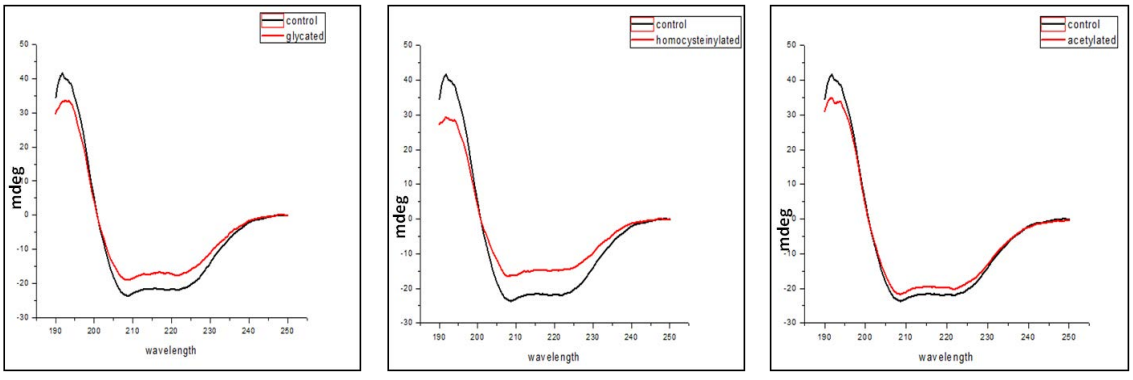
Using this criteria, 60 glycated peptides corresponding to 36 modifications on albumin were identified, since different peptides were detected for the same modified residue as well as same residue was detected with one or more of the glycation modifications. For example, the peptide **K***QTALVELVK was identified with all three glycation modifications (Appendix 1). In similar manner, 12 modified peptides for 12 acetylation sites, 3 modified peptides corresponding to 3 homocysteinylation sites, 14 modified peptides corresponding to 9 HNE carbonylation sites and 1 modified peptide for 1 modification site on nitrated albumin were identified (Table 2.1).

Table 2. 1 Modified residues identified for each modification

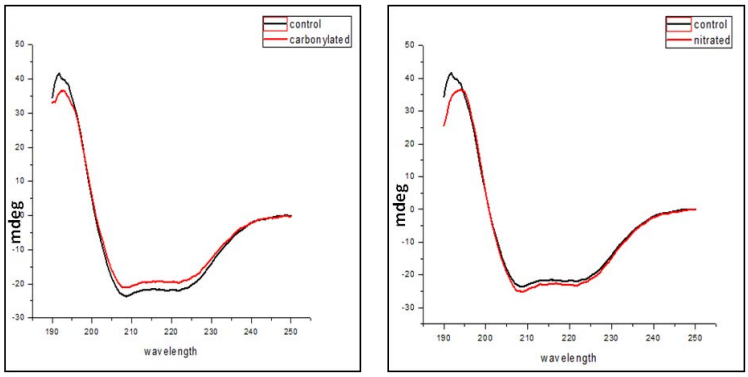
Sr. No	Modifications	# modified peptides	Modified Residues
1	Glycation	60	K12, K51, K64, K73, K93, K105, K137, K159, K162, K181, K205, K212, K225, K233, K240, K262, K323, K351, K359, K378, K402, K414, K466, K475, K519, K524, K525, K541, K545, K557, K564, K574, R18, R117, R337, R445
2	Homocysteinylation	3	K12, K351, K525
3	Acetylation	12	K93, K205, K212, K225, K262, K274, K281, K351, K359, K475, K541, K573
4	HNE carbonylation	14	H39, H67, H146, H242, H288, H338, H367, H464, H510
5	Nitration	1	Y138

2.3.5. CD Spectroscopy

CD spectroscopy is widely used to study the secondary structure or conformation of macromolecules. Particularly in case of proteins, it has been used to study the change in secondary structure due to environmental conditions or interaction with other molecules. Far UV CD spectra can be used to predict the percentages of each secondary structural element in the protein structure such as helix, sheet, turns and random coils[5]. The CD spectra of unmodified and modified albumin show the characteristic alpha helical structure which is slightly distorted after modification (Figure 2.5).



(a) **(b)** **(c)**

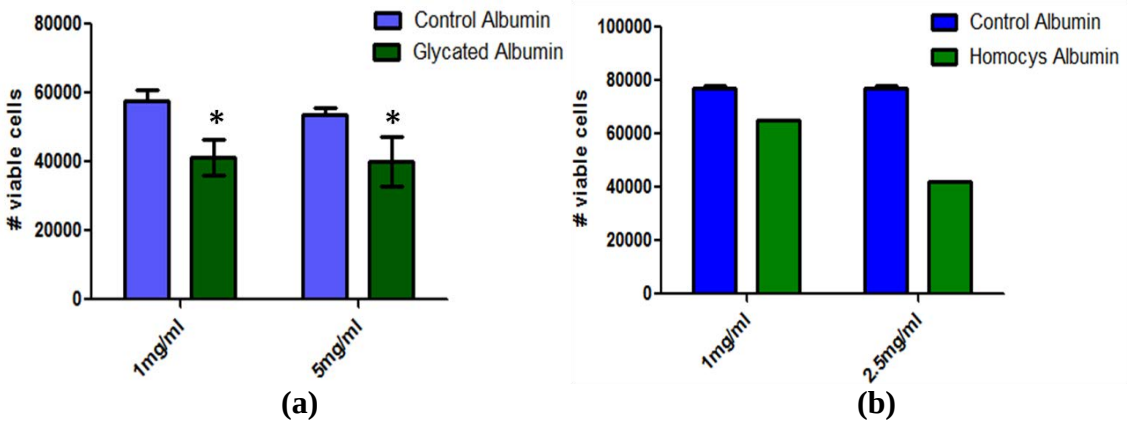


(d) (e)

Figure 2. 5: CD spectra of modified albumin
CD spectroscopy shows a distortion in the alpha helical structure of serum albumin after modification

2.3.6. Effect of Glycated Albumin on HUVECs: Viability assay

Trypan blue exclusion assay was performed after 24h treatment of HUVECs with different concentrations of modified HSA to study the effect on viability. Many of the modified proteins led to a reduction in endothelial cell viability at high concentrations (Figure 2.6).



(a) (b)

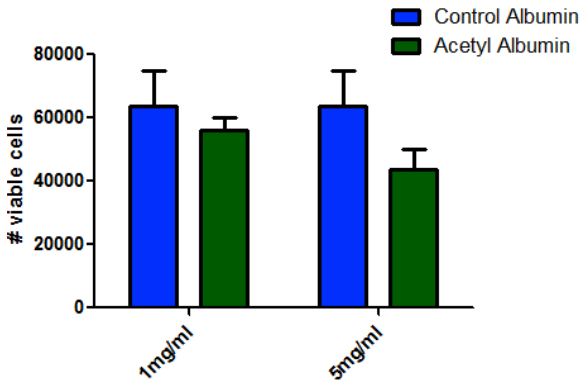


Figure 2. 6: Effect of modified albumin on viability of HUVEC
Trypan blue exclusion assay after 24h incubation with control unmodified and **(a)** glycated **(b)** homocysteinylated and **(c)** acetylated albumin; data represented as average number of viable cells ±SEM (* represents *p*-value <0.05)

2.4. Discussion

In this chapter, we repot the modification albumin *in vitro* with different physiologically relevant modifying agents. For few of the agents like peroxynitrite and HNE, the molar ratios of modifying agent to albumin used were higher as compared to their reported plasma concentrations. However, such concentrations are possibly achieved *in vivo* under conditions of high oxidative stress wherein these compounds are formed. For example, local concentrations of peroxynitrite adjacent to activated macrophages in culture is reported to approach a concentration of about 50nM which almost reduces to zero as distance from the cells increases[6]. Additionally, these compounds are less stable *in vitro*. For example, peroxynitrite is relatively stable under basic conditions but is protonated at physiological pH to form peroxynitrous acid, which decomposes with a half-life of less than 1s at 37°C[7]. Therefore, short incubation times for such reactions necessitated the use of higher concentrations to effect modification of albumin. MALDI mass spectrometry and spectroscopic techniques were used to determine optimum concentration and incubation time that lead to maximum modification. However, since many modifications also lead to protein aggregation and precipitation, the modifier concentration at which maximum extent of modification was observed without significant loss in solubility was considered as optimum and used for further studies.

Historically protein modifications have been identified and quantified by spectroscopic techniques which are not very specific and by immunochemical detection methods like ELISA. One of the earlier methods used for measurement of glycation was fructosamine assay which involves reduction of alkaline Nitro blue tetrazolium salts by fructosamines or ketoamines formed in the reaction followed by colorimetric detection[8]. However, this assay only measures the early products of glycation reaction and not the AGEs which are formed later via molecular rearrangements. Therefore, fructosamine assay does not provide an accurate measurement of the level of protein modification. Currently mass spectrometric techniques are being widely used for identification and quantification of protein modifications since they provide high specificity in identification of different modifications as well as high sensitivity for quantification. It is, however, dependant on a number of factors such as stability of the modification during analysis, peptide mass shift as a result of the modification as well as overall abundance of modified peptide[9]. Also, some PTMs affect the cleavage efficiency proteases like trypsin to generate large peptides and can also affect the ionization of the peptide. Therefore, all such factors need to be carefully addressed when using mass spectrometry for PTM analysis. We have used high resolution accurate mass spectrometry using Hybrid Quadrapole Orbitrap which has high sensitivity that allows identification of even low abundant modified peptides as well as high mass accuracy to identify the modification sites on human serum albumin.

Mass spectrometry has been previously used to identify glycation sites on serum albumin and a number of other plasma proteins[10, 11]. Earlier studies have used peptide mass fingerprinting (PMF) and ESI MS/MS[12] coupled with phenylboronic acid affinity chromatography to identify glycated peptides in albumin from *in vitro* glycation reactions as well as plasma samples of diabetic patients[13]. A recent study by Korwar *et al*. used different mass spectrometric approaches *viz*., parallel reaction monitoring or PRM, SWATH and MS^e, to identify glycated peptides from serum albumin. They could identify about 50 modified peptides containing amadori, CML and CEL modifications[14]. Our mass spectrometry analysis of *in vitro* glycated albumin for the same modifications yielded 60 modified peptides that correspond to 36 modification sites on albumin. In clinical plasma, however, lesser number of glycated peptides of albumin are reported to be identified, possibly since the incubation time for *in vitro* reactions is much longer than the plasma half life of albumin (90 days vs 21

days), as well as due to fact that plasma may contain a number of other factors that might interfere with the glycation reaction.

Similar mass spectrometric studies with *in vitro* homocysteinylated albumin have earlier reported 6 modified lysine residues, *viz*., K525 (K*QTALVELVK), K4 (DAHK*SEVAHR), K12 (FK*DLGEENFK), K137 (K*YLYEIAR), K159 (HPYFYAPELLFFAK*R), K205 (CASLQK*FGER) and K212 (AFK*AWAVAR)[15]. Of these, K525 has been reported to be stable and most predominant site of homocysteinylation[16], which was also observed in our analysis, although the total number of modifications that we could identify was lesser than those reported previously. Apart from K525, we could also identify the modification at K12, and at novel site K351 (LA**K***YETTLEK) which was not earlier reported to be homocysteinylated.

In vitro acetylation of plasma proteins by aspirin has been initially reported by monitoring the incorporation of radiolabelled aspirin into protein[17, 18 19]. More recently, HSA acetylated by aspirin has been characterized by MALDI MS/MS and QTRAP MS/MS mass spectrometry which led to the identification of 26 modified lysines[20]. We could identify 9 of these previously reported modified sites *viz*., K205 (**C**ASLQ**K*** FGER), K225 (FP**K*** AEFAEVSK), K541 (AT**K*** EQLK), K93 (ETYGEMAD**CC**A**K*** QEPER), K212 (AF**K*** AWAVAR), K281 (E**CC**E**K*** PLLEK), K351 (LA**K*** TYETTLEK), K262 (ADLA**K*** YI**C**ENQDSISSK) and K475 (VT**K*****CC**TESLVNR), in addition to three other sites, K274, K359 and K573 not reported earlier. *In vitro* carbonylation of albumin by HNE has been reported to lead to formation of Michael adducts at both histidine and lysine residues[21], however, lysine modifications have been observed only at higher HNE levels [22]. It is possible that we could not detect any lysine modifications in our analysis due to lesser concentration of HNE used. Out of the 9 histidine modifications that we could identify, 4 have been reported earlier, *viz*., H67 (SL**H*** TLFGDK), H510 (EFNAETFTF**H*** ADI**C**TLSEK), H242 (V**H*** TE**C*****C*** HGDLLE**C*** ADDR) and H367 (**CC**AAADP**H*** E**C**YAK) among which, H67, H510 and H242 have been reported to be most prone to modification by HNE[23]. The modified peptide **H*** PYFYAPELLFFAK was identified even in control unmodified albumin, therefore, was excluded from the analysis.

The nitro group of nitro-tyrosine reportedly gets fragmented in MALDI-MS leading to loss of one or both oxygen atoms as a result of which the Δm of 46Da is rarely observed[24]. In our MALDI analysis as well, we were unable to see a significant mass shift for nitrated albumin. There could be two possible reasons for this observation, one being the aforementioned fragmentation of nitro group in laser ionization. The other reason could be that extent of modification was low which led to a smaller mass shift in the peak of nitrated albumin and therefore, could not be resolved in linear mode of acquisition. Using HPLC and peptide sequencing, Jiao *et al*., have reported peroxynitrite mediated nitration to occur at Y411 and Y138 of albumin[25]. In our analysis also we could detect the peptide **Y***LYEIR with nitration at Y138. Modified **Y***TK with nitration at Y411, which although reported to be the most prone site for nitration, was not detected possibly due to its very small size.

Most of these chemical modifications tend to occur at surface exposed residues of albumin, that also form a part of its drug/fatty acid binding sites[23]. Such chemical modifications, therefore, also can affect the drug binding capacity of albumin[26]. Modifications also affect the surface charge distribution of albumin and thereby, its secondary structure and formation of partially unfolded or molten globule state of albumin is reported after homocysteinylation[27]as well as glycation modifications[28], although the process is slower for glucose mediated glycation[29], [30]. Our CD spectroscopic analysis also indicated a distortion in alpha helical structure of albumin after glycation and homocysteinylation, while other modifications also lead to a slight distortion in the CD spectrum. Such modified proteins are detected by pattern recognition receptors[31] that can mediate an inflammatory response in cells[32]. For few modifications like glycation, the effect on cell viability and function has been widely studied in endothelial cells[33] as well as other cells[34-36]. However, for other modifications very few studies on effect on cell viability and function have been reported even though their accumulation has been observed in a number of diseases[37], [38]. We have studied some of these modified proteins with respect to their effect on endothelial cells in the forthcoming chapters.

2.5. Conclusion

Human serum albumin was modified *in vitro* using a number of modifying agents and the extent of modification was characterized using mass spectrometry. Most agents modify

multiple residues on albumin and can potentially distort the secondary structure of albumin. They also affect viability of endothelial cells in culture. Since a number of these modified proteins are observed in clinical plasma and have been reported to be associated with the development of different diseases, it is important to further study their mechanism at molecular level.

2.6. Future Directions

Since accumulation of modified proteins have been associated with a number of conditions, it is important to understand the effect of these proteins on cell physiology as well as their molecular mechanism by identifying the receptors and signaling pathways involved. In context of vascular diseases, the effect of such modified proteins on endothelial functions and the possibility of inhibiting their effect to prevent endothelial dysfunction need to be explored.

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Chapter 3: Study of Endothelial Dysfunction in Response to Glycated Albumin via RAGE and its Involvement in Diabetic Vascular Complications

3.1. Background

Diabetes has currently become a global pandemic affecting about 8.5% of the world's population [1]. It is a metabolic disorder that is mainly characterized by increased blood glucose levels, either due to insufficient (Type I diabetes) or ineffective (Type II diabetes) insulin. 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 are one of the most predominant complications of diabetes and comprise atherosclerosis leading to myocardial infarction or stroke, and diabetic cardiac myopathy [3]. Diabetic patients have a greater risk of developing heart disease and resultant death as compared to non-diabetics, with more than 65% of Type 2 diabetic patients dying of cardiovascular disease [4]. Even with controlled blood glucose levels diabetics have a higher risk of cardiovascular diseases which indicates there are other underlying factors that contribute towards their development [5]. 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 has been associated with the development of diabetic cardiovascular complications [6-9]. Even other complications of diabetes like nephropathy and retinopathy involve abnormal functioning of vascular endothelial cells. 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, including increased protein glycation [11]. The elevated levels of glucose and glucose derived intermediates in blood in diabetes leads to increase in plasma protein glycation [12 13]. Protein glycation has been widely recognized as important in diabetes and in the development of various diabetic complications. It is reported that diabetic patients with

CVD have even higher levels of plasma AGEs as compared to other diabetic patients [14, 15]; therefore, it is possible that plasma AGEs might play a role in development of cardiovascular complications in such patients.

Interaction of AGEs with their receptor RAGE can negatively affect endothelial cell physiology in diabetes predisposing to cardiovascular disease [16, 17] and this has been shown in clinical studies wherein diabetics show higher RAGE expression [18] and AGE localization near atherosclerotic plaques [19]. Animal models of diabetic atherosclerosis have also shown improved regression of atherosclerotic plaques after RAGE knockout [20]. 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 this chapter, we report the effect of glycated proteins on endothelial cell function. We have looked at effect of glycated HSA on apoptosis, oxidative stress in HUVECs as well as we have performed total cell differential proteomics using SWATH MS platform to look at the global changes in proteomic level.

3.2. Materials & Methods

3.2.1. Reagents

All chemicals were procured from Merck (formerly Sigma-Aldrich) unless otherwise stated. Antibodies used were anti-p65 Nf-κB (Santacruz) and anti NRF-1 (Abcam) and secondary antibodies tagged to Alexafluor were from Merck (formerly Sigma-Aldrich). 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.

3.2.2. Reactive Oxygen Species detection

Serum starved HUVECs were treated with 3mg/ml Con-HSA and Gly-HSA for 24h. After treatment, cells were washed with PBS and incubated with 5μM 2',7'-dichlorofluorescein diacetate (DCFH-DA) in phenol red free medium for 15 minutes 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 Accurie C6 flow cytometer (BD Biosciences, USA) using 488nm excitation laser and detection on green channel (530/30 nm). Fluorescence images were also acquired on a FLoid Cell Imaging Station (Thermo Fisher Scientific, USA) for qualitative assessment of fluorescent cells.

3.2.3. Apoptosis assay

HUVECs were seeded on 6-well plates and allowed to grow up to 80% confluence followed by 3h serum starvation. Cells were then treated with 3mg/ml Con-HSA and Gly-HSA and harvested using trypsin after 24h. 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 1X Annexin Binding Buffer and incubated with Annexin V-FITC and Propidium iodide followed by flow cytometry acquisition on BD Accuri C6 Cytometer using 488nm 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.

3.2.4. Total cell protein extraction

HUVECs treated with 3mg/ml Con-HSA and Gly-HSA for 24h were harvested and total cell proteins were extracted using mass spectrometry compatible detergent RapiGest^{TM} SF (Waters Corporation, USA). RapiGest^{TM}, at a final concentration of 2% in 50mM Ammonium bicarbonate, with Protease Inhibitor cocktail (P8340 Sigma-Aldrich) was added to the cells followed by incubation on ice for 30 minutes with intermittent vortexing. Cell debris and protein aggregates were removed by centrifugation at 17,000 rpm at 4°C for 1h and the supernatant was collected. Bradford's assay was used for protein quantification.

3.2.5. SWATH based Proteomics

100µg of extracted protein was subjected to proteolytic digestion by trypsin and the digested peptides were desalted using ZipTip$^{®}$ pipette tips (Merck Millipore, USA). 3.5µg digest was

injected onto a C18 reverse phase column (dimensions: 100×0.3mm, 3µm, 120 Å) of a microLC 200 liquid chromatography system (Eksigent Technologies, USA) coupled to a Triple TOF 5600 mass spectrometer (SCIEX, USA). Peptides were separated over a 95 minute gradient of 3- 40% acetonitrile in water with 0.1% formic acid at a flow rate of 8µL/min.

For label free quantification using SWATH, spectral library was first generated by acquiring all samples in Information Dependant Acquisition (IDA) mode over a peptide mass range of 400-1250 m/z. Accumulation time for MS was 250ms while that for MS/MS was 70ms. For SWATH MS acquisitions, the peptide mass range was split into 34 overlapping windows of 25Da each. Peptide fragmentation was performed using rolling collision energy and MS/MS was performed over a mass range of 100-2000 m/z. Six replicate runs were acquired for each of the three biological replicates of each treatment. The IDA data was searched against UniProt *Homo sapiens* database (release 2017_10; 20,231 reviewed entries) using ProteinPilot™ version 5.0 software. The result generated was used in PeakView v2.2 software as spectral library and SWATH runs were processed using 50 ppm error, 4min retention time window, 99% confidence and 1% False Discovery Rate (FDR). The processed data was further exported to MarkerView™ v1.2.1 for quantitative and statistical analysis. The data across the runs was normalized using total area sum. The raw mass spectrometric data is deposited at the public repository PeptideAtlas (PASS01138).

3.2.6. Bioinfomatic analysis

Gene ontology enrichment analysis for the proteins showing altered abundance was performed using the online bioinformatics tool DAVID [21]. Protein-protein interaction (PPI) network construction and clustering was performed using Cytoscape 3.2, an open source network visualization software [22]. Transcription factor prediction was performed using iRegulon plug-in of Cytoscape [23].

3.2.7. Immunofluorescence

Cells were seeded on coverslips, serum starved and treated with Con-HSA and Gly-HSA. After treatment, cells were fixed with 4% paraformaldehyde for 20 minutes and permeabilized with 0.2% Triton X100 for 30 minutes. Fixed and permeabilized cells were then washed with PBS thrice and incubated with anti-p65 Nf-κB or anti- NRF-1 antibody in

2% Normal Horse Serum (NHS) for 45 minutes. After further washes, secondary anti-rabbit antibody tagged to Alexafluor (1:1000 dilution in NHS) along with phalloidin (1:500 dilution in NHS) and DAPI was added to the coverslips and incubated for 45 minutes. Coverslips were then washed and mounted on glass slides using mounting medium. Cells were visualized under Axiovert 200M inverted fluorescence microscope (Zeiss, Germany) under blue, red and green channels. 10ng/ml TNF-α was used as positive control for Nf-κB translocation.

3.2.8. Quantitative PCR

RNA was extracted from cells after 5h of treatment with 3mg/ml Con-HSA and Gly-HSA using PureLink® RNA mini kit. 2ug RNA was given DNase treatment (RiboPure™ RNA purification kit) and further used for cDNA synthesis using Applied Biosystems™ High Capacity cDNA Reverse Transcription kit. cDNA synthesis was checked by PCR amplification using DHR gene specific primers. Quantitative PCR was performed using Taqman probes procured from Thermo Scientific on an AriaMx Real time PCR system (Agilent Technologies, USA). β-actin was used as the reference gene to calculate relative expression levels of genes of interest.

3.2.9. Statistical Analysis

All experiments were performed in triplicates. Statistical significance was calculated using student's t-test and p-value<0.05 was considered as statistically significant. Data is expressed as mean ± S.E.M.

3.3. Results

3.3.1. ROS assay

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 2',7'-dichlorofluorescein (DCF) that can be detected by flow cytometry. An increased ROS production in cells was detected after Gly-HSA treatment as seen by the increase in their mean fluorescence intensity in flow cytometry (Figure 3.1b). Fluorescence microscopic analysis also showed higher number of cells showing DCF fluorescence after Gly-HSA treatment as compared to Con-HSA (Figure 3.1c).

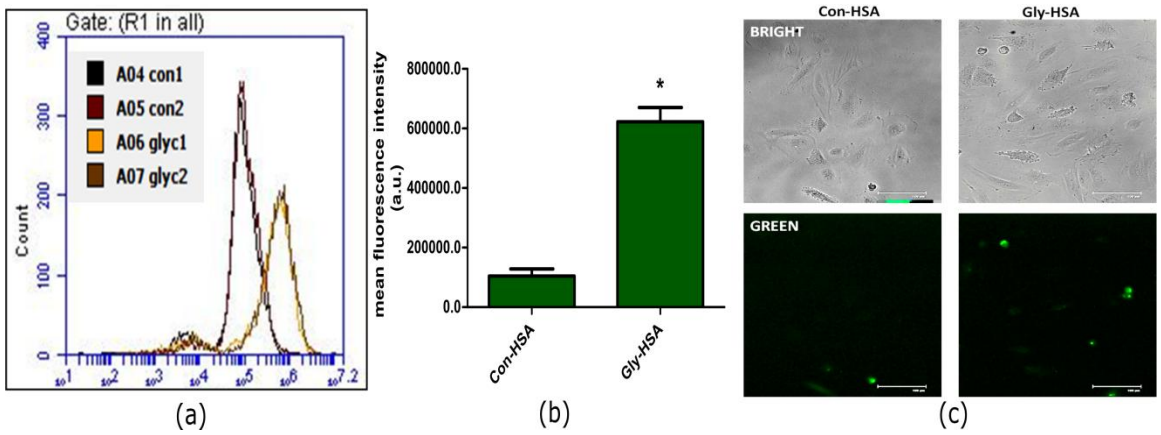


Figure 3.1: ROS assay after Gly HSA treatment
(a) histogram shows increase in DCF fluorescence in cells treated with Gly-HSA **(b)** bar graph showing increase in the mean fluorescence intensity of Gly-HSA treated cells **(c)** fluorescence microscopy shows more number of cells with green DCF fluorescence on Gly-HSA treatment. (* indicates *p*-value<0.05)

3.3.2. 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 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. After 24h treatment with Gly-HSA, there was an increase in the number of both early and late apoptotic cells as seen by flow cytometry (Figure 3.2). Since oxidative stress and apoptosis are one of the initial signs of endothelial dysfunction, above observations demonstrate that glycated HSA treatment is leading to dysfunction in HUVECs.

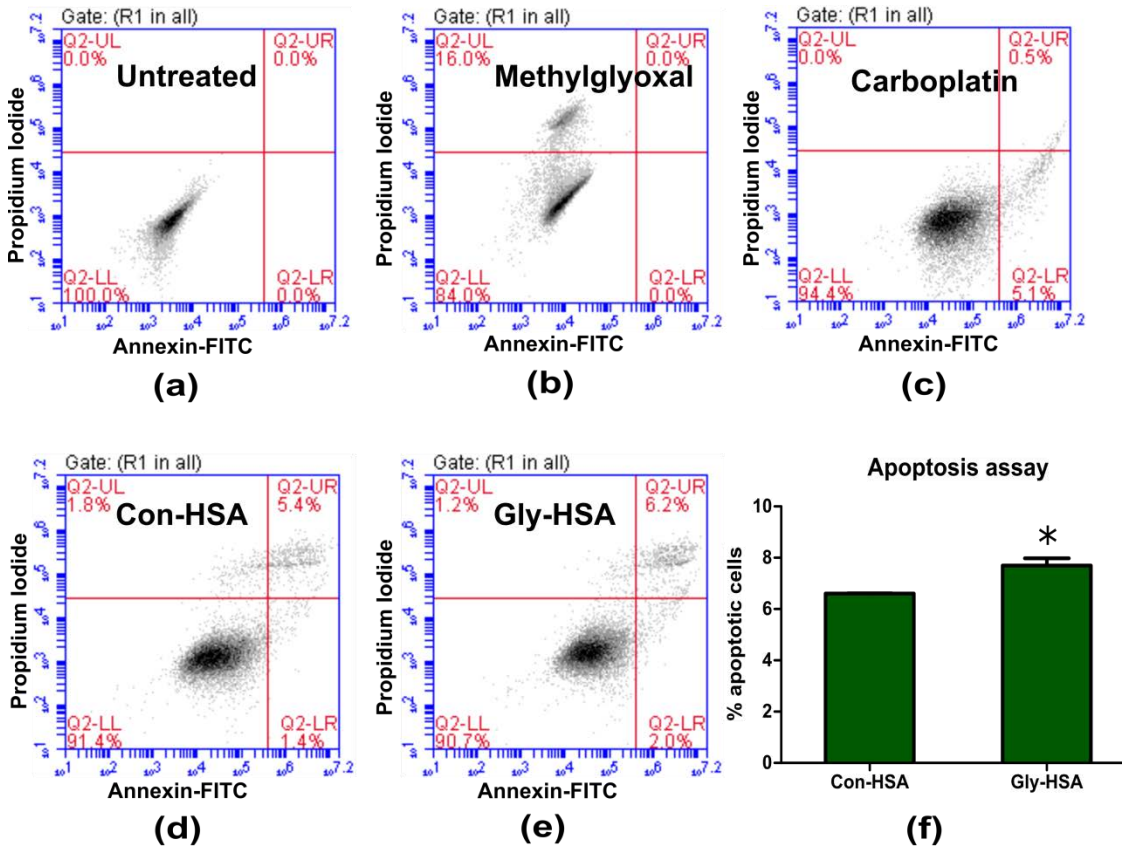


Figure 3.2: Apoptosis assay by flow cytometry
Scatter plots show the number of cells in the early apoptotic phase (lower right quadrant: LR) and late apoptotic (upper right quadrant: UR) phase for (a) no treatment (b) methylgyloxal (c) carboplatin (d) control HSA (e) glycated HSA; bar graph (f) shows increase in the percentage of apoptotic cells; data expressed as percentage of total number of cells ± S.E.M (* indicates p-value<0.05)

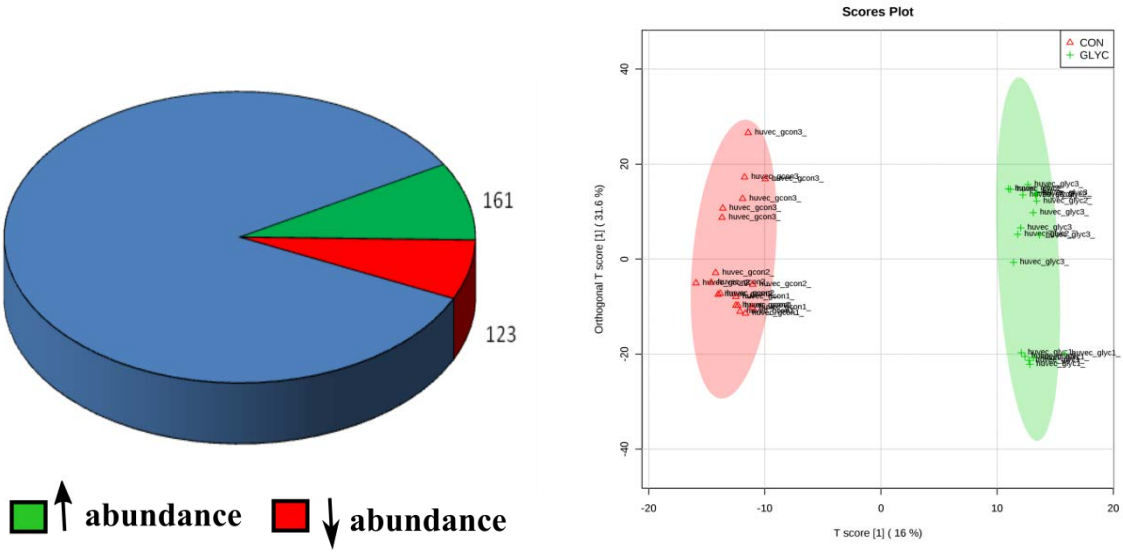
3.3.3. Glycated HSA Induces Expression of Proteins Involved in Endothelial Dysfunction

3.3.3.1.SWATH proteomics

SWATH or Sequential Window Acquisition of all Theoretical masses is an untargeted label free quantitative technique wherein the MS acquisition mass range is split into windows of 25 Da each and all the ions which fall into the same window are fragmented simultaneously in data independent manner (DIA or MS/MS^{ALL}). Quantification is then based on peak areas of extracted ion chromatograms (XIC) of peptide fragments. It requires development of a spectral library beforehand by data dependent (DDA) or information dependent acquisition

(IDA) of MS/MS spectra, which is then used to match the fragment ions or transitions observed in DIA to their respective peptides/proteins and perform quantification [24].

IDA led to identification of 1860 proteins by ProteinPilot™ with at least 2 peptides. These proteins were further used as spectral library for quantification by SWATH. 161 proteins showed more than 1.3 fold higher abundance while 123 proteins showed lower abundance with p-value<0.05 after treatment with Gly-HSA (Figure 3.3a). All replicate runs of one treatment cluster together in the oPLSDA plot constructed using MetaboAnalyst web tool [25], which indicates reproducibility of the SWATH runs (Figure 3.3b).



(a) **(b)**

Figure 3.3: SWATH proteomics of Con and Gly HSA treated cells
(a) Pie chart shows that out of the 1860 total proteins identified in IDA, 161showed higher abundance and 123 showed reduced abundance in Gly-HSA treated cells as compared to Con-HSA treated ones by SWATH **(b)** oPLSDA plot of all replicate runs shows reproducibility between replicates of each treatment

3.3.3.2.Gene Ontology

Gene ontology analysis using DAVID showed that a number of proteins with altered abundance are involved in important endothelial functions such as cell adhesion, leukocyte migration, angiogenesis and blood coagulation (Figure 3.4).

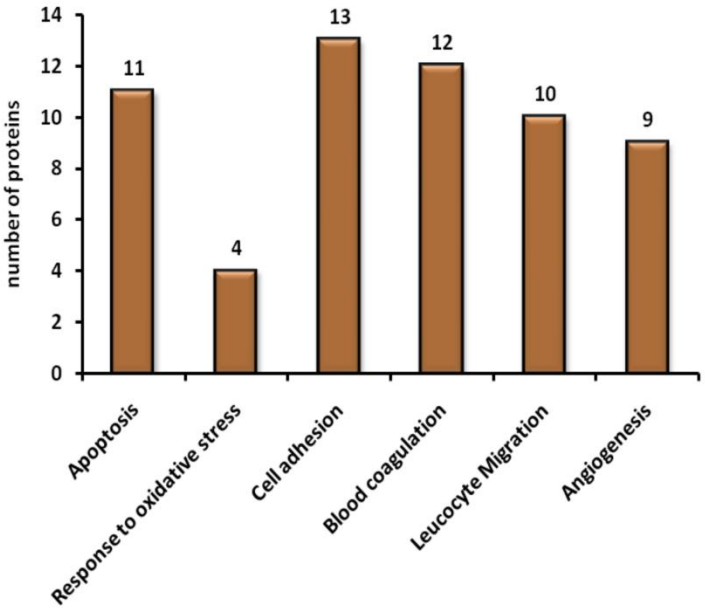


Figure 3.4: GO analysis of differentially abundant proteins
A number of important endothelial functions are seen to be enriched by gene ontology analysis

Some examples include proteins associated with cell adhesion like ICAM1, with blood coagulation like vWF and plasminogen activator inhibitor (PAI-1), and with angiogenesis like caveolin-1 and MMP-14. ICAM-1 interacts with integrins to facilitate leukocyte endothelial transmigration and increased ICAM-1 expression is associated with atherosclerosis and coronary heart disease [26]. PAI-1 is a serine protease inhibitor serpin E1 that inhibits fibrinolysis by blocking conversion of plasminogen to plasmin by plasminogen activators [27], and increased levels of PAI-1 have been associated with increased risk of atherosclerosis [28]. The list of proteins involved in specific endothelial functions is given in Table 3.1.

Table 3. 1 Differentially expressed proteins involved in endothelial function after Gly-HSA treatment

Sr. No.	Accession no.	Protein name	Gene Symbol	p-value	Fold Change
Apoptosis					
1	P05556	Integrin beta-1	ITGB1	2.70E-03	1.82
2	P10809	60 kDa heat shock protein, mitochondrial	HSPD1	9.83E-08	0.59
3	P00441	Superoxide dismutase [Cu-Zn]	SOD1	1.14E-02	2.40
4	Q07021	Complement component 1 Q subcomponent-	C1QBP	8.51E-03	0.46

Sr. No.	Accession no.	Protein name	Gene Symbol	p-value	Fold Change
		binding protein, mitochondrial			
5	P00338	L-lactate dehydrogenase A chain	LDHA	1.00E-04	1.72
6	O00429	Dynamin-1-like protein	DNM1L	2.19E-02	1.66
7	Q13501	Sequestosome-1	SQSTM1	3.67E-03	4.23
8	Q16678	Cytochrome P450 1B1	CYP1B1	3.50E-02	2.73
9	P62753	40S ribosomal protein S6	RPS6	1.69E-03	1.72
10	P35222	Catenin beta-1	CTNNB1	3.20E-04	2.24
11	P63000	Ras-related C3 botulinum toxin substrate 1	RAC1	1.91E-03	1.97
Response to reactive oxygen species					
1	P99999	Cytochrome c	CYCS	8.15E-03	0.50
2	P00441	Superoxide dismutase [Cu-Zn]	SOD1	1.14E-02	2.40
3	P04179	Superoxide dismutase [Mn], mitochondrial	SOD2	3.65E-09	4.72
4	P07237	Protein disulfide-isomerase	P4HB	4.64E-03	0.39
Cell adhesion					
1	P17813	Endoglin	ENG	2.31E-07	0.42
2	P05362	Intercellular adhesion molecule 1	ICAM1	5.77E-05	20.24
3	O00592	Podocalyxin	PODXL	4.09E-03	3.27
4	P35222	Catenin beta-1	CTNNB1	3.20E-04	2.24
5	P63000	Ras-related C3 botulinum toxin substrate 1	RAC1	1.91E-03	1.97
6	P13598	Intercellular adhesion molecule 2	ICAM2	7.28E-05	0.33
7	P06756	Integrin alpha-V	ITGAV	1.20E-03	3.25
8	P17301	Integrin alpha-2	ITGA2	3.56E-02	2.67
9	P04275	von Willebrand factor	VWF	2.85E-07	0.48
10	Q16678	Cytochrome P450 1B1	CYP1B1	3.50E-02	2.73
11	P16070	CD44 antigen	CD44	3.68E-02	3.20
12	P07996	Thrombospondin-1	THBS1	8.50E-04	0.35
13	Q13201	Multimerin-1	MMRN1	1.74E-07	0.45
Coagulation					
1	Q13547	Histone deacetylase 1	HDAC1	1.82E-02	2.38
2	P13987	CD59 glycoprotein	CD59	5.34E-06	2.72
3	P60953	Cell division control protein 42 homolog	CDC42	1.69E-03	1.87
4	P47755	F-actin-capping protein subunit alpha-2	CAPZA2	1.06E-02	1.55
5	P17301	Integrin alpha-2	ITGA2	3.56E-02	2.67
6	P49257	Protein ERGIC-53	LMAN1	1.35E-05	2.92
7	O95340	Bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthase 2	PAPSS2	2.88E-02	3.92
8	P50148	Guanine nucleotide-binding protein G(q) subunit alpha	GNAQ	1.33E-03	0.29
9	P04275	von Willebrand factor	VWF	2.85E-07	0.48
10	Q13201	Multimerin-1	MMRN1	1.74E-07	0.45
11	P63000	Ras-related C3 botulinum toxin substrate 1	RAC1	1.91E-03	1.97
12	P10644	cAMP-dependent protein kinase type I-alpha	PRKAR1A	5.68E-03	0.31

Sr. No.	Accession no.	Protein name	Gene Symbol	p-value	Fold Change
		regulatory subunit			
Leukocyte migration					
1	P62937	Peptidyl-prolyl cis-trans isomerase A	PPIA	2.89E-03	0.50
2	P06756	Integrin alpha-V	ITGAV	1.20E-03	3.25
3	P05556	Integrin beta-1	ITGB1	2.70E-03	1.82
4	Q92896	Golgi apparatus protein 1	GLG1	1.16E-03	2.55
5	P05362	Intercellular adhesion molecule 1	ICAM1	5.77E-05	20.24
6	P35613	Basigin	BSG	8.33E-05	2.26
7	O00592	Podocalyxin	PODXL	4.09E-03	3.27
8	P14174	Macrophage migration inhibitory factor	MIF	7.11E-06	2.81
9	P16070	CD44 antigen	CD44	3.68E-02	3.20
10	Q03135	Caveolin-1	CAV1	1.42E-02	3.35
Angiogenesis					
1	P06576	ATP synthase subunit beta, mitochondrial	ATP5B	3.89E-05	0.25
2	P06756	Integrin alpha-V	ITGAV	1.20E-03	3.25
3	Q9Y696	Chloride intracellular channel protein 4	CLIC4	1.05E-03	3.99
4	P05121	Plasminogen activator inhibitor 1	SERPINE1	5.46E-05	3.40
5	Q16678	Cytochrome P450 1B1	CYP1B1	3.50E-02	2.73
6	P06744	Glucose-6-phosphate isomerase	GPI	2.13E-03	1.70
7	P50281	Matrix metalloproteinase-14	MMP14	1.83E-02	4.56
8	P98160	Basement membrane-specific heparan sulfate proteoglycan core protein	HSPG2	2.80E-04	2.52
9	Q03135	Caveolin-1	CAV1	1.42E-02	3.35

3.3.3.3.Protein-protein Interaction Study

Understanding protein-protein interactions is essential to understand cell physiology and, interaction networks are mathematical representations of such interactions. Mutually interacting proteins and those involved in similar function and pathway tend to be densely connected to each other in an interaction map and form close clusters. Protein protein interactions of the differentially abundant proteins observed in the SWATH data were mapped to visualize relationships between them. Differentially abundant proteins involved in endothelial functions such as coagulation, cell adhesion and leukocyte migration are seen as clusters indicating that these functions are affected on treatment with Gly-HSA (Figure 3.5).

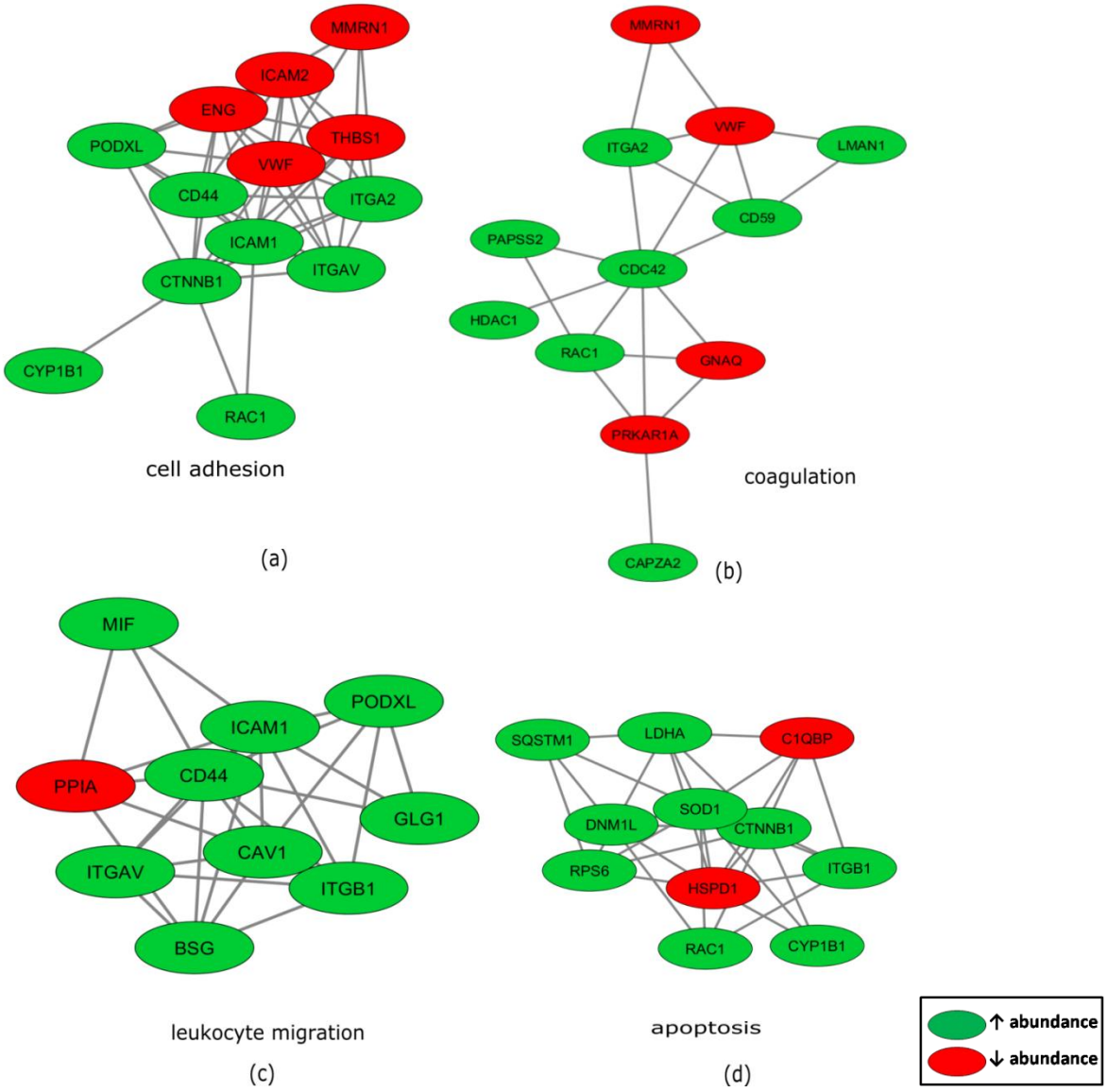


Figure 3.5: PPI network clusters of differentially abundant proteins
Clustering identified proteins involved in specific endothelial functions having differential abundance; green indicates >1.3fold higher abundance and red indicates >1.3fold lesser abundance in Gly-HSA treated cells

3.3.3.4.Transcription Factor Prediction

iRegulon application in cytoscape identifies transcription factors associated with the proteins by analyzing their binding motifs in the gene promoter regions. Analysis of the proteins showing differential abundance in our study shows that many of them are regulated by RelA or Nf-κB, which is known to operate downstream of RAGE (Figure 3.6). This indicates the

involvement of RAGE in inducing endothelial dysfunction [29]. Another key transcription factor observed to be involved is NRF1 which regulates many mitochondrial genes.

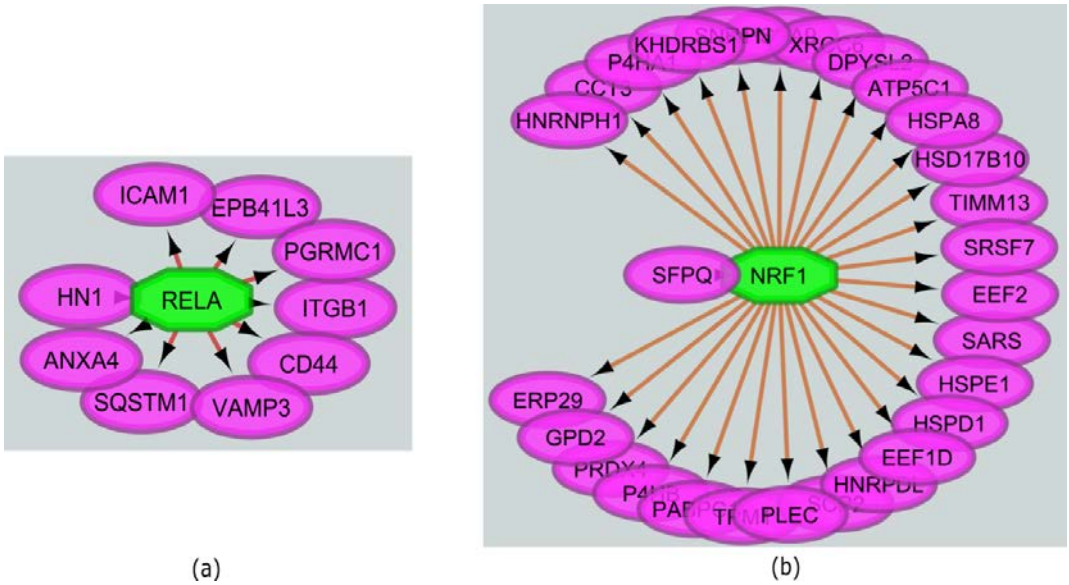
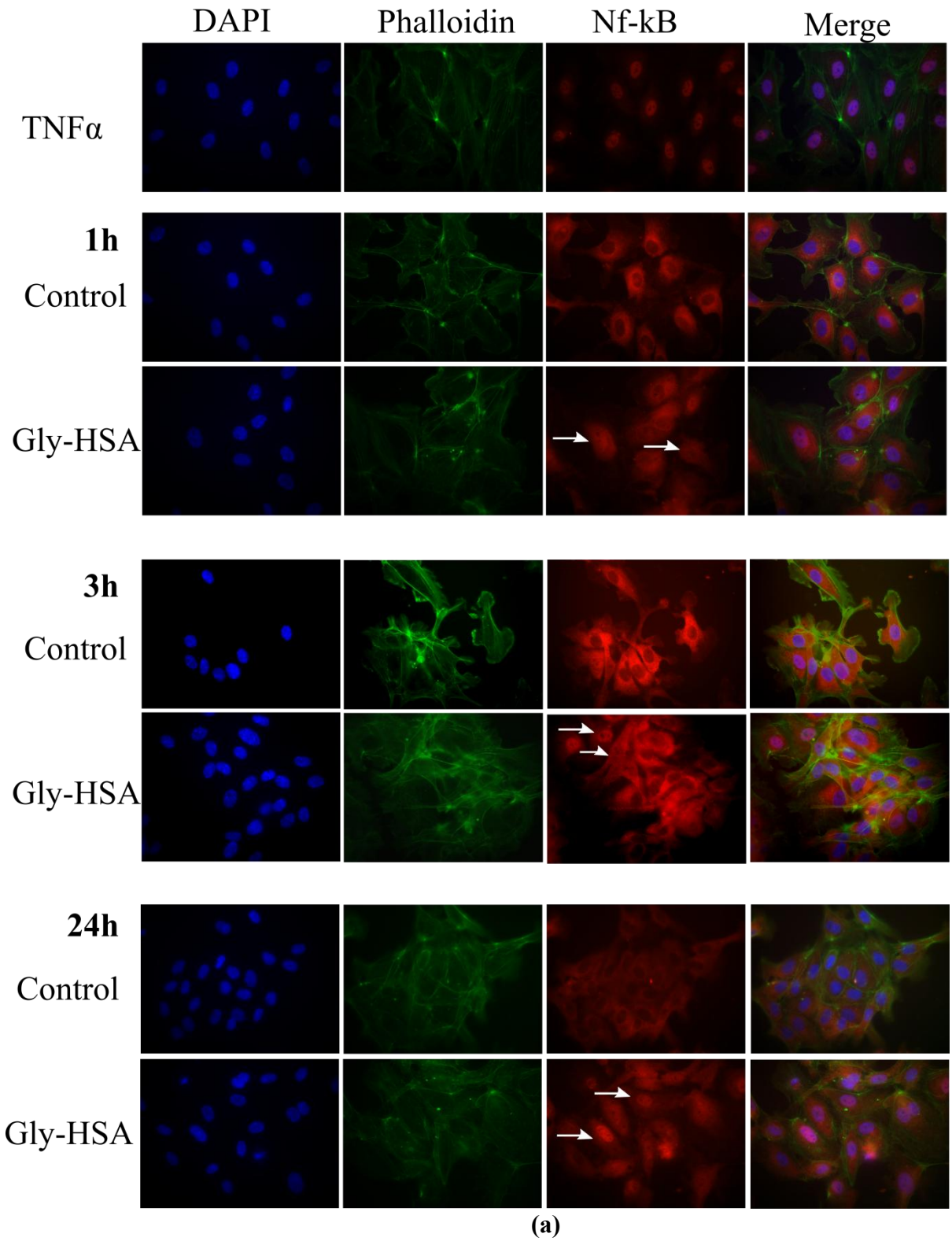


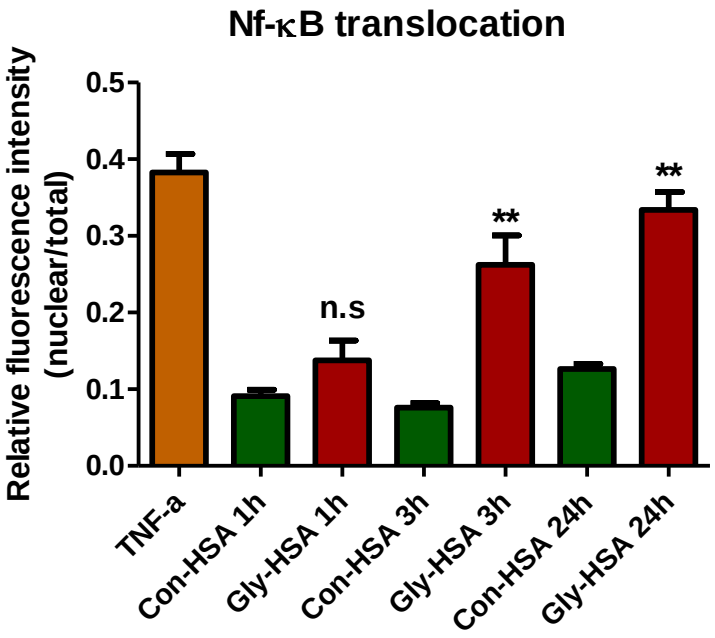
Figure 3.6: Transcription factor prediction by iRegulon
(a) RelA or Nf-κB and (b) Nrf-1 are seen to be important regulators of many of the differentially abundant proteins

3.3.3.5.Validation of Transcription Factors by Nuclear Translocation Assays

Nuclear factor kappa B is a transcription factor widely implicated in responses to a number of stimuli such as infections, inflammatory cytokines and oxidative stress. It is normally present as an inactive complex with its inhibitor IκB (Inhibitor of Nf-κB) in the cytoplasm. Phosphorylation of IκB in response to activating stimuli leads to release of Nf-κB dimers from the complex and their translocation to the nucleus where they activate transcription of target genes [30]. Visualization of translocation Nf-κB subunits to the nucleus can thus serve as an indicator of its activation. Activation of Nf-κB in response to Gly-HSA treatment was confirmed by checking the nuclear translocation of its subunit p65 by immunofluorescence. Treatment with glycated albumin for different time intervals showed translocation of p65 to nucleus and the extent of Nf-κB translocation was compared to TNF-α, which was used as positive control (Figure 3.7a).


DAPI
Phalloidin
Nf-kB
Merge
TNFα
1h
Control
Gly-HSA
3h
Control
Gly-HSA
24h
Control
Gly-HSA

(a)

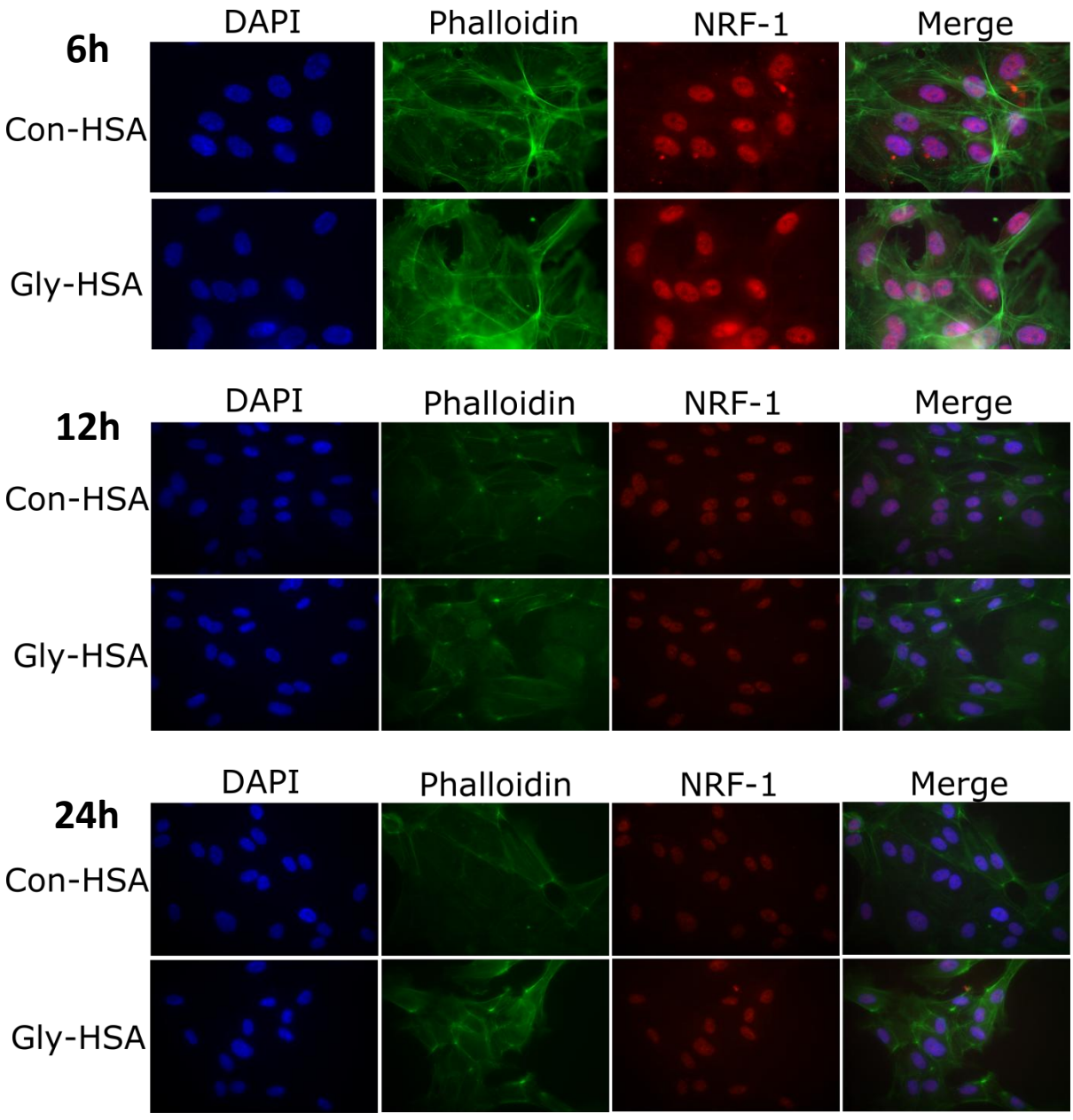


(b)

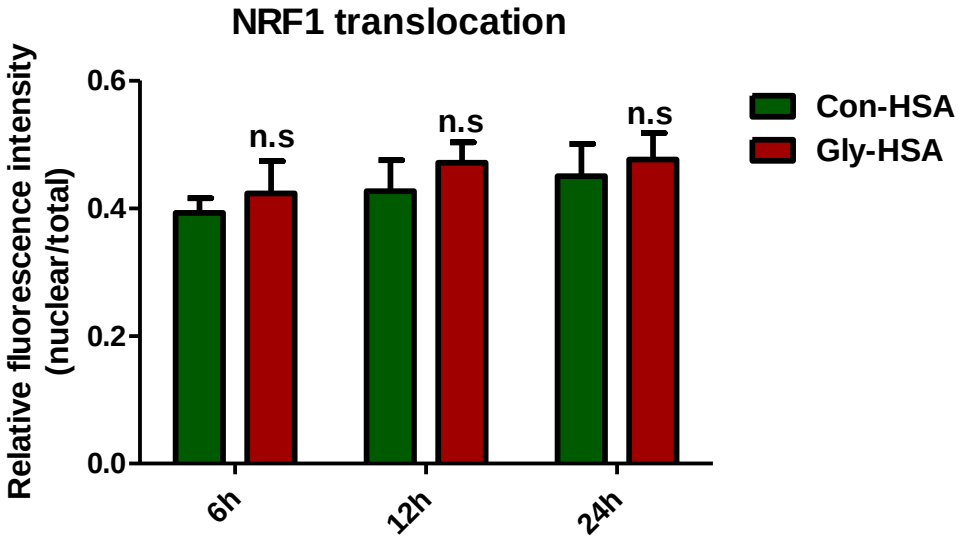
Figure 3.7: Immunofluorescence for Nuclear Translocation of Nf-κB
(a) Images of HUVECs treated with Gly-HSA and TNF-α and stained using anti p65 Nf-κB antibody and anti-rabbit secondary antibody tagged to Alexafluor 594 (red), phalloidin tagged to Alexafluor 488 (green) and DAPI (blue) show nuclear translocation of Nf-κB after treatment **(b)** Bar graph quantifying translocation also shows increase in nuclear localization of Nf- κB after Gly-HSA treatment; data expressed as ratio of nuclear fluorescence to that of total cell ± S.E.M (** indicates *p*-value<0.01)

Nuclear Respiratory Factor (NRF1) is a transcription factor involved in the expression of respiratory subunits and mitochondrial transcription factors along. Although a number of proteins regulated by NRF-1 are seen to be downregulated in SWATH data, no difference in its translocation was observed after treatment with control and glycated albumin (Figure 3.7b). Our prediction of NRF1 to be one of the possible regulatory factors involved based on the analysis of the upstream sequences of the genes and any promoter binding motifs present therein using iRegulon application of Cytoscape. However, transcription of genes is usually under control of multiple transcription factors and down-regulation or reduction in nuclear localization of one of the transcription factors, in this case NRF1, may not be the only factor affecting the gene expression. It is possible that there are other

regulatory factors involved downstream of AGE-RAGE and therefore, we are unable to observe any change in the nuclear localization of NRF1.



(a)



(b)

Figure 3.8: Immunofluorescence for Nuclear Translocation of NRF1
(a) Images of HUVECs treated with Gly-HSA and stained using anti NRF1 antibody and anti-rabbit secondary antibody tagged to Alexafluor 594 (red), phalloidin tagged to Alexafluor 488 (green) and DAPI (blue) show similar nuclear translocation of NRF1 with and without treatment **(b)** Bar graph quantifying translocation also shows no change in nuclear localization of NRF1 after Gly-HSA treatment; data expressed as ratio of nuclear fluorescence to that of total cell ± S.E.M

3.3.4. Quantitative real time PCR

3.3.4.1. RNA extraction

Total RNA extracted from HUVECs show the 28s and 18s rRNA bands on 1% agarose gel electrophoresis (Figure 3.8). Genomic DNA is also seen as a faint band, which was later removed by DNase treatment.

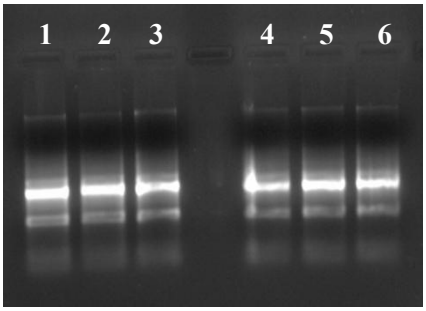


Figure 3.9: RNA extraction from Con and Gly HSA treated cells
Agarose gel electrophoresis of total RNA isolated from HUVEC treated with Con and Gly-HSA for 6h; **Lanes 1-3**: RNA from Con-HSA treated cells, **Lanes 4-6**: RNA from Gly-HSA treated cells

3.3.4.2.cDNA Synthesis and quality check

The cDNA obtained by reverse transcription PCR was checked by PCR amplification using DHR forward and reverse primers resulting in an amplicon of about 400bp as expected (Figure 3.9).

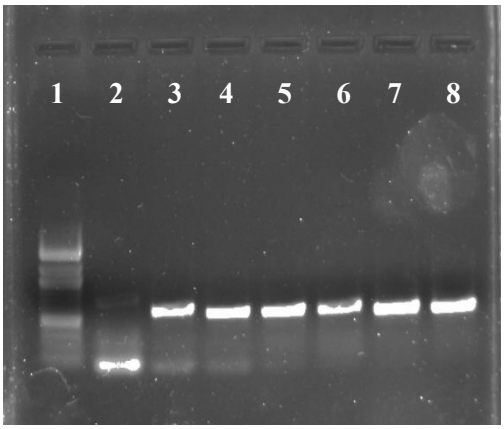
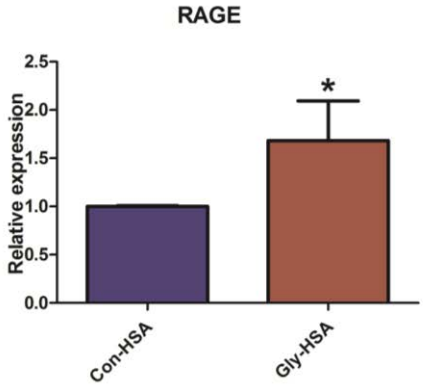


Figure 3.10: cDNA quality control check
Agarose gel electrophoresis of cDNA PCR using DHR primers for cDNA quality check; **Lane 1:** 100bp ladder, **Lane 2:** Negative control, **Lane 3-5:** PCR of cDNA from Con-HSA treated cells, **Lane 6-8:** PCR of cDNA from Gly-HSA treated cells

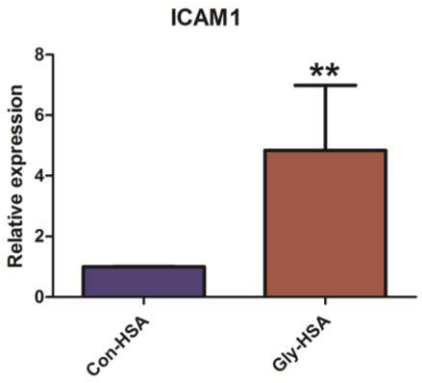
3.3.4.3.Real-time PCR

To understand the correlation of observed proteomic changes with RAGE, we checked the expression levels of RAGE by quantitative real time PCR. Intercellular adhesion molecule-1 (ICAM1) and von Willebrand's factor (vWF) were selected as representative genes from our

observed set of proteins showing differential abundance along with endothelial nitric oxide synthase (eNOS), endothelin-1 (ET-1) and vascular endothelial growth factor receptor 2 (VEGFR-2) which have been shown to be affected by RAGE in endothelial cells [31] {Pala, 2005 #606 [32]. We could observe an increase in the expression of RAGE as well as ICAM-1, vWF, ET-1 and VEGFR-2 after Gly-HSA stimulation while eNOS showed reduced expression (Figure 3.10). ET-1 and VEGFR-2 are involved in vasoconstriction and angiogenesis respectively. ET-1 is a potent vasoconstrictor and it's over-expression contributes to progression of atherosclerosis [33]. VEGFR-2 functions as the receptor for vascular endothelial growth factor or VEGF and promotes endothelial proliferation and migration and vascular permeability. It has been shown to be over-expressed in retinal and renal vascular endothelial cells in diabetes [34, 35]. eNOS is involved in generating nitric oxide (NO) in blood vessels, which is a potent vasodilator. eNOS is also involved in regulating thrombosis, cell adhesion and migration and angiogenesis, and is reported to show reduced expression in endothelial dysfunction induced by AGEs [36, 37].



(a)



(b)

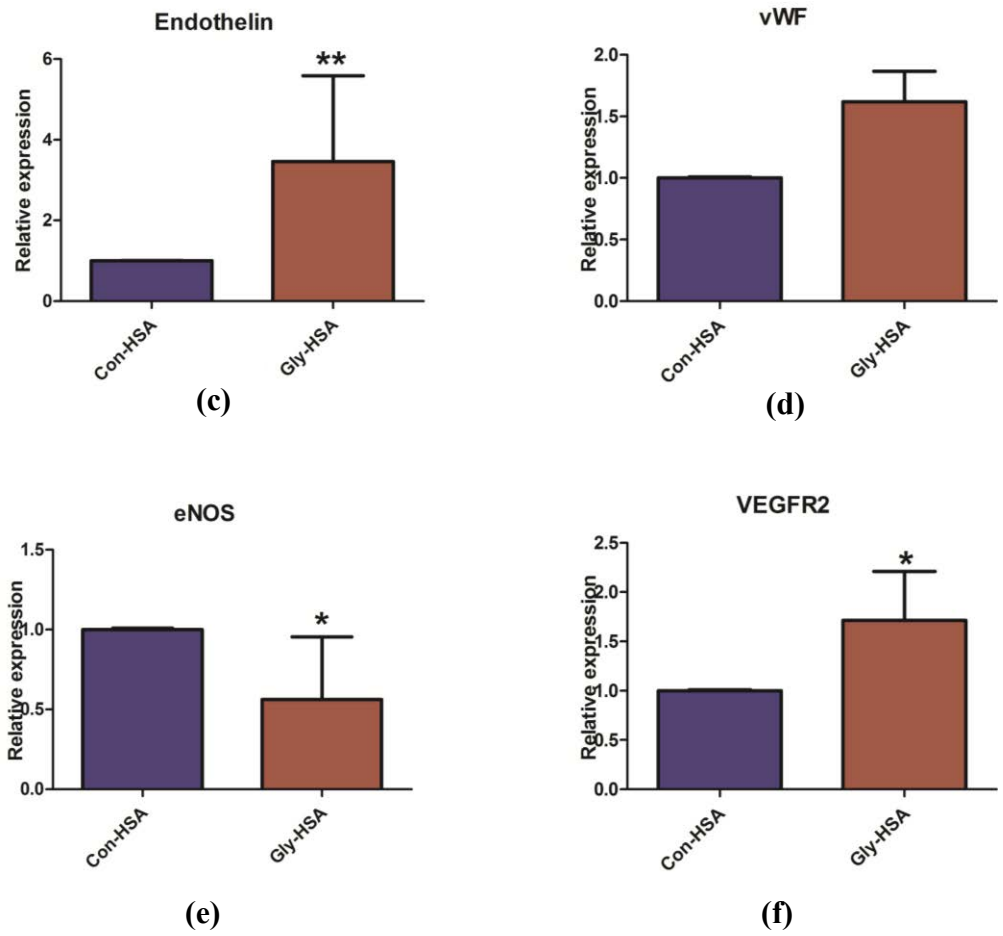


Figure 3.11: Relative fold change in expression observed in qPCR analysis
Fold change of **(a)** RAGE **(b)** eNOS **(c)** ICAM-1 **(d)** vWF **(e)** endothelin-1 **(f)** VEGFR-2 expression after Gly-HSA treatment. Values are expressed as relative fold change with respect to Con-HSA treated cells. β-actin was used as reference gene. (* indicates *p*-value<0.05, ** indicates *p*-value<0.01)

3.4. Discussion

In order to understand the overall changes in endothelial cell function in response to glycated albumin treatment and associate the observations to the development of vascular complications in diabetes, we have used MS based proteomics tools in conjunction with cell based assays. MS based proteomics can be performed using untargeted approaches such as SWATH or MS^E or using targeted approaches like MRM which require isotopic labeled standards of the peptides which are to be quantified. Untargeted approach or shotgun proteomics is useful when high throughput and maximum coverage of proteome is desired as

against targeted proteomics approach which can be used when a limited number of proteins/peptides need to be quantified [38]. Since endothelial function involves a number of proteins associated with a variety of functions, the former can be a useful approach to understand the processes involved in development of endothelial dysfunction.

There have been few previous attempts to use proteomics to understand endothelial function [39]. The first study on HUVECs proteome was reported by using 2D electrophoresis followed by MALDI based identification of proteins [40]. A similar approach was used to identify proteins expressed in response to stimulation by pro-inflammatory cytokines [41]. There have been some serum proteomic studies to identify markers of endothelial dysfunction [42]. However, there are very few studies on the endothelial proteome in response to modified proteins or in context of diabetes. One of the recent studies describes the effect of high glucose on S-nitrosylation of proteins in endothelial cells under diabetic conditions [43]. Many of the earlier studies on endothelial cells under diabetic conditions have mainly looked at specific aspects of endothelial function and the proteins and signaling cascades involved therein.

In our study using SWATH MS, we could observe altered abundance of many proteins that are known to be associated with endothelial function. Some of these proteins have been previously studied in context of AGEs and are seen to follow a similar trend in our study as reported earlier. For example, ICAM1 and PAI-1 that are earlier reported to show increased expression in response of AGEs, are seen to have higher abundance in our proteomic data as well [44, 45]. We could also find altered abundance of some proteins which have not been associated with AGEs earlier, such as MMP14 (mitochondrial-mmp1) which activates MMP2 by binding TIMP2 [46]. MMP-2 is involved in neovascularization and has been recently shown to be elevated in serum of patients with type-1 diabetes in association with increased CML levels [47]. Higher MMP-14 levels, as observed in our study, could be responsible for the increased MMP-2 activation and secretion in diabetic conditions. Other proteins not reported to be altered in vascular endothelial cells in response to glycated albumin include podocalyxin, which regulates vascular permeability [48] and is reported to be elevated in urine of diabetic nephropathy patients [49]. Elevated secretion of podocalyxin in response to AGEs might play a role in the pathogenesis of diabetic nephropathy and

albuminuria. Inflammatory proteins like S100, which function downstream to RAGE, are observed to be up-regulated in proteomic data.

In real time quantitative PCR validation, over-expression of ICAM1 observed correlates with the increased abundance observed in proteomic data. On the contrary, vWF showed decreased abundance in proteomic data while it was seen to be over expressed at mRNA level after Gly-HSA treatment. This anomaly could be as a result of its increased secretion which occurs under inflammatory conditions. In fact, increased serum levels of vWF are considered as biomarker for endothelial dysfunction and are associated with increased risk of cardiovascular disease [50]. This is also supported by the observed increase in abundance of proteins associated with exocytosis of vWF from endothelial cells, such as VAMP3 or Vesicle associated membrane protein-3 [51]. Similar reduced abundance at protein level was also observed for few other secretory proteins like thrombospondin-1, endoplasmin and multimerin-1.

Nf-κB and NRF-1 are seen to regulate a number of differentially expressed proteins in our study by iRegulon. Additionally, nuclear localization of Nf-κB after treatment with glycated HSA validated its the involvement in the observed proteomic changes. Interaction of AGEs with RAGE has been shown to induce oxidative stress and inflammatory response [29, 52] and Nf-κB is known to be a major regulator operating downstream of RAGE. Activation of Nf-κB observed, therefore, indicates the involvement of RAGE in inducing endothelial dysfunction [29]. Also, earlier studies have reported diminished NRF-1 action in conjunction with elevated RAGE expression to be involved in neurodegeneration leading to diabetic neuropathy [53]. A similar trend in AGE induced endothelial dysfunction can be observed in our study as most of the NRF-1 regulated proteins have reduced abundance after Gly-HSA treatment. Based on bioinformatics, immunofluorescence and qPCR analysis to support the proteomic observations, RAGE appears to be an important mediator of endothelial dysfunction and it can be useful to study its inhibition in diabetes to control endothelial dysfunction before it progresses to more severe vascular complications although further studies to prove its efficacy need to be carried out.

3.5. Conclusion

In this chapter, we could identify a change in abundance of various proteins involved in endothelial dysfunction on AGE treatment, as well as an up-regulation of the RAGE receptor and some of its downstream targets. Thus AGEs can negatively affect endothelial function which predisposes to cardiovascular complications and inhibition of its receptor RAGE can be a useful therapeutic approach in future.

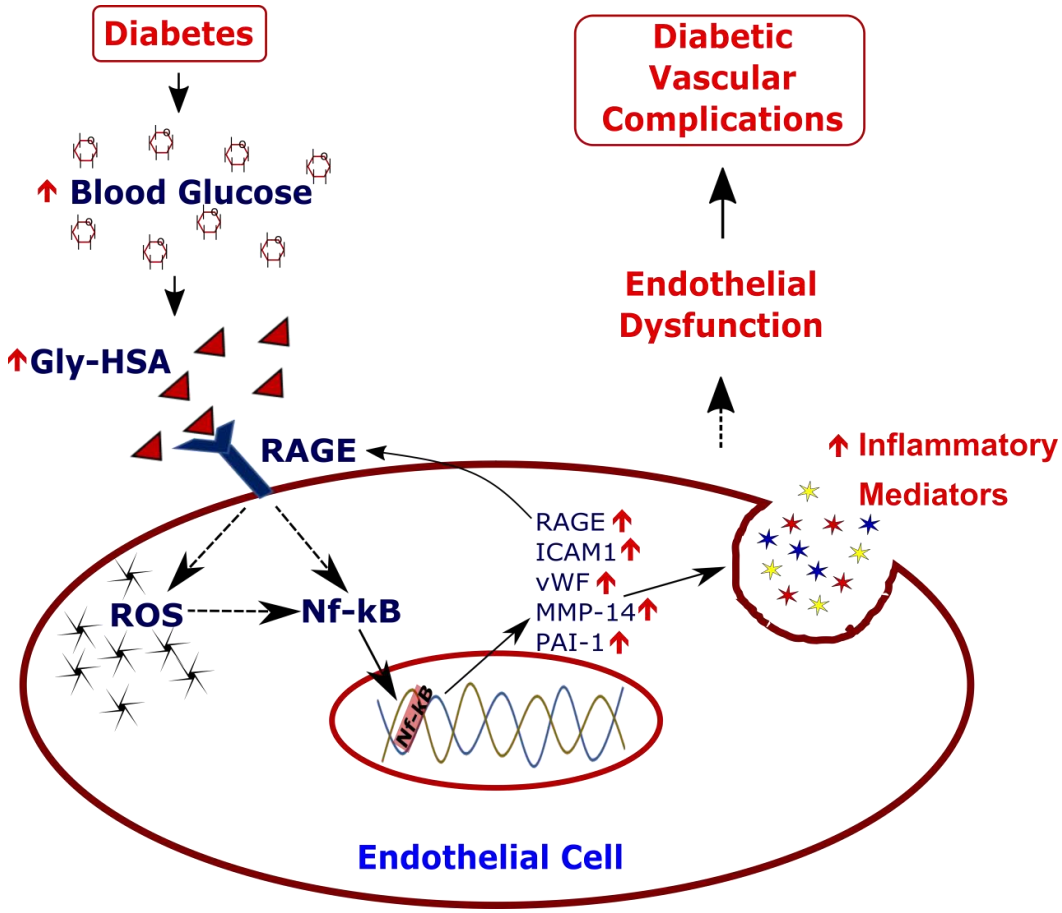


Figure 3.12: Summary Figure

3.6. Future Directions

In our study, we could find reduced cellular abundance of a number of secretory proteins which can be associated with endothelial dysfunction such as vWF, multimerin, thrombospondin and so on. The enhanced secretion of these proteins can be confirmed by

their detection and quantification in the supernatent cell culture medium of AGE treated cells. Likewise, a comprehensive secretomic study of endothelial cells after glycated albumin treatment would be useful to validate our observations and also identify other secretory proteins that might be affected.

Also, to confirm the involvement of RAGE in inducing endothelial dysfunction, validation studies in the presence of agents that block RAGE receptor, such as anti-RAGE antibody, need to be performed. Angiotensin Receptor blockers (ARBs) have been shown to downregulate RAGE expression via PPAR-γ activation and consequently, inhibit the negative effects of AGEs [54, 55]. Whether ARBs or other drugs that can inhibit RAGE expression and/or signaling, can ameliorate AGE induced endothelial dysfunction needs to be further studied.

3.7. References

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Chapter 4: Study of Endothelial Response to Homocysteinylated Albumin

4.1. Background

Hyperhomocystenemia (HHcy) is defined as the increase in plasma Hcy levels above 15μM, with levels above 100μM reported in severe cases[1]. It can result from excess intake of methionine rich food or genetic disorders associated with deficiency of cystathionine β synthase (cbs) or methylenetetrahyfolate reductase (Mthfr)[2]. An estimated 1 in 70 people have HHcy[3] and it is most commonly associated with vitamin B12 or folate deficiency[4]. Vitamin B6 deficiency has also been shown to increase plasma Hcy levels[5].

HHcy has been associated with a number of diseases such as atherosclerosis[6], Alzheimer's disease and other forms of dementia[7], cancer[8], pre-eclampsia[9] and other complications associated with pregnancy. It was first reported to be associated with atherosclerosis by McCully in 1996[10] and currently, HHcy[11] along with folate[12] and B12 deficiency[13] are considered as independent risk factors for atherosclerosis and coronary artery disease. Elevated Hcy has also been associated with increased mortality in CVD patients[14]. Although there are many studies that show beneficial effects of vitamin supplementation on lowering plasma Hcy and subsequent reduction in CVD risk[15 16 17], not all clinical studies of vitamin supplementation report improved cardiovascular outcomes[18 19]. Therefore, it is important to understand the mechanism of Hcy toxicity to design better prognostic therapies.

In plasma, Hcy is bound either to cysteine residues on proteins or, to free cysteine and other homocysteine molecules via disulfide bridges[20]. The major plasma circulatory form of Hcy is the N-linked homocysteine or homocystamide. An increase in plasma Hcy levels leads to a simultaneous increase in N-homocysteinylated protein levels[21]. Increase in the levels of such modified proteins has also been associated with the development of cardiovascular disease[22] and homocysteinylated albumin has been detected in atherosclerotic lesions of $ApoE^{-/-}$ mice with HHcy[23]. Therefore, it is possible that Hcy modified proteins mediate some of the deleterious effects associated with HHcy.

To further investigate this, we have studied the effect of homocysteinylated albumin on HUVECs so as to determine whether it plays a role in development of endothelial dysfunction.

4.2. Materials & Methods

4.2.1. Synthesis of Homocysteinylated albumin

Fatty acid free bovine serum albumin was used to synthesize homocysteinylated albumin using the protocol described in Chapter 2. Control unmodified albumin was synthesized by incubating BSA under similar conditions in the absence of homocysteine thiolactone. Both reactions were dialyzed overnight against phosphate buffer, concentrated using 30kDa molecular weight cut-off centrifugal filters and filter sterilized using syringe filters before using for cell culture.

4.2.2. Cell culture and treatments

HUVECs were grown in culture as previously described. Cells were serum starved for 3h prior to treatments. For oxidative stress assay, cells were serum starved and pre-treated with 10μM DCFH-DA in phenol red free medium for 45 minutes followed by treatment with control unmodified and homocys- albumin for 15min. Fluorescence of DCF was analyzed using flow cytometry as well as fluorescence microscopy. Apoptosis assay was performed using and Annexin-V FITC Propidium iodide staining after 24h of treatment using similar protocol as described in Chapter 3.

4.2.3. SWATH Proteomics

Differential proteomics was performed for cells treated with control unmodified and homocys-albumin using SWATH-MS. Total cell proteins were extracted in Rapigest and digested with trypsin using the protocol as described in Chapter 3. Equal amounts of digested and desalted peptides from each sample were individually acquired on SCIEX TripleTOF 5600 mass spectrometer in IDA mode and analyzed together on ProteinPilot software. The spectral ion library thus generated was further used for SWATH analysis. At least 4 replicate SWATH runs for each sample were acquired and used for quantification using Peakview software. The acquisition and analysis parameters were the same as those mentioned in Chapter 3.

4.2.4. Bioinformatic Analysis

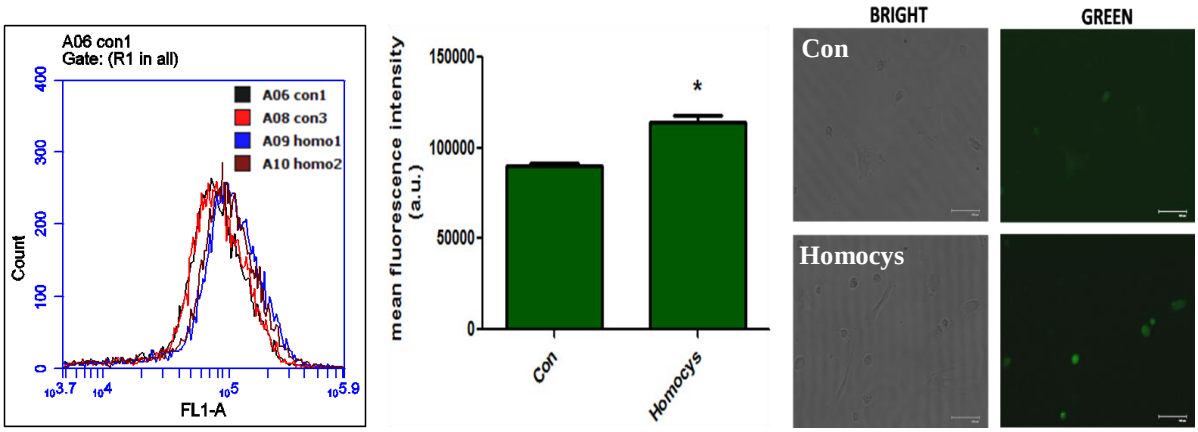
Differentially abundant proteins obtained by SWATH were analyzed for Gene Ontology and functional annotation using the online tool DAVID. Further, PPI network was generated using STRING version 10.5[24] and imported into Cytoscape v3.2 for visualization and analysis. Regulatory factors for the differential proteins were predicted using iRegulon application of Cytoscape.

4.3. Results

4.3.1. Effect of Homocysteinylated Albumin on HUVECs

4.3.1.1. Oxidative stress

Treatment of HUVECs with homocys-albumin led to an increase in ROS as seen by increase in mean cellular fluorescence in flow cytometry (Figure 4.1 a and b) as well as increase in the number of cells showing DCF fluorescence using fluorescence microscopy (Figure 4.1c).

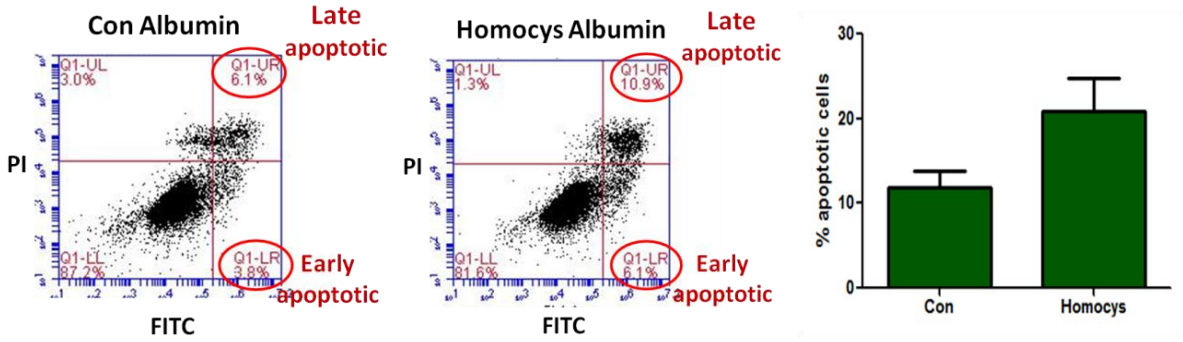


(a) **(b)** **(c)**

Figure 4. 1: ROS Assay after Homocys-albumin treatment
(a) histogram shows increase in DCF fluorescence in cells treated with homocys-albumin **(b)** bar graph showing increase in the mean fluorescence intensity of cells treated with homocys-albumin **(c)** fluorescence microscopy shows more number of cells with green DCF fluorescence after homocys-albumin treatment (* indicates *p*-value<0.05).

4.3.1.2. Apoptosis assay

24h treatment with homocys-albumin led to an increase in the percentage of both early and late apoptotic cells as seen by flow cytometry after Annexin V-FITC and PI staining (Figure 4.2).

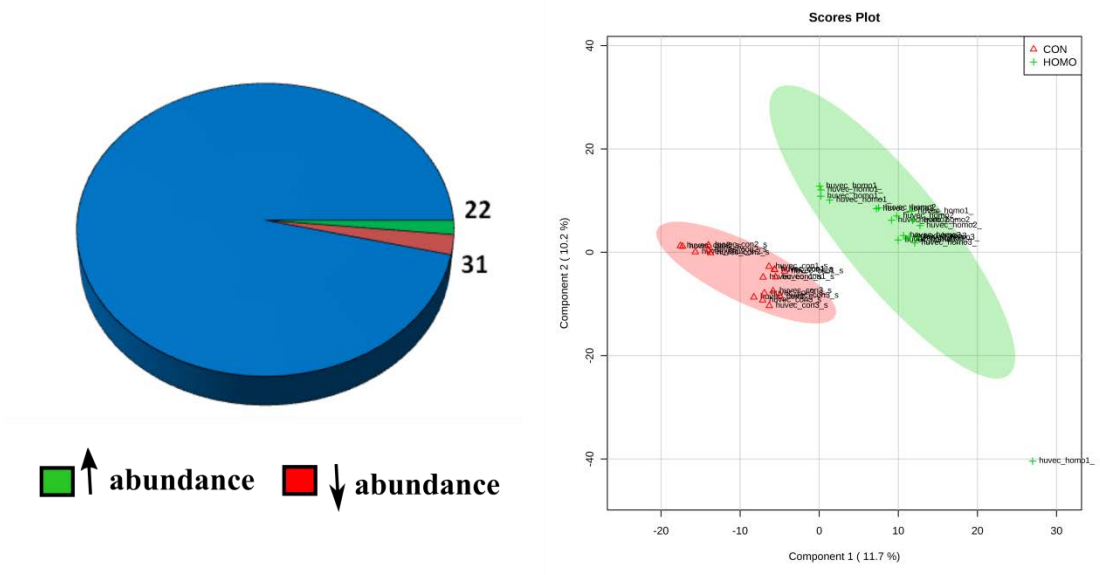


(a) **(b)**

Figure 4. 2: Apoptosis Assay after Homocys-albumin treatment
(a) Scatter plots show increase in the number of cells in the early apoptotic phase (lower right quadrant: LR) and late apoptotic (upper right quadrant: UR) phase after homocys albumin treatment as compared to control unmodified Con-albumin treatment **(b)** Bar graph shows the percentage of apoptotic cells; data expressed as percentage of total number of cells ±SEM

4.3.2. Differential Proteomics of HUVECs treated with Homocysteinylated Albumin

IDA led to identification of 1410 proteins by ProteinPilot software, which were further used as spectral library for SWATH quantification. 22 proteins showed a minimum of 1.3 fold increase in abundance while 31 showed similar decrease in abundance with p-value <0.05 after 24h treatment with homocys- albumin (Figure 4.3a). PCA of all replicate runs showed good reproducibility between the replicate runs of each treatment (Figure 4.3b).



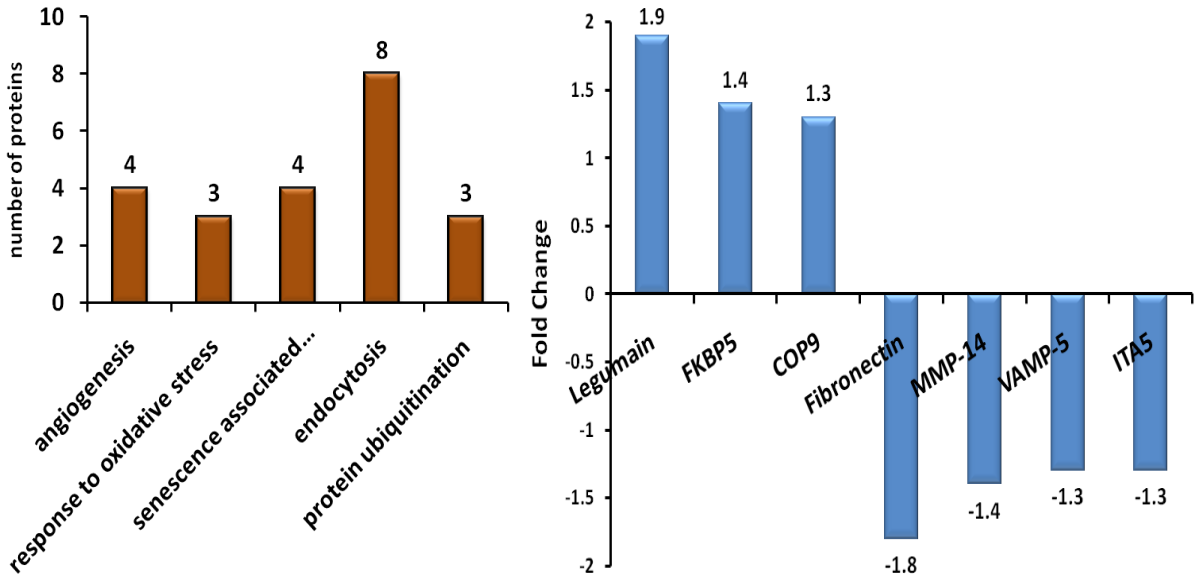
(a) **(b)**

Figure 4. 3: Differential SWATH Proteomics

(a) Pie chart indicates that 22 proteins showed increased abundance, while 31 proteins showed decreased abundance after treatment with homocys albumin, out of the 1410 proteins identified **(b)** PCA plot shows reproducibility among replicate runs of each treatment

4.3.3.Bioinformatic Analysis

Gene ontology analysis using DAVID indicated that a number of proteins showing altered abundance are involved in processes such as oxidative stress, endocytosis and protein ubiquitination (Figure 4.4a). Also, proteins like fibronectin, MMP-14 and integrin alpha V involved in endothelial functions like angiogenesis and cell adhesion show reduced abundance (Figure 4.4b).



(a) **(b)**

Figure 4. 4: Gene Ontology and Fold Change of Differentially Abundant Proteins
(a) Bar graph of functional annotation of the differentially abundant proteins using DAVID shows the specific functions that are enriched and number of proteins involved in each function (b) Bar graph shows fold change of few differentially abundant proteins

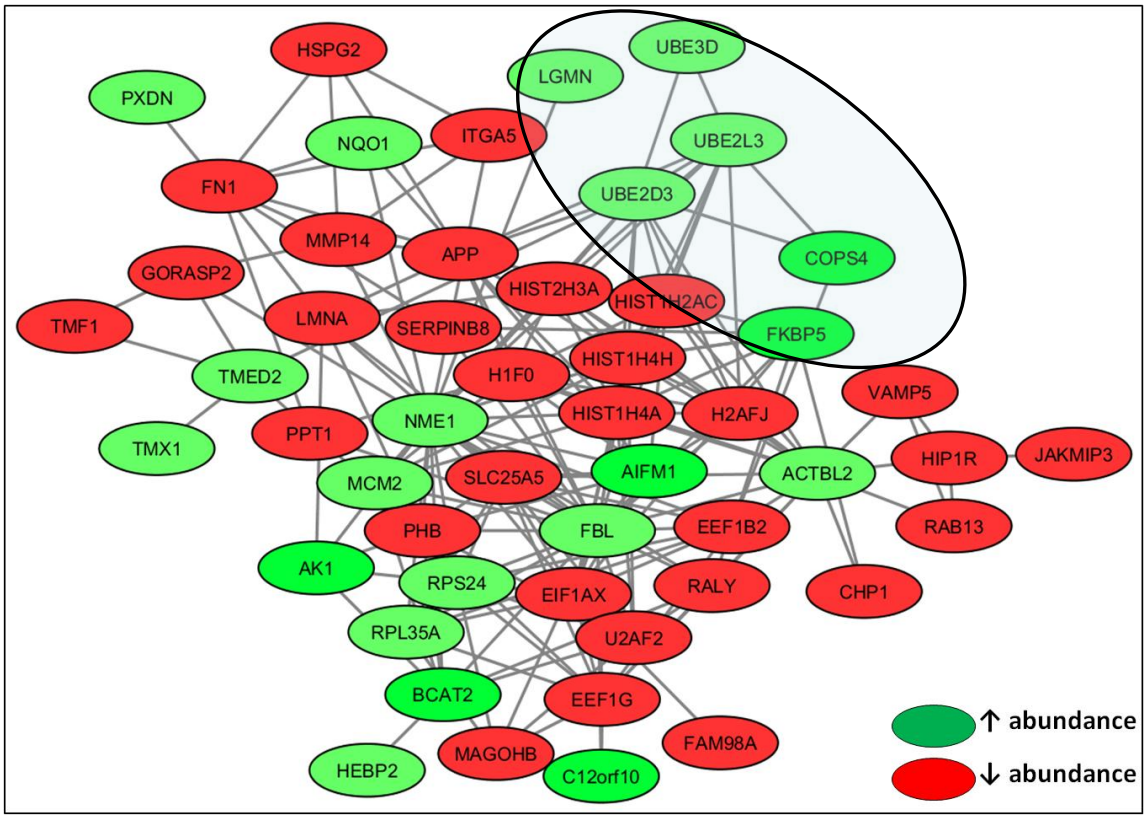
Some of the proteins showing reduced abundance are seen to belong to the functional class of Senescence Associated Secretory Proteins or SASPs (Figure 4.4a). SASPs are proteins released by senescent cells under oxidative stress that induce inflammation in nearby cells and thereby form a link between oxidative stress and endothelial dysfunction. Increased levels of such proteins has been observed in diabetes and metabolic disorders which are characterized by chronic subclinical low grade inflammation or metainflammation i.e. metabolically triggered inflammation[25]. The reduced cellular abundance of these proteins observed in our data might be a result of their elevated secretion from cells. List of proteins belonging to specific functional classes is given in Table 4.1.

Table 4. 1: Differentially expressed proteins and their functional annotation

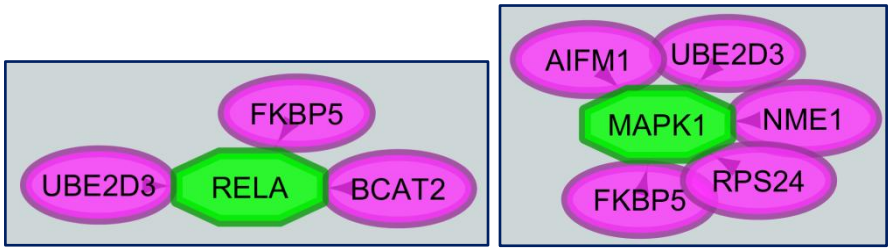
Sr. No.	Gene Symbol	Protein Name	Accession	*p*-value	Fold Change
Angiogenesis					
1	FINC	Fibronectin	P02751	3.63E-11	0.55
2	ITA5	Integrin alpha-5	P08648	2.40E-07	0.74
3	MMP14	Matrix metalloproteinase-14	P50281	2.77E-05	0.69
4	PGBM	Basement membrane-specific heparan sulfate proteoglycan core protein	P98160	3.10E-08	0.75
Protein Ubiquitination					
16	UB2L3	Ubiquitin-conjugating enzyme E2 L3	P68036	0.02376	1.40
17	UBE3D	E3 ubiquitin-protein ligase E3D	Q7Z6J8	0.00476	1.85
18	UB2D3	Ubiquitin-conjugating enzyme E2 D3	P61077	0.00193	1.41
Senescence Associated Secretory Phenotype					
19	H2AJ	Histone H2A.J	Q9BTM1	0.01841	0.69
20	H2A1C	Histone H2A type 1-C	Q93077	0.00745	0.75
21	H32	Histone H3.2	Q71DI3	0.00951	0.60
22	H4	Histone H4	P62805	0.00015	0.61
Response to Oxidative Stress					
5	PXDN	Peroxidasin homolog	Q92626	0.00407	1.35
6	A4	Amyloid-beta A4 protein	P05067	0.0001	0.59
7	MMP14	Matrix metalloproteinase-14	P50281	2.77E-05	0.69
Endocytosis					
8	A4	Amyloid-beta A4 protein	P05067	0.0001	0.59
9	TMX1	Thioredoxin-related transmembrane protein 1	Q9H3N1	0.01492	1.34
10	RAB13	Ras-related protein Rab-13	P51153	0.00262	0.67
11	HIP1R	Huntingtin-interacting protein 1-related protein	O75146	7.72E-06	0.74
12	PPT1	Palmitoyl-protein thioesterase 1	P50897	0.04295	0.75
13	TMED2	Transmembrane emp24 domain-containing protein 2	Q15363	0.0246	1.62
14	VAMP5	Vesicle-associated membrane protein 5	O95183	0.02985	0.73
15	NDKA	Nucleoside diphosphate kinase A	P15531	0.001	1.55

PPI network of the differentially abundant proteins indicates that they have been reported to be mutually interacting and possibly involved in common biochemical functions

(Figure 4.5a). Particularly, a number of proteins involved in ubiquitination and proteolysis like ubiquitin ligases UBE2D3, UBE2L3 and UBE3D, COPS4 and FKBP5 are seen among up-regulated proteins. Regulatory factor identification by iRegulon application in Cytoscape identified that a number of differentially abundant proteins can activate Nf-κB and MAPK signaling cascades (Figure 4.5b).



(a)



(b)

Figure 4. 5: Bioinformatic Analysis

(a) Protein-protein Interaction Network of all proteins showing differential abundance after Homocys-albumin treatment (b) Regulatory Factor Prediction by iRegulon

4.4. Discussion

Even though a large number of studies associate HHcy with different disorders, the exact mechanisms for its effects are not fully understood. A number of studies report the toxicity of free Hcy on cells by various different mechanisms. Free Hcy can be transported into endothelial cells via the alanine-serine-cysteine (ASC) transporter, sodium dependant cysteine transporter and large branched chain neutral amino acids transporter systems[26]. Additionally, *in vitro* studies have identified the two glutamate receptors, AMDA and NMDA as potential receptors for uptake of Hcy[27]. Hcy treatment to human endothelial cells in culture, leads to increase in VCAM-1, ICAM-1, E-selectin and P-selectin levels[28 29]. Oxidative stress is one of the underlying mechanisms by which HHcy leads to endothelial dysfunction[30] and Hcy leads to oxidative stress by activating NADPH oxidase and further activates of Nf-κB transcription factor in HUVECs[28]. Hcy has also been reported to activate p66shc by hypomethylation of its promoter leading to oxidative stress and ED[31]. The involvement of oxidative stress in development of endothelial dysfunction due to Hcy has also been reported in a number of clinical studies[32 33]. An alternative mechanism reported for Hcy toxicity is unfolded protein response or UPR. Hcy as well as its cyclic thiolactone form, lead to UPR and endoplasmic reticulum-stress (ER-stress) in endothelial cells[34] and a subsequent increase in VEGF expression[35]. Further, Hcy conversion to Hct in the endothelial cell itself can lead to toxicity via consequent modification of cellular proteins thereof [36].

However, free Hcy is not the major form in which it is present in the plasma. The level of free Hcy in blood is about 35nM, which is about 0.3% total plasma homocysteine. About 10-20% Hcy is present in its oxidized form as Hcy-Hcy or Hcy-Cys dimers but the major form of plasma Hcy is as its protein linked homocystamide. The total concentration of protein N-linked homocysteine in blood is about 15 μM in normal individuals which is about 80-90%, of the total Hcy in blood. Out of this, Hcy bound to serum albumin in the form of homocystamide is about 2.8μM which is about 25% of total plasma Hcy. Therefore, it is possible that in HHcy the elevated levels of Hcy modified plasma proteins is recognized by pattern recognition receptors, similar to those reported for other modified proteins. The inflammatory response elicited by such pattern recognition receptors could be involved in the development of ED and subsequent CVD. We have tried to explore this aspect of Hcy

toxicity using N-homocysteinylated serum albumin to treat HUVECs and study their response.

Currently, there are very few studies on effect of homocys-protein on endothelial cells. One study has shown that S-homocysteinylated LDL affects proliferation and viability of human umbilical vein endothelial cells[37]. Treatment of purified N-homocysteinylated-albumin to the human endothelial cell line EAhy926 reportedly lead to an over-expression of ICAM-1, VCAM-1 along with MCP-1 and a resultant increase in adhesion of monocytes to the endothelial monolayer[38]. Microarray analysis of HUVECs treated with N-homocysteinylated albumin by Gurda *et al* showed differential expression of 47 genes of which many were involved in blood coagulation and atherosclerosis[39]. In our study, we have performed total cell differential quantitative proteomics using SWATH-MS platform to understand the effects of N-homocysteinylated albumin on HUVECs at proteome level. Although we could not observe the proteins reported by Gurda *et al* in our study, we could see altered expression of a number of proteins involved in ED and cardiovascular disease.

Among the proteins that were found to show altered abundance after homocys-albumin treatment is fibronectin, a high molecular weight glycoprotein of the extracellular matrix involved in cell adhesion, angiogenesis, wound healing and platelet aggregation[40]. Its expression has been reported to increase in CVD, possibly due to its involvement in thrombogenesis and atherosclerosis[41]. Similar to the other secretory proteins observed in our cellular proteomic data, the reduced abundance of fibronectin could be as a result of its increased secretion from the cells. ITGA5 or integrin alpha V belongs to the alpha chain family of integrins that are heterodimeric integral membrane proteins. ITGA5, as a dimer with ITGB1 or integrin beta I, acts as the receptor for fibronectin and is thus involved in cell adhesion[42]. Interestingly, ITGA5 also showed lower abundance after homocys-albumin treatment which could correspond to its elevated export to the cellular membrane. Another protein associated with atherosclerosis[43] and showing higher abundance in our study is legumain, a cysteine protease reported to be responsible for plaque instability[44]. Although it is believed to be secreted from activated macrophages in atherosclerotic plaques, it is also expressed in endothelial cells where it is involved in angiogenesis via activation of proMMP-2[45].

Analogous to the reports for Hcy toxicity, homocys-albumin is also seen to mediate oxidative stress in endothelial cells in our study as seen by the ROS assays and differential abundance of proteins involved in oxidative stress. Oxidative stress can lead to activation of Nf-κB[46] and correspondingly, proteins regulated by Nf-κB such as SASPs[47] are also observed in our study. At the same time, few regulators of Nf-κB are also observed to show increased abundance in our study. FKBP5 is an HSP-90 co-chaperone which impacts on the stability of IκB and on phosphorylation of Nf-κB, and enhances DNA binding of Nf-κB[48]. Similarly ubiquitin conjugating enzyme UBE2L3 also activates NF-κB by ubiquitinating its inhibitor NEMO or IKKγ[49]. UBE2D3 a homolog of UBE2L3, is also seen to be up-regulated in proteomics data, however it has not been reported to activate NF-κB[50]. Thus, our proteomic data suggests that N-homocysteinylated albumin can affect endothelial cell function via oxidative stress and possibly Nf-κB activation.

4.5. Conclusion

In this study, we could observe differential abundance of a number of proteins in response to N-homocysteinylated albumin. Thus, our study indicates that homocysteinylated plasma proteins could play a role in the development of vascular complications of HHcy by regulating various pathways such as oxidative stress, Nf-κB activation and proteolysis. Further studies are needed to elucidate the exact mechanism and determine whether inhibition of protein homocysteinylation can prevent the adverse consequences of HHcy.

4.6. Future Directions

In our study, we could identify differential expression of a number of proteins involved in cellular function. The observed change in their expression needs to be further validated by western blots or gene expression analysis. Also, the mechanism by which homocysteinylated albumin exerts the effects is not known. Identification of proteins which interact with the Hcy modified protein would be essential to determine if there are any scavenger receptors that bind to such modified proteins. This can be carried out by co-immunoprecipitation of endothelial membrane proteins with homocysteinylated albumin and further, mass spectrometric identification of the interacting proteins.

There are very few earlier reports of quantification of Hcy proteins in plasma samples of cardiovascular patients. Correlation of elevated plasma levels of Hcy modified peptides in CVD patients with HHcy will support the role of modified proteins in development of such complications. Targeted quantification of N-homocysteinylated peptides in clinical plasma of patients can be a useful approach to quantify and correlate protein homocysteinylation levels in cardiovascular diseases.

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Chapter 5: Study of Modulation of Insulin Signal Transduction by Oxidized LDL via LOX-1

5.1. Background

Diabetes and insulin resistance have been associated with increased risk of endothelial dysfunction[1] and atherosclerosis[2]. Moreover, insulin resistance increases the risk of atherosclerosis even in the absence of hyperglycemia[3]. Particularly, obesity associated insulin resistance has been observed to lead to endothelial dysfunction [4, 5]. It has also been observed that insulin resistance in vascular endothelium precedes its development in other insulin sensitive tissues like liver, skeletal muscle and adipose tissue[6], which is possible partly because the vascular endothelium controls the delivery of insulin to other tissues and impairment of endothelial response to insulin can affect insulin transport to peripheral tissues[7].

Although the principle function of insulin is to regulate glucose and lipid metabolism, it effects a myriad of functions in different cell types[8]. In endothelial cells, for example, insulin leads to angiogenesis [9] and prevents apoptosis [10] and ROS formation via Akt2 phosphorylation[11]. It also mediates NO production via induction of fatty acid synthase which promotes eNOS palmitoylation and translocation to the cell membrane[12] as well as via eNOS phosphorylation[13], leading to consequent vasodilation[14, 15] and decreased expression of adhesion molecules[16]. Conversely, insulin has also been reported to show pro-atherogenic effects such as stimulation of endothelin-1 release[17], expression of PAI-1[18] and increased expression of adhesion molecules[19].

"Insulin resistance" can refer to either down-regulated insulin receptor signaling or hyperinsulinemia-mediated excessive insulin receptor signaling[20]. The pro-atherogenic effects of insulin as a result of hyperinsulinemia[21] has been debated[22]. At the same time, perturbed insulin signaling in endothelial cells has reportedly lead to atherogenesis[23]. Factors like over-nutrition[24] [25] and hypertension[26] have been reported to lead to vascular inflammation and impaired endothelial insulin signaling, which in turn, leads to further inflammation and endothelial dysfunction. Activation of TLR4 or Toll-like receptor 4, a pattern recognition receptor (PRR) has been implicated in the development of endothelial insulin resistance in obesity[27]. Similarly, other

mechanisms that perturb insulin signaling in endothelial cells can lead to vascular insulin resistance and consequent, endothelial dysfunction and atherosclerosis.

Another PRR widely studied in atherosclerosis is LOX1. Although primarily, the uptake of oxidized LDL by LOX1 has been associated with the formation of atherosclerotic plaques[28], oxLDL binding to LOX1 also mediates inflammatory signaling[29]. It is therefore, possible that elevated plasma LDL levels, which are a risk factor for atherosclerosis, lead to increased inflammatory signaling of LOX1 and consequent development of vascular insulin resistance, endothelial dysfunction and CVD. To further study this hypothesis, we have expressed human LOX1 protein in HEK293 cells and monitored LOX1 signaling mediated by oxLDL binding and its cross-talk with insulin signaling.

5.2. Materials and Methods

5.2.1. Reagents

All chemicals were procured from Merck (formerly Sigma-Aldrich) unless otherwise specified. Antibodies used were p-Akt, total Akt, from Cell Signaling Technologies, LOX-1, tubulin and HRP tagged secondary antibodies.

5.2.2. Isolation of LDL

18ml of whole blood was collected into a centrifuge tube containing 2ml of 3.8% trisodium citrate. The mixture was centrifuged at 3000rpm for 10 minutes at 4°C and the suparnatent plasma was collected. The plasma was then mixed with OptiPrep™ Density Gradient medium in a ratio of 4:1 v/v and layered with 1ml HEPES Buffered Saline in Beckmann centrifuge tubes. They were then centrifuged at 100000g for 3 h at 16°C. The yellow orange layer of LDL formed after centrifugation was carefully removed using a syringe and dialyzed overnight against PBS in dark. Protein concentration was estimated using BCA method.

5.2.3. Oxidation of LDL

Oxidized LDL was prepared by incubating the isolated LDL with 5µM $CuSO_4$ at 37°C for 24 h in dark. The reaction was stopped after 24 h by addition of 100µM (1:1000 ratio) EDTA and 20µM BHT (1:1000 ratio) and unreacted $CuSO_4$ was removed by dialysis. For immunofluorescence assays, oxLDL was labeled with DiI, a lipophilic orange-red fluorescent

dye (Thermofisher) by incubation at 37°C for 16h followed by centrifugation and dialysis to remove the unreacted dye.

5.2.4. Cell Culture

HEK293 cells expressing human LOX1 gene under tetracycline inducible system was used for the study. These cells were generated using Flp-in T-rex HEK293 cells which contain an FRT region (Flippase Recognition Target) and a Zeocin resistance gene downstream to SV40 promoter and a tetracyline repressor gene (TetR) under control of CMV promoter in their genomic sequence. The Flp-in T-rex cells were transfected with two plasmids, one containing FLAG tagged human *lox*-1 gene downstream to FRT region, hygromycin resistance gene, CMV promoter and tetracycline oppressor region, and another plasmid containing flippase gene under CMV promoter. Homologous recombination between the two FRT sites mediated by flippase leads to the insertion of hygromycin resistance gene and the FLAG tagged human *lox*1 gene with CMV promoter and tetracycline oppressor into the genomic sequence of HEK293 cells (Figure 5.1). The transfected cells can thus be selected based on their hygromycin resistance. Such LOX1 expressing and Empty Vector transfected HEK293 cells were grown in high glucose DMEM containing 10% fetal bovine serum, penicillin streptomycin antibiotic mix, 15µg/ml blasticidin and 50µg/ml hygromycin at 37°C in 5% CO_2.

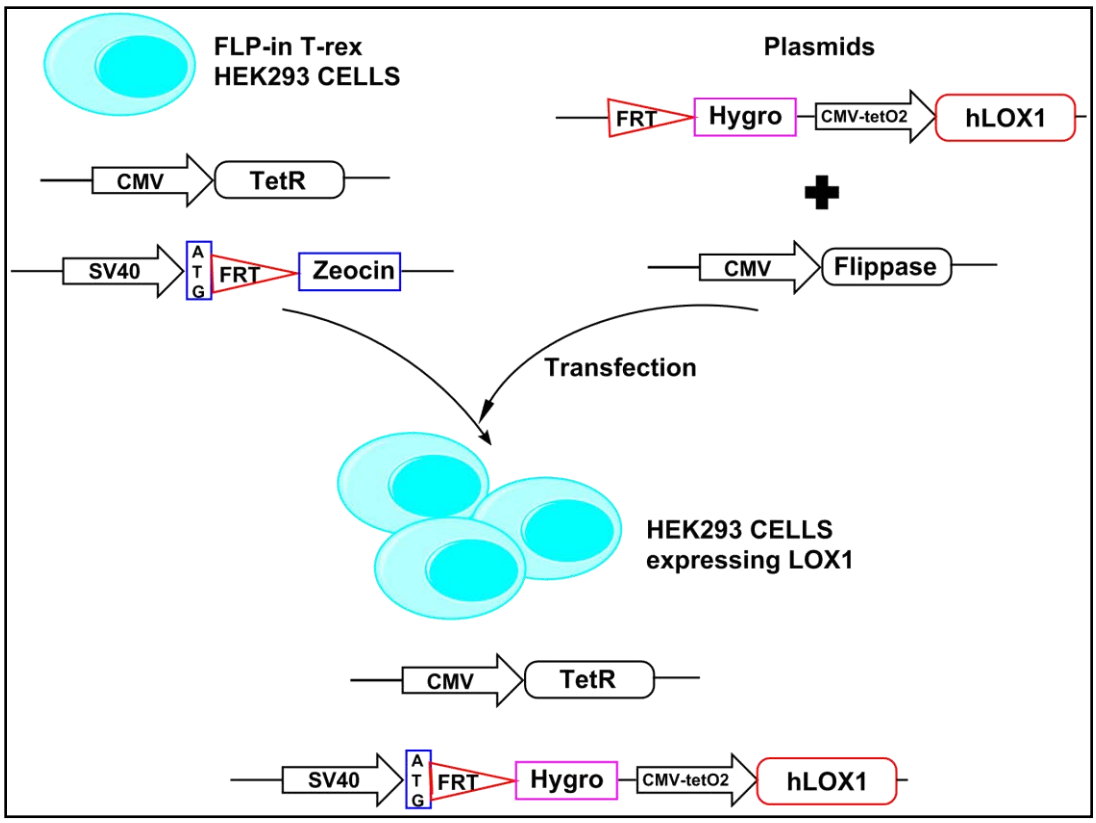


Figure 5. 1: HEK293 cells tetracycline inducible expression system for human LOX1

5.2.5. Western Blotting

For western blot, cells were lysed in 2% SDS followed by short sonication cycle and total cell proteins were quantified by BCA assay. 15µg cell lysate was separated on 12% SDS PAGE and transferred to nitrocellulose membrane using wet transfer system. Following transfer, the membrane was stained with Ponceaeu S solution to confirm equal transfer of protein. The membrane was blocked using 5% skimmed milk at ambient temperature for 1h. Primary antibody incubation was performed overnight at 4°C with gentle rocking. This was followed by washes with PBS-T and secondary antibody incubation for 1h at room temperature on a rocker shaker. After incubation, membrane was washed with PBS-T and developed using chemiluminescence based detection.

5.2.6. Immunofluorescence

Cells were seeded on poly-L-lysine coated coverslips and LOX1 expression was induced with tetracycline. oxLDL stained with fluorescent lipophilic dye DiI was added to cells on ice for

different time intervals followed by fixation of cells for 20 minutes at 37°C using Sigmafix (10% formalin). Cells were then washed with PBS and incubated with 5% BSA (v/w in PBS) for 1h at 37°C. Primary antibody diluted in 1% BSA was added to the cells and incubated at room temperature overnight. Further, cells were washed with PBS and incubated with secondary antibody conjugated to FITC, and DAPI in PBS for 2-3h at room temperature. The cells were then washed again with PBS and mounted on glass slides using mounting medium. Cells were visualized on an EVOS® Cell Imaging System (Thermo Fisher Scientific, USA).

5.3. Results

5.3.1. Expression of LOX-1 in HEK293 cells

In the transfected HEK293 cells, in the absence of tetracyline, TetR binds to the upstream region of human *lox*1 and prevents its expression. On addition of tetracycline, it binds to TetR and allows expression of LOX1 via the CMV promotor. LOX1 expression after adding tetracycline (1µg/ml) was monitored by western blot after different time intervals. Expression of LOX1 was seen HEK293 LOX1 cells only after tetracycline treatment and not in its absence or in empty vector transfected cells treated with tetracycline (Figure 5.2).

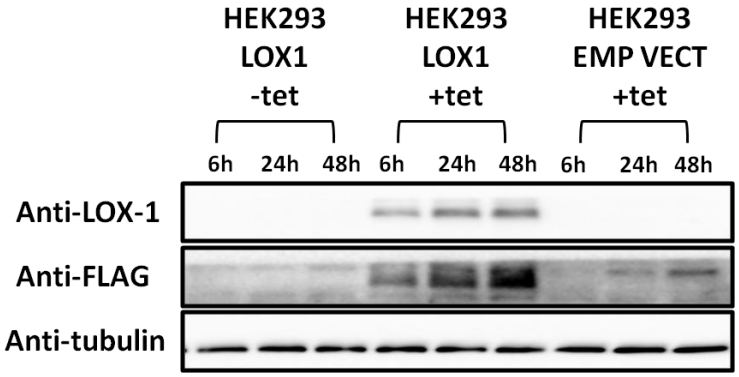


Figure 5. 2 Tetracycline induced expression of LOX1 in HEK293 cells
Western blot using anti-LOX1, anti-FLAG and anti-tubulin antibody without and with tetracycline treatment at different time intervals.

Additionally, in order to check for any expression of native LOX1 in HEK293 cells in response to oxLDL, tetracycline induced and uninduced cells were treated with 10µg/ml oxLDL at indicated time points. Here too, expression of LOX1 was observed only after tetracycline

induction and not in the uninduced cells indicating no detectable expression of native LOX1 in response to oxLDL in HEK293 cells (Figure 5.3).

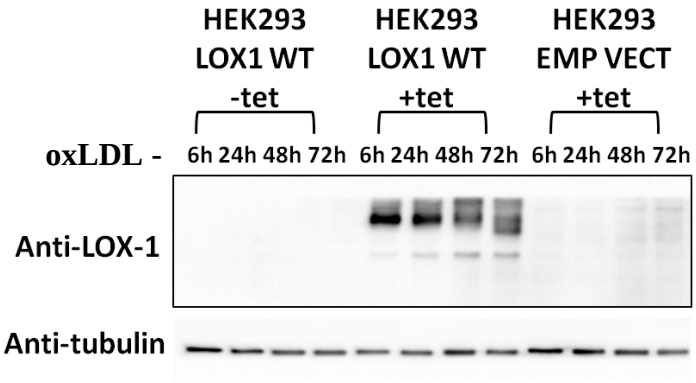


Figure 5. 3 LOX1 expression in presence of ox-LDL
Western blot of induced and uninduced HEK293 cells after oxLDL treatment

5.3.2. Uptake of oxLDL by LOX-1

oxLDL binding to LOX1 is reported to induce its internalization[30], which was monitored by immunofluorescence using anti-FLAG antibody and fluorescently labeled oxLDL. Different time intervals of oxLDL treatment from zero to thirty minutes lead to its binding to LOX1 and subsequent internalization as seen by their co-localization in the immunofluorescence images (Figure 5.4).

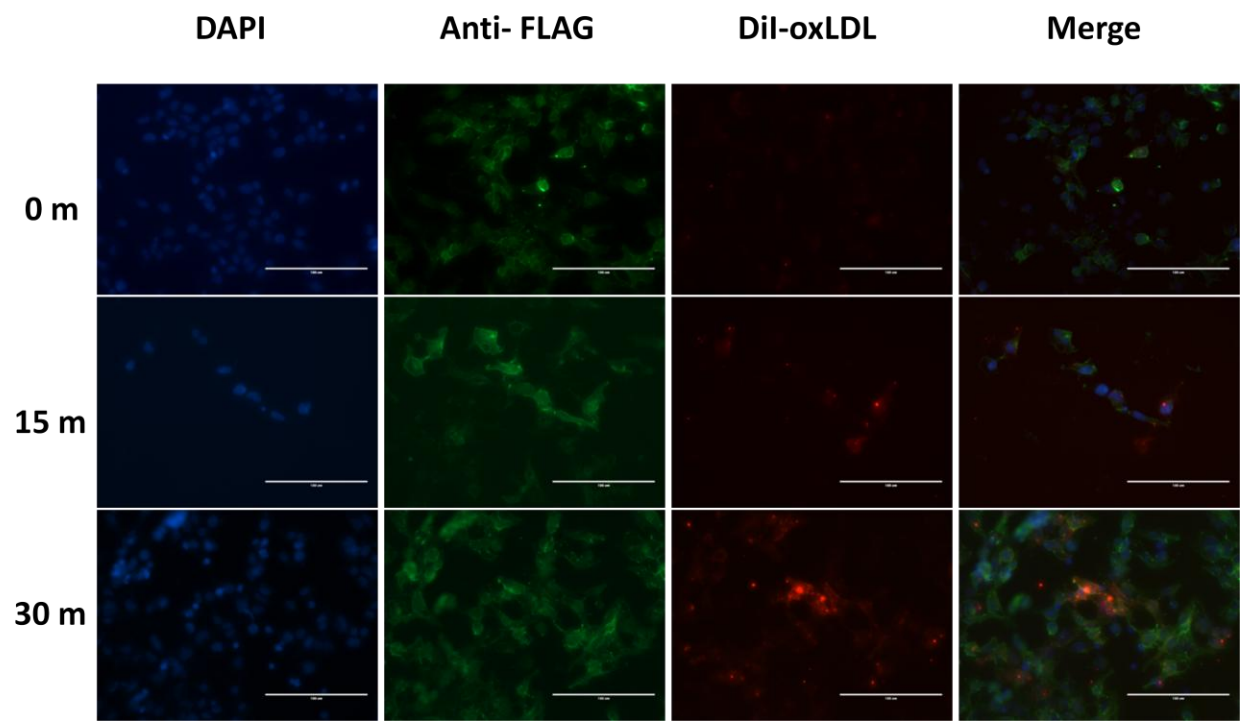
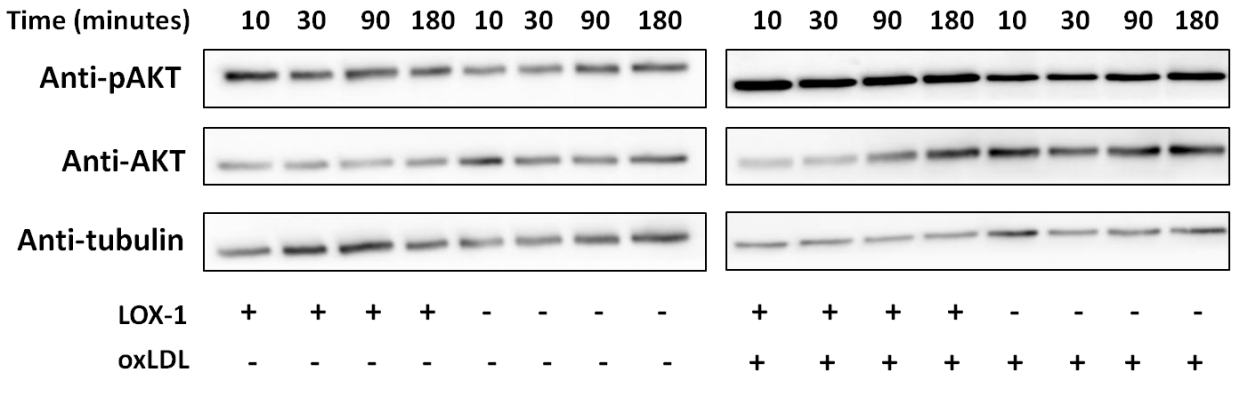


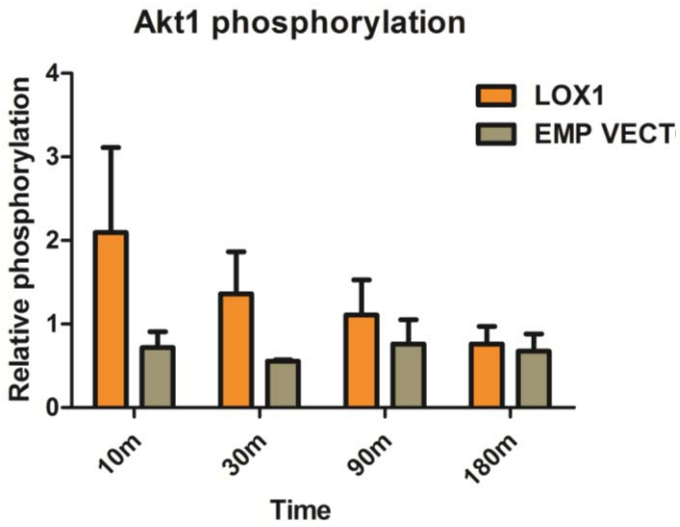
Figure 5. 4 LOX-1 mediated uptake of oxLDL by HEK293 cells
Immunofluorescence images after treatment with DiI labelled oxLDL (red) for different time intervals and staining with anti-FLAG primary and anti-mouse secondary antibody tagged to FITC (green), and DAPI (blue)

5.3.3.Induction of LOX-1 signaling by oxLDL

Treatment of oxLDL at different time intervals with 10μg/ml led to phosphorylation of Akt at Ser473 in HEK293 LOX1 cells but not in empty vector transfected cells as seen in the western blots (Figure 5.5a). Akt phosphorylation after different time intervals of treatment were compared to that without oxLDL treatment to determine the relative phosphorylation levels (Figure 5.5b)



(a)



(b)

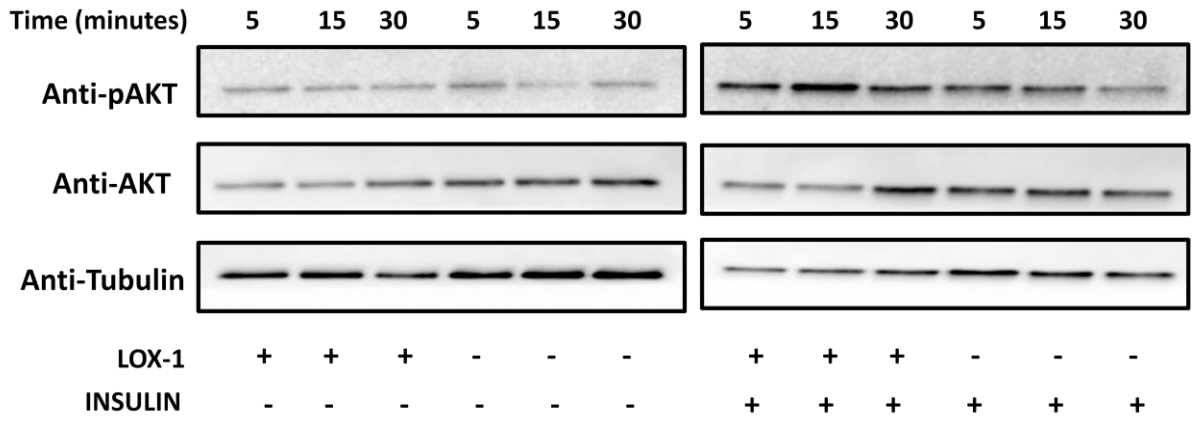
Figure 5. 5 LOX-1 pAkt signaling in response to oxLDL treatment

(a) Western blot for phospho Akt1 (Ser473), total Akt and tubulin in HEK293 cells and Empty vector transfected cells after oxLDL treatment for different time intervals **(b)** Quantification of the same; data expressed as average relative phosphorylation with respect to that of no treatment ±SEM

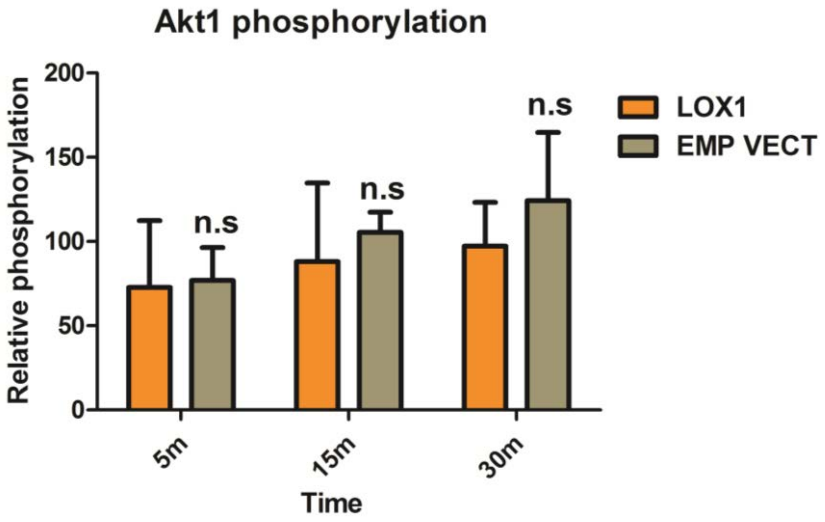
5.3.4.Modulation of Insulin Signaling by LOX-1

5.3.4.1. Insulin Signaling in HEK293 cells

Monitoring phosphorylation of Akt at Ser473 serves as a measure of activation of insulin signaling cascade. HEK293 LOX1 cells and empty vector transfected cells were treated with 100nM insulin for indicated time-points followed by western blot. Time-dependant increase in Akt phosphorylation was seen in both cells (Figure 5.6a). The levels of phosphorylation were calculated relative to that without insulin treatment (Figure 5.6b).



(a)



(b)

Figure 5. 6 Insulin signaling in HEK293 cells

(a) Western blot for phospho Akt1 (Ser473), total Akt and tubulin in HEK293 cells and Empty vector transfected cells after insulin treatment for different time intervals **(b)** Quantification of the same; data expressed as average relative phosphorylation with respect to that of no treatment ±SEM

5.3.4.2. oxLDL alters Insulin Signaling

To monitor the cross-talk between oxLDL and insulin signaling, cells were pre-treated with oxLDL for 30 minutes followed by insulin treatment over indicated time-points. Insulin mediated phosphorylation of Ser473 of Akt was then monitored by western blot and compared to cells without oxLDL pre-treatment (Figure 5.7a). oxLDL lead to a marked decrease in insulin dependant Akt phosphorylation in HEK293 LOX1 cells (Figure 5.7b) which was not observed in empty vecter transfected HEK293 cells (Figure 5.7c).

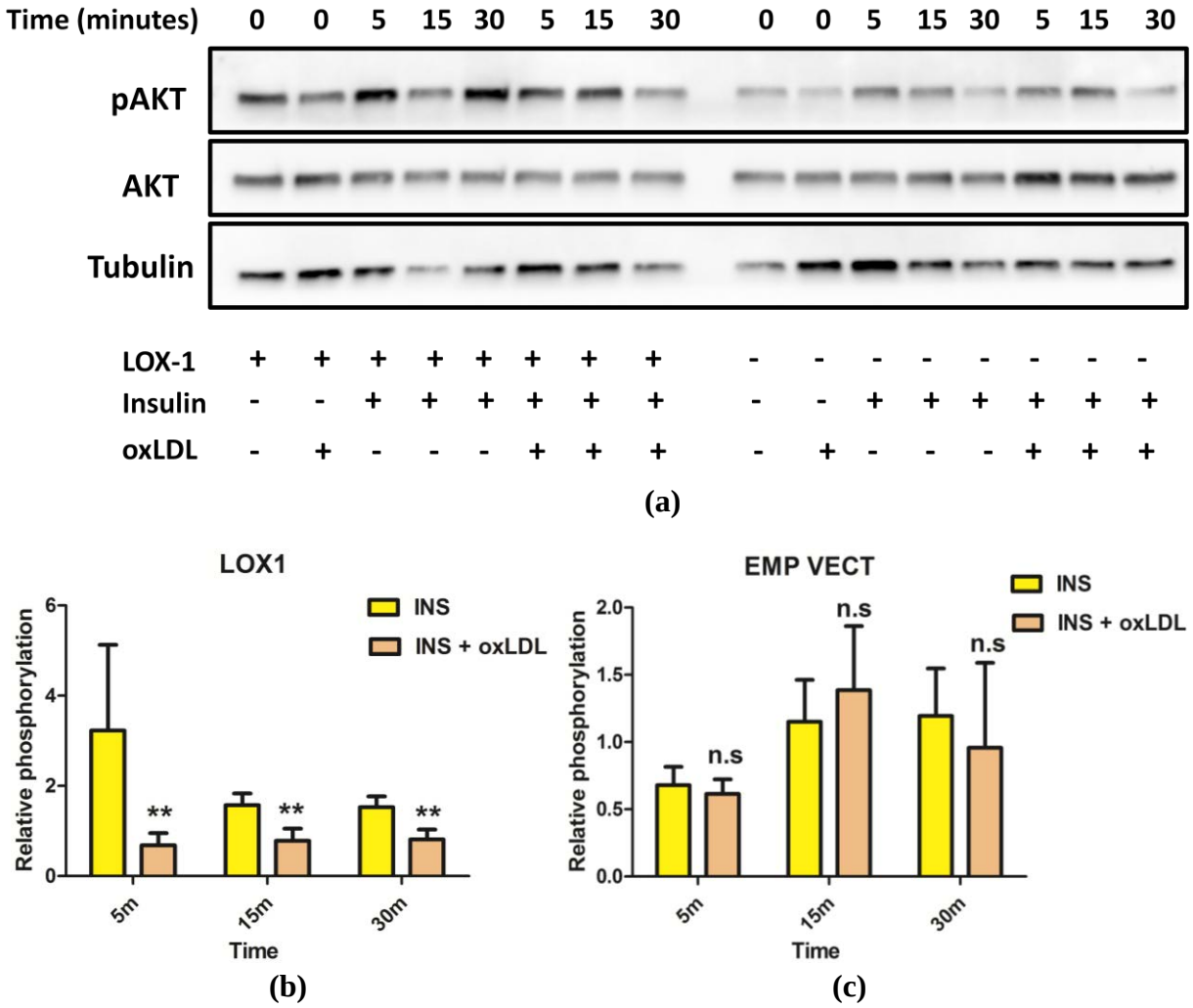


Figure 5. 7: LOX-1 mediated suppression of insulin Akt signaling
(a) Western blot for phospho Akt1 (Ser473) in HEK293 cells in response to insulin with and without oxLDL pre-treatment **(b)**, **(c)** Quantification of the same; data expressed as average relative phosphorylation with respect to that of no treatment ±SEM (** *p*-value < 0.001)

5.4. Discussion

oxLDL is has been well reported as risk factor and a biomarker for atherosclerosis[31, 32] and cardiovascular risk[33, 34]. Formation of oxLDL *in vivo* occurs via a number of mechanisms[35, 36] including copper or iron catalyzed oxidation[37], oxidation by oxidative free radicals like superoxide[38] and peroxynitrite[39] or via oxidizing enzymes such as lipoxygenase[40] and myeloperoxidase[41]. Formation of oxLDL is believed to occur in the arterial wall during atherogenesis[42], however, it may get released into circulation due to plaque rupture[43]. LDL

oxidation stimulates its uptake and accumulation by macrophages and leads to the formation of foam cells, which are the first hallmark of an atherosclerotic lesion and also a major source of inflammatory mediators involved in atherogenesis[44]. The major receptor involved in uptake of oxLDL is LOX1[45], although it also recognized by other scavenger receptors belonging to class A and B[46]. Moreover, LOX1 expression has also been shown to increase in conditions associated with atherosclerosis such as hypertension[47], diabetes[48], and hyperlipidemia[49] and interaction of oxLDL with LOX-1 is reported to be one of the major mechanisms leading to atherosclerosis[50].

Previous studies on LOX1 have shown that oxLDL binding regulates oxidative stress via activation of NADPH oxidase[51] and further leads to reduced nitric oxide production[52] as well as activation of p38 MAPK and NF-κB in endothelial cells[53, 54]. It reportedly inhibits Akt phosphorylation and thereby downregulates the expression and activation of eNOS[55]. It also leads to activation of protein kinase C (PKC) and increased expression of CD40, the key activator of inflammatory response in endothelial cells[56] which may further lead to the activation of caspases and apoptosis in endothelial cells[57]. Thus, oxLDL via LOX1 leads to endothelial dysfunction and injury, the initiating point for atherosclerosis. LOX-1 also mediates the increased expression and activity of collagenase or MMP-1 and stromelysin or MMP-3[58] leading to destabilization of the atherosclerotic plaque. In vascular smooth muscle cells as well, oxLDL binding to LOX1 has been shown to lead to apoptosis via Bax/Bcl-2 signaling[59] and via ER stress, JNK and p38 signaling[60], leading further to plaque destabilization and contributing to CVD. Interestingly, increased expression of LOX1 has been observed in adipose tissue in association with insulin resistance[61] as well as with expression of pro-inflammatory cytokines[62] in two separate studies. Since inflammation is one of the mechanisms which can lead to insulin resistance[63], the pro-inflammatory action of LOX1 can also contribute towards insulin resistance in endothelial cells. Therefore, we attempted to study whether a similar cross-talk also exists between insulin signaling and LOX-1 in endothelial cells.

Insulin binding to its receptor IR leads to the recruitment of IR substrate-1 and -2 (IRS) and phosphoinositide 3-kinase (PI3K) to its cytoplasmic tail. PI3K forms phosphatidylinositol-3,4,5-trisphosphate in the plasma membrane, which in turn recruits 3-phosphoinositide-dependent protein kinase 1 (PDK1). PDK1 activates various isoforms of protein kinase B (PKB) or Akt, which mediate the metabolic actions of insulin such as GLUT-4 translocation, expression

of enzymes involved in glycogen synthesis and lipid synthesis and so on[20]. The endothelial actions of insulin also involves activation of PI3K/Akt leading to phosphorylation of eNOS at Ser1177 and generation of NO[15]. It has already been shown that loss of Akt1 promotes atherosclerosis via enhanced expression of pro-inflammatory genes in Akt1 knock-out mice[64]. Therefore, the inhibitory effect of LOX1 on insulin dependent Akt signaling observed in our study may contribute towards insulin resistance in endothelial cells and consequent CVD.

5.5. Conclusion

We have used HEK293 over-expressing human LOX1 as a system to study LOX1 signaling after oxLDL binding as well as its cross-talk with insulin signaling. The inhibitory effect of LOX-1 on Akt signaling led to reduced insulin dependent Akt1 phophorylation, which can possibly lead to development of insulin resistance and endothelial dysfunction. However, further studies are required to establish this hypothesis.

5.6. Future Directions

Functional implications of reduced insulin dependent phosphorylation on endothelial function need to be studied further using endothelial cell models with LOX1 over-expression and down-regulation. Also, determining whether inhibition of oxLDL and LOX1 binding can improve insulin resistance and endothelial function will allow use of LOX1 inhibitors as a novel therapeutic approach to improve insulin sensitivity and vascular function in conditions like obesity, metabolic syndrome, diabetes and dyslipidemia. PPARγ agonists have been well reported to improve insulin sensitivity by improving insulin signaling[65]. Moreover, they have also been reported to inhibit LOX1 expression in response to TNF-α[66]. Therefore, it would be interesting to see if they can potentially improve vascular condition in insulin resistance and delay the progression of cardiovascular complications.

5.7. References

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Appendix 1: Modified peptides identified by LC-MS/MS

Glycation

Sr. No	Sequence	# PSMs	XCorr	z	MH+ [Da]	RT [min]	# Missed Cleavages	Modification site	Modifications
1	AA**C***LLP**K***LDELRDEGK	29	2.62	4	1990.02	35.45	2	K7	Amadori
2	ADD**K***ET**C***FAEEGK	23	1.84	3	1661.69	15.67	1	K4	Amadori
3	ADLA**K***YI**C***ENQDSISSK	57	3.41	3	2103.98	31.12	1	K5	Amadori
4	ADLA**K***YI**C***ENQDSISSK	16	3.25	3	1999.92	33.91	1	K5	Carboxymethyl
5	AEFAEVS**K***LVTDLTK	19	2.52	3	1722.89	59.09	1	K8	Carboxyethyl
6	AEFAEVS**K***LVTDLTK	29	3.02	3	1708.90	53.50	1	K8	Carboxymethyl
7	AF**K***AWAVAR	27	2.37	3	1181.63	26.56	1	K3	Amadori
8	AF**K***AWAVAR	15	1.84	3	1077.59	29.78	1	K3	Carboxymethyl
9	AT**K***EQL**K***AVMDDFAAFVEK	35	2.08	4	2361.16	54.12	2	K3,K7	Carboxymethyl, Amadori
10	AT**K***EQL**K***AVMDDFAAFVEK	10	2.18	4	2257.12	56.68	2	K3,K7	Carboxymethyl, Carboxymethyl
11	AVMDDFAAFVE**K***C*C***K	18	2.35	3	1848.80	44.47	1	K12	Carboxymethyl
12	AWAVA**R***LSQR	6	1.62	3	1319.71	27.69	1	R6	Amadori
13	**C***ASLQ**K***FGER	26	1.56	3	1357.64	19.17	1	K6	Amadori
14	**C***ASLQ**K***FGER	13	1.98	3	1253.60	21.68	1	K6	Carboxymethyl
15	EFNAETFTFHADI**C***TLSE**K***ER	41	3.70	3	2603.18	42.53	1	K19	Carboxymethyl
16	EQL**K***AVMDDFAAFVEK	42	3.31	3	2002.97	51.54	1	K4	Amadori
17	EQL**K***AVMDDFAAFVEK	17	3.09	3	1898.92	54.48	1	K4	Carboxymethyl
18	ETYGEMAD**C***C*A**K***QEPERNE**C***FLQHK	24	2.21	5	3292.37	26.52	2	K12	Amadori
19	ETYGEMAD**C***C*A**K***QEPERNE**C***FLQHK	4	2.90	5	3188.31	27.69	2	K12	Carboxymethyl
20	F**K***DLGEENFK	53	1.94	3	1388.66	24.46	1	K2	Amadori
21	FP**K***AEFAEVSK	22	2.77	3	1414.71	26.89	1	K3	Amadori
22	FP**K***AEFAEVS**K***LVTDLTK	77	2.51	4	2347.22	51.94	2	K3,K11	Amadori, Amadori
23	HPYFYAPELLFFA**K***R	16	2.61	3	2061.05	49.81	1	K14	Amadori

24	HPYFYAPELLFFA**K*** R	9	1.98	4	1971.02	52.88	1	K14	Carboxyethyl
25	HPYFYAPELLFFA**K*** R	37	3.84	3	1957.00	52.59	1	K14	Carboxymethyl
26	**K*** LVAASQAALGL	9	2.64	2	1303.75	34.16	1	K1	Amadori
27	**K*** LVAASQAALGL	7	3.13	2	1199.70	36.73	1	K1	Carboxymethyl
28	**K*** QTALVELVK	60	2.67	3	1290.75	28.48	1	K1	Amadori
29	**K*** QTALVELVK	10	2.64	3	1200.72	32.10	1	K1	Carboxyethyl
30	**K*** QTALVELVK	25	3.20	3	1186.71	31.66	1	K1	Carboxymethyl
31	**K*** VPQVSTPTLVEVSR	44	3.79	3	1802.00	31.24	1	K1	Amadori
32	**K*** VPQVSTPTLVEVSR	32	2.61	3	1711.93	35.79	1	K1	Carboxyethyl
33	**K*** VPQVSTPTLVEVSR	48	3.14	3	1697.95	33.52	1	K1	Carboxymethyl
34	**K*** YLYEIAR	18	2.14	3	1113.59	27.74	1	K1	Carboxymethyl
35	LA**K*** TYETTLEK	52	3.49	3	1458.76	18.88	1	K3	Amadori
36	LA**K*** TYETTLEK	24	2.69	3	1354.71	21.12	1	K3	Carboxymethyl
37	LA**K*** TYETTLE**K*C*C*** AAADPHE**C*** YAK	66	3.46	5	3154.41	24.94	2	K3,K11	Amadori, Amadori
38	L**K*C*** ASLQK	14	1.51	3	1109.59	11.76	1	K2	Amadori
39	LVNEVTEFA**K*** T**C*** VADESAEN**C*** DK	69	4.57	3	2791.23	35.47	1	K10	Amadori
40	LV**R*** PEVDVM**C*** TAFHDNEETFLK	13	2.81	4	2812.32	44.32	0	R3	Amadori
41	LVTDLT**K*** VHTE**C*C*** HGDLLE**C*** ADDR	60	3.87	4	3019.34	31.77	1	K7	Amadori
42	MP**C*** AEDYLSVVLNQL**C*** VLHE**K*** TPVSDR	53	3.06	4	3335.60	59.12	1	K21	Amadori
43	MP**C*** AEDYLSVVLNQL**C*** VLHE**K*** TPVSDR	45	4.40	4	3231.56	60.84	1	K21	Carboxymethyl
44	NE**C*** FLQH**K*** DDNPNLPR	27	2.34	4	2158.99	24.07	1	K8	Amadori
45	NYAEA**K*** DVFLGMFLYEYAR	73	3.86	3	2462.17	55.84	1	K6	Amadori
46	NYAEA**K*** DVFLGMFLYEYAR	7	3.14	3	2358.11	59.45	1	K6	Carboxymethyl
47	QI**K*K*** QTALVELVK	61	2.80	3	1822.05	26.85	2	K3,K4	Amadori, Amadori
48	QN**C*** ELFEQLGEY**K*** FQNALLVR	32	3.59	4	2761.35	50.14	1	K13	Amadori
49	QN**C*** ELFEQLGEY**K*** FQNALLVR	27	4.17	3	2657.31	51.91	1	K13	Carboxymethyl
50	**R*** HPDYSVVLLLR	12	3.63	3	1629.90	40.19	1	R1	Amadori
51	RHPYFYAPELLFFA**K*** R	34	2.77	4	2217.16	46.38	2	K15	Amadori
52	RHPYFYAPELLFFA**K*** R	25	4.57	4	2113.11	49.05	2	K15	Carboxymethyl
53	**R*** MP**C*** AEDYLSVVLNQL**C*** VLHEK	34	3.38	4	2836.37	60.12	1	R1	Amadori
54	SLHTLFGD**K*** L**C*** TVATLR	84	3.90	4	2094.10	40.06	1	K9	Amadori

55	T**C*** VADESAEN**C*** D**K*** SLHTLFGDK	30	3.38	4	2555.11	34.68	1	K13	Carboxymethyl
56	VFDEF**K*** PLVEEPQNLIK	48	2.37	3	2207.15	45.97	0	K6	Amadori
57	VFDEF**K*** PLVEEPQNLIK	27	3.35	3	2103.10	48.35	0	K6	Carboxymethyl
58	VT**K*C*C*** TESLVNR	80	2.82	3	1628.77	17.16	1	K3	Amadori
59	VT**K*C*C*** TESLVNR	24	2.90	3	1524.72	18.61	1	K3	Carboxymethyl
60	Y**K*** AAFTE**C*C*** QAADK	14	2.63	3	1720.74	20.58	1	K2	Carboxymethyl

Homocysteinylation

Sr. No	Sequence	# PSMs	XCorr	z	MH+ [Da]	RT [min]	# Missed Cleavages	Modification site	Modifications
1	**K*** VPQVSTPTLVEVSR	7	2.00	3	1756.97	19.29	1	K1	HCysThiolactone
2	**K*** QTALVELVK	2	1.73	2	1245.73	19.01	1	K1	HCysThiolactone
3	LA**K*** TYETTLEK	3	1.45	3	1413.73	14.27	1	K3	HCysThiolactone

Carbonylation

Sr. No	Sequence	# PSMs	XCorr	z	MH+ [Da]	RT [min]	# Missed Cleavages	Modification site	Modification
1	ALVLIAFAQYLQQ**C*** PFED**H*** VK	374	4.03	3	2646.40	35.46	0	H19	HNE
2	**C***C*AAADP**H***E**C*** YAK	7	1.45	3	1708.72	16.22	0	H8	HNE
3	EFNAETFTF**H*** ADI**C*** TLSEK	25	2.52	3	2416.14	28.85	0	H10	HNE
4	**H*** PDYSVVLLLR	17	4.69	3	1467.85	22.27	0	H1	HNE
5	**H*** PDYSVVLLLRLAKTYETTLEK	27	4.52	5	2745.53	28.73	2	H1	HNE
6	**H*** PYFYAPELLFFAKR	3	3.31	4	2055.10	25.98	1	H1	HNE
7	MP**C*** AEDYLSVVLNQL**C*** VL**H*** EK	93	2.11	3	2674.33	36.87	0	H19	HNE
8	R**H*** PDYSVVLLLR	41	4.58	3	1623.96	26.88	1	H2	HNE
9	R**H*** PYFYAPELLFFAK	60	5.22	3	2055.11	31.35	1	H2	HNE
10	RMP**C*** AEDYLSVVLNQL**C*** VL**H*** EK	15	1.69	4	2830.43	36.80	1	H20	HNE
11	S**H*C*** IAEVENDEMPADLPSLAADFVESK	19	5.37	3	3130.46	30.49	0	H2	HNE
12	SL**H*** TLFGDK	8	2.20	3	1173.65	25.50	0	H3	HNE

13	V**H*TE**C*C***HGDLLE**C***ADDR	61	3.67	3	2242.95	18.33	0	H2	HNE
14	V**H*TE**C*C***HGDLLE**C***ADDRADLAK	109	3.40	4	2741.23	18.99	1	H2	HNE

Nitration

Sr. No	Sequence	# PSMs	XCorr	z	MH+ [Da]	RT [min]	# Missed Cleavages	Modification site	Modification
1	**Y***LYEIAR	35	1.63	2	972.48	19.00	0	Y1	Nitro

Acetylation

Sr. No	Sequence	PLGS Score	z	MH+ (Da)	RT	Missed Cleavages	Modification Site	Modifications
1	LA**K***TYETTLEK	8.447	2.00	1338.7152	24.35	1	K3	Acetyl
2	AT**K***EQLK	8.2737	1.93	859.4884	17.84	1	K3	Acetyl
3	FP**K***AEFAEVSK	7.8666	2.00	1294.6678	28.80	1	K3	Acetyl
4	ADLA**K***YI**C***ENQDSISSK	8.898	2.10	1983.9329	29.65	1	K5	Acetyl
5	TYETTLE**K*C*C***AAADPHE**C***YAK	8.0084	3.00	2560.079	24.82	1	K8	Acetyl
6	**C**ASLQ**K***FGER	8.751	2.00	1237.5995	25.21	1	K6	Acetyl
7	YI**C***ENQDSISS**K***LK	9.0546	2.00	1726.8317	26.76	1	K12	Acetyl
8	ET**C***FAEEG**K***K	8.8113	1.98	1240.5515	20.33	1	K9	Acetyl
9	ETYGEMAD**C*C***A**K***QEPER	9.1427	2.20	2115.8418	23.13	1	K12	Acetyl
10	E**C*C***E**K***PLLEK	7.8396	2.00	1347.6284	24.80	1	K5	Acetyl
11	VT**K*C*C***TESLVNR	7.7904	2.01	1508.7197	22.70	1	K3	Acetyl
12	AF**K***AWAVAR	7.7162	2.00	1061.5891	29.53	1	K3	Acetyl

Appendix 2: Differentially abundant proteins in HUVEC after Gly-HSA treatment

Sr. No	Gene Symbol	Protein Name	Accession	*p*-value	Fold Change
1	ICAM1	Intercellular adhesion molecule 1	P05362	5.77E-05	20.24096
2	SODM	Superoxide dismutase [Mn], mitochondrial	P04179	3.65E-09	4.723621
3	SPCS2	Signal peptidase complex subunit 2	Q15005	0.01829	4.514596
4	CD44	CD44 antigen	P16070	0.03675	3.195435
5	VDAC1	Voltage-dependent anion-selective channel protein 1	P21796	0.00114	5.47831
6	TMED9	Transmembrane emp24 domain-containing protein 9	Q9BVK6	0.00024	3.88557
7	SQSTM	Sequestosome-1	Q13501	0.00367	4.228536
8	1A69	HLA class I histocompatibility antigen, A-69 alpha chain	P10316	2.03E-09	7.535251
9	MYO1C	Unconventional myosin-Ic	O00159	0.04397	3.799967
10	PAI1	Plasminogen activator inhibitor 1	P05121	5.46E-05	3.400402
11	COPB	Coatomer subunit beta	P53618	0.01395	3.042634
12	CLIC4	Chloride intracellular channel protein 4	Q9Y696	0.00105	3.989235
13	LMAN1	Protein ERGIC-53	P49257	1.35E-05	2.916478
14	ILK	Integrin-linked protein kinase	Q13418	0.05183	2.600135
15	MYG1	UPF0160 protein MYG1, mitochondrial	Q9HB07	0.02546	2.405061
16	TMOD3	Tropomodulin-3	Q9NYL9	7.86E-05	2.990414
17	PODXL	Podocalyxin	O00592	0.00409	3.265005
18	SODC	Superoxide dismutase [Cu-Zn]	P00441	0.0114	2.402104
19	MMP14	Matrix metalloproteinase-14	P50281	0.0183	4.563727
20	SORCN	Sorcin	P30626	0.00904	2.267286
21	CAV1	Caveolin-1	Q03135	0.01422	3.350593
22	ITA2	Integrin alpha-2	P17301	0.03556	2.665498
23	VAMP3	Vesicle-associated membrane protein 3	Q15836	0.00425	1.806659
24	DREB	Drebrin	Q16643	0.02178	2.411742
25	ANXA4	Annexin A4	P09525	0.0159	2.036689
26	UGPA	UTP--glucose-1-phosphate uridylyltransferase	Q16851	0.03107	1.755062
27	S10A6	Protein S100-A6	P06703	1.7E-06	2.193405
28	VPS35	Vacuolar protein sorting-associated protein 35	Q96QK1	0.02355	1.805632
29	LA	Lupus La protein	P05455	0.01574	1.738587
30	FPPS	Farnesyl pyrophosphate synthase	P14324	0.02735	1.916265
31	C1TC	C-1-tetrahydrofolate synthase, cytoplasmic	P11586	0.00891	1.87873
32	RAC1	Ras-related C3 botulinum toxin substrate 1	P63000	0.00191	1.972941
33	VDAC2	Voltage-dependent anion-selective channel protein 2	P45880	0.00033	3.373027
34	COPB2	Coatomer subunit beta'	P35606	0.04172	1.573074
35	URP2	Fermitin family homolog 3	Q86UX7	0.01681	1.64211
36	EFHD2	EF-hand domain-containing protein D2	Q96C19	3.58E-05	3.063579
37	ITB1	Integrin beta-1	P05556	0.0027	1.815945
38	RAB1B	Ras-related protein Rab-1B	Q9H0U4	0.0281	1.49917

39	1433F	14-3-3 protein eta	Q04917	0.02105	1.656893
40	EDF1	Isoform 3 of Endothelial differentiation-related factor 1	O60869-3	0.02366	1.634201
41	RLA1	60S acidic ribosomal protein P1	P05386	0.04712	20.22736
42	TBA1B	Tubulin alpha-1B chain	P68363	0.03781	9.313704
43	ACTBL	Beta-actin-like protein 2	Q562R1	0.0014	5.341919
44	ACTS	Actin, alpha skeletal muscle	P68133	0.02351	4.077245
45	IPO5	Importin-5	O00410	0.01086	3.174185
46	ACTC	Actin, alpha cardiac muscle 1	P68032	0.00811	3.417696
47	RECQ1	ATP-dependent DNA helicase Q1	P46063	0.00768	2.662088
48	RS24	40S ribosomal protein S24	P62847	0.04865	2.725348
49	AHSA1	Activator of 90 kDa heat shock protein ATPase homolog 1	O95433	0.03128	3.001072
50	RFA3	Replication protein A 14 kDa subunit	P35244	0.04488	2.772527
51	HDAC1	Histone deacetylase 1	Q13547	0.01821	2.375203
52	CBR1	Carbonyl reductase [NADPH] 1	P16152	0.01651	3.276714
53	RL10	60S ribosomal protein L10	P27635	0.01314	2.216677
54	UB2L3	Ubiquitin-conjugating enzyme E2 L3	P68036	0.01738	2.741598
55	TBB3	Tubulin beta-3 chain	Q13509	0.03935	2.556583
56	PSA4	Proteasome subunit alpha type-4	P25789	0.03099	2.351957
57	CP1B1	Cytochrome P450 1B1	Q16678	0.03499	2.731848
58	TOLIP	Toll-interacting protein	Q9H0E2	0.04637	2.145267
59	HDGF	Hepatoma-derived growth factor	P51858	0.00713	2.472877
60	PAPS2	Bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthase 2	O95340	0.02883	3.919229
61	RUXE	Small nuclear ribonucleoprotein E	P62304	0.05144	1.713218
62	PPM1F	Protein phosphatase 1F	P49593	0.04987	2.946553
63	DPP3	Dipeptidyl peptidase 3	Q9NY33	0.00166	2.093835
64	PRS4	26S proteasome regulatory subunit 4	P62191	0.00929	1.937758
65	RS27L	40S ribosomal protein S27-like	Q71UM5	0.05332	2.148119
66	PA1B2	Platelet-activating factor acetylhydrolase IB subunit beta	P68402	6.92E-06	2.244056
67	ACTG	Actin, cytoplasmic 2	P63261	0.00144	1.867436
68	TBA1C	Tubulin alpha-1C chain	Q9BQE3	0.02754	2.215804
69	RL32	60S ribosomal protein L32	P62910	0.00051	1.816502
70	NAGK	N-acetyl-D-glucosamine kinase	Q9UJ70	0.01223	1.785502
71	LIS1	Platelet-activating factor acetylhydrolase IB subunit alpha	P43034	0.04655	1.623553
72	PGM2	Phosphoglucomutase-2	Q96G03	0.02449	1.694239
73	MARCS	Myristoylated alanine-rich C-kinase substrate	P29966	0.00155	1.855946
74	PGBM	Basement membrane-specific heparan sulfate proteoglycan core protein	P98160	0.00028	2.517245
75	CDC42	Cell division control protein 42 homolog	P60953	0.00169	1.866395
76	BUB3	Mitotic checkpoint protein BUB3	O43684	0.01539	1.815156
77	RAB7A	Ras-related protein Rab-7a	P51149	4.57E-05	1.743098

78	CSRP1	Cysteine and glycine-rich protein 1	P21291	0.02333	2.688004
79	RS23	40S ribosomal protein S23	P62266	5.47E-06	1.877169
80	ACTN4	Alpha-actinin-4	O43707	2.52E-07	1.593878
81	TPM1	Isoform 5 of Tropomyosin alpha-1 chain	P09493-5	0.03129	2.117622
82	AAMDC	Mth938 domain-containing protein	Q9H7C9	0.04328	2.63373
83	SSRD	Translocon-associated protein subunit delta	P51571	0.00067	2.669088
84	SYAC	Alanine--tRNA ligase, cytoplasmic	P49588	0.01068	1.776834
85	G6PI	Glucose-6-phosphate isomerase	P06744	0.00213	1.7046
86	IF2B3	Insulin-like growth factor 2 mRNA-binding protein 3	O00425	0.00606	1.655347
87	RL29	60S ribosomal protein L29	P47914	0.02859	2.167646
88	ERF3A	Isoform 3 of Eukaryotic peptide chain release factor GTP-binding subunit ERF3A	P15170-3	0.05362	1.880877
89	COPE	Coatomer subunit epsilon	O14579	0.04941	2.232055
90	RS6	40S ribosomal protein S6	P62753	0.00169	1.721315
91	SGTA	Small glutamine-rich tetratricopeptide repeat-containing protein alpha	O43765	0.04844	2.130753
92	IPYR	Inorganic pyrophosphatase	Q15181	0.00027	1.850132
93	TYB4	Thymosin beta-4	P62328	0.04325	2.189354
94	CNDP2	Cytosolic non-specific dipeptidase	Q96KP4	0.02893	2.469008
95	6PGL	6-phosphogluconolactonase	O95336	0.00863	2.410028
96	PFKAL	ATP-dependent 6-phosphofructokinase, liver type	P17858	0.03992	2.447967
97	COPD	Coatomer subunit delta	P48444	0.03148	1.622134
98	MVP	Major vault protein	Q14764	0.00079	1.644603
99	HS71B	Heat shock 70 kDa protein 1B	P0DMV9	0.00212	1.822235
100	EHD4	EH domain-containing protein 4	Q9H223	0.01393	1.713978
101	PSMD1	26S proteasome non-ATPase regulatory subunit 1	Q99460	0.04651	1.385545
102	COR1C	Coronin-1C	Q9ULV4	0.00039	1.590825
103	NUCB1	Nucleobindin-1	Q02818	0.03442	1.953299
104	E41L3	Band 4.1-like protein 3	Q9Y2J2	0.00704	2.050896
105	STRAP	Serine-threonine kinase receptor-associated protein	Q9Y3F4	0.00946	1.420293
106	STIP1	Stress-induced-phosphoprotein 1	P31948	0.00555	1.468973
107	PSA3	Proteasome subunit alpha type-3	P25788	0.00491	1.645025
108	RS19	40S ribosomal protein S19	P39019	0.01386	1.452783
109	PSB2	Proteasome subunit beta type-2	P49721	0.0025	1.424686
110	PSD12	26S proteasome non-ATPase regulatory subunit 12	O00232	0.02278	1.447876
111	SYTC	Threonine--tRNA ligase, cytoplasmic	P26639	0.02026	1.697275
112	MIF	Macrophage migration inhibitory factor	P14174	7.11E-06	2.814128
113	MYADM	Myeloid-associated differentiation marker	Q96S97	8.46E-06	3.1236
114	GSTO1	Glutathione S-transferase omega-1	P78417	0.00012	2.440885
115	AKAP2	A-kinase anchor protein 2	Q9Y2D5	5.33E-07	2.303475
116	PHB	Prohibitin	P35232	2.47E-05	3.132465
117	CD59	CD59 glycoprotein	P13987	5.34E-06	2.724328
118	GBB1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	P62873	0.00014	2.616662

119	CSN2	COP9 signalosome complex subunit 2	P61201	1.8E-06	2.159531
120	GSLG1	Golgi apparatus protein 1	Q92896	0.00116	2.552877
121	TYB10	Thymosin beta-10	P63313	3.32E-06	2.102192
122	G6PD	Glucose-6-phosphate 1-dehydrogenase	P11413	9.96E-07	2.122525
123	FKB1A	Peptidyl-prolyl cis-trans isomerase FKBP1A	P62942	1.39E-06	2.064066
124	CHSP1	Calcium-regulated heat-stable protein 1	Q9Y2V2	5.52E-05	2.19605
125	NP1L4	Nucleosome assembly protein 1-like 4	Q99733	0.01241	2.172553
126	PUR4	Phosphoribosylformylglycinamidine synthase	O15067	0.00484	2.122447
127	RS5	40S ribosomal protein S5	P46782	2.15E-05	1.97976
128	CASP3	Caspase-3	P42574	2.58E-05	2.103754
129	UBC9	SUMO-conjugating enzyme UBC9	P63279	0.01515	2.041214
130	TCP4	Activated RNA polymerase II transcriptional coactivator p15	P53999	3.84E-05	1.990177
131	JUPI1	Jupiter microtubule associated homolog 1	Q9UK76	0.00122	1.974904
132	ENOG	Gamma-enolase	P09104	3.12E-06	2.053557
133	PGRC1	Membrane-associated progesterone receptor component 1	O00264	0.00906	2.292907
134	HINT1	Histidine triad nucleotide-binding protein 1	P49773	4.72E-05	1.923384
135	GAPR1	Golgi-associated plant pathogenesis-related protein 1	Q9H4G4	0.01846	3.098419
136	RHOC	Rho-related GTP-binding protein RhoC	P08134	1.24E-06	1.867514
137	DDAH2	N(G),N(G)-dimethylarginine dimethylaminohydrolase 2	O95865	4.25E-05	1.927592
138	ALDR	Aldose reductase	P15121	1.1E-06	1.866982
139	SF3B3	Splicing factor 3B subunit 3	Q15393	0.00231	2.367408
140	ITAV	Integrin alpha-V	P06756	0.0012	3.251561
141	COTL1	Coactosin-like protein	Q14019	2.12E-05	2.125049
142	CTNB1	Catenin beta-1	P35222	0.00032	2.238417
143	VATB2	V-type proton ATPase subunit B, brain isoform	P21281	7.63E-05	2.413993
144	PP1A	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	P62136	4.19E-05	1.943009
145	LDHA	L-lactate dehydrogenase A chain	P00338	0.0001	1.716947
146	BASP1	Brain acid soluble protein 1	P80723	8.3E-06	1.869395
147	PML	Protein PML	PML	2.46E-05	2.082701
148	DNM1L	Dynamin-1-like protein	O00429	0.02189	1.660079
149	MARE1	Microtubule-associated protein RP/EB family member 1	Q15691	0.00205	1.596139
150	BASI	Basigin	P35613	8.33E-05	2.255466
151	ARL8A	ADP-ribosylation factor-like protein 8A	Q96BM9	6.32E-05	1.683445
152	ARPC5	Actin-related protein 2/3 complex subunit 5	O15511	0.00691	1.557139
153	LAMP1	Lysosome-associated membrane glycoprotein 1	P11279	1.01E-05	1.555533
154	SH3G1	Endophilin-A2	Q99961	0.01489	1.615728
155	RS18	40S ribosomal protein S18	P62269	0.00059	1.479771
156	RB11A	Isoform 2 of Ras-related protein Rab-11A	P62491-2	0.01285	1.442815
157	H13	Histone H1.3	P16402	9.24E-05	1.474754

158	PLIN3	Perilipin-3	O60664	0.05371	1.560074
159	RAP1B	Ras-related protein Rap-1b	P61224	0.00186	1.575612
160	CAZA2	F-actin-capping protein subunit alpha-2	P47755	0.0106	1.55413
161	RUXF	Small nuclear ribonucleoprotein F	P62306	0.00728	1.857592
162	ENPL	Endoplasmin	P14625	0.00102	0.5702
163	TCPG	T-complex protein 1 subunit gamma	P49368	0.02443	0.453458
164	CATB	Cathepsin B	P07858	0.00367	0.507901
165	EFTU	Elongation factor Tu, mitochondrial	P49411	4.23E-06	0.584283
166	HSP7C	Heat shock cognate 71 kDa protein	P11142	0.00809	0.505387
167	SRSF3	Serine/arginine-rich splicing factor 3	P84103	1.06E-05	0.512206
168	GRP75	Stress-70 protein, mitochondrial	P38646	0.0005	0.315422
169	DDX5	Probable ATP-dependent RNA helicase DDX5	P17844	2.36E-05	0.638751
170	KAD4	Adenylate kinase 4, mitochondrial	P27144	0.01357	0.611697
171	PDIA1	Protein disulfide-isomerase	P07237	0.00464	0.394304
172	PDIA6	Protein disulfide-isomerase A6	Q15084	3.88E-05	0.240473
173	PABP1	Polyadenylate-binding protein 1	P11940	0.00794	0.454759
174	TPM1	Isoform 5 of Tropomyosin alpha-1 chain	P09493-5	0.03129	0.39025
175	ERP29	Endoplasmic reticulum resident protein 29	P30040	0.00216	0.301585
176	CH60	60 kDa heat shock protein, mitochondrial	P10809	9.83E-08	0.585809
177	GLSK	Isoform 3 of Glutaminase kidney isoform, mitochondrial	O94925-3	5.1E-05	0.568844
178	AATM	Aspartate aminotransferase, mitochondrial	P00505	0.00025	0.63217
179	DX39B	Spliceosome RNA helicase DDX39B	Q13838	0.00013	0.520517
180	PGH1	Prostaglandin G/H synthase 1	P23219	9.73E-05	0.504908
181	MMRN1	Multimerin-1	Q13201	1.74E-07	0.447411
182	LMNA	Prelamin-A/C	P02545	9.22E-06	0.277218
183	DECR	2,4-dienoyl-CoA reductase, mitochondrial	Q16698	4.85E-05	0.439365
184	OAT	Ornithine aminotransferase, mitochondrial	P04181	0.00024	0.424374
185	CHCH2	Coiled-coil-helix-coiled-coil-helix domain-containing protein 2	Q9Y6H1	3.25E-07	0.160514
186	SIAS	Sialic acid synthase	Q9NR45	0.02751	0.501557
187	PLEC	Plectin	Q15149	0.01878	0.534487
188	CISY	Citrate synthase, mitochondrial	O75390	0.03208	0.706356
189	HYOU1	Hypoxia up-regulated protein 1	Q9Y4L1	0.00093	0.493776
190	CATD	Cathepsin D	P07339	0.0292	0.513366
191	MDHM	Malate dehydrogenase, mitochondrial	P40926	2.17E-07	0.55156
192	ECI1	Enoyl-CoA delta isomerase 1, mitochondrial	P42126	0.00915	0.539415
193	F213A	Redox-regulatory protein FAM213A	Q9BRX8	1.54E-05	0.637543
194	TCPZ	T-complex protein 1 subunit zeta	P40227	0.00181	0.344352
195	HNRH1	Heterogeneous nuclear ribonucleoprotein H	P31943	0.04899	0.26355
196	XRCC6	X-ray repair cross-complementing protein 6	P12956	0.00402	0.469123
197	SUCB2	Succinate--CoA ligase [GDP-forming] subunit beta, mitochondrial	Q96I99	0.03487	0.678976
198	ROA3	Heterogeneous nuclear ribonucleoprotein A3	P51991	0.00341	0.402347

199	SERPH	Serpin H1	P50454	2.45E-06	0.111533
200	RCN1	Reticulocalbin-1	Q15293	0.01539	0.741789
201	EIF3A	Eukaryotic translation initiation factor 3 subunit A	Q14152	0.01787	0.656694
202	ECHA	Trifunctional enzyme subunit alpha, mitochondrial	P40939	1.55E-05	0.493958
203	GNS	N-acetylglucosamine-6-sulfatase	P15586	0.00018	0.595143
204	IF5	Eukaryotic translation initiation factor 5	P55010	0.00656	0.541542
205	TLN1	Talin-1	Q9Y490	0.0003	0.522567
206	PDIA4	Protein disulfide-isomerase A4	P13667	0.00336	0.612823
207	PLOD1	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	Q02809	0.03044	0.4181
208	SPEE	Spermidine synthase	P19623	0.03499	0.598683
209	HCD2	3-hydroxyacyl-CoA dehydrogenase type-2	Q99714	0.00265	0.656101
210	FHOD3	Isoform 4 of FH1/FH2 domain-containing protein 3	Q2V2M9-4	0.00426	0.389507
211	FLNB	Filamin-B	O75369	0.00419	0.520856
212	C1QBP	Complement component 1 Q subcomponent-binding protein, mitochondrial	Q07021	0.00851	0.460389
213	EF2	Elongation factor 2	P13639	0.03874	0.525581
214	SPRC	SPARC	P09486	0.00977	0.475629
215	GLU2B	Glucosidase 2 subunit beta	P14314	0.01116	0.426422
216	IDHP	Isocitrate dehydrogenase [NADP], mitochondrial	P48735	3.84E-07	0.475996
217	FUBP2	Far upstream element-binding protein 2	Q92945	1.76E-06	0.669546
218	RALY	RNA-binding protein Raly	Q9UKM9	9.27E-05	0.200491
219	CATZ	Cathepsin Z	Q9UBR2	0.00337	0.669725
220	TBB6	Tubulin beta-6 chain	Q9BUF5	0.00086	0.487212
221	ECHM	Enoyl-CoA hydratase, mitochondrial	P30084	7.23E-06	0.322516
222	CYC	Cytochrome c	P99999	0.00815	0.504006
223	CALU	Calumenin	O43852	0.00612	0.490565
224	GPDM	Glycerol-3-phosphate dehydrogenase, mitochondrial	P43304	4.82E-06	0.469771
225	GANAB	Neutral alpha-glucosidase AB	Q14697	2.98E-06	0.1322
226	FLNC	Filamin-C	Q14315	0.00435	0.669076
227	EF1D	Elongation factor 1-delta	P29692	0.00023	0.593029
228	LETM1	Mitochondrial proton/calcium exchanger protein	O95202	0.00349	0.358702
229	TSP1	Thrombospondin-1	P07996	0.00085	0.35092
230	KAP0	cAMP-dependent protein kinase type I-alpha regulatory subunit	P10644	0.00568	0.307714
231	ALBU	Serum albumin	P02768	0.02734	0.501365
232	APMAP	Adipocyte plasma membrane-associated protein	Q9HDC9	5.46E-07	0.411766
233	MAP1B	Microtubule-associated protein 1B	P46821	0.00052	0.578003
234	SFPQ	Splicing factor, proline- and glutamine-rich	P23246	1.65E-07	0.579818
235	HNRPR	Heterogeneous nuclear ribonucleoprotein R	O43390	7.87E-05	0.380578
236	PPIA	Peptidyl-prolyl cis-trans isomerase A	P62937	0.00289	0.505086
237	NUCB2	Nucleobindin-2	P80303	0.02451	0.470185
238	GRP78	78 kDa glucose-regulated protein	P11021	0.00385	0.124557
239	SLIRP	SRA stem-loop-interacting RNA-binding protein, mitochondrial	Q9GZT3	0.00144	0.54194

240	LEG1	Galectin-1	P09382	2.86E-05	0.553285
241	ALDH2	Aldehyde dehydrogenase, mitochondrial	P05091	0.00061	0.608122
242	CH10	10 kDa heat shock protein, mitochondrial	P61604	3.98E-05	0.559171
243	P3H3	Prolyl 3-hydroxylase 3	Q8IVL6	0.00013	0.400737
244	LPPRC	Leucine-rich PPR motif-containing protein, mitochondrial	P42704	6.55E-05	0.556782
245	C1TM	Monofunctional C1-tetrahydrofolate synthase, mitochondrial	Q6UB35	0.00025	0.31889
246	AL1A1	Retinal dehydrogenase 1	P00352	0.00015	0.301713
247	TIM13	Mitochondrial import inner membrane translocase subunit Tim13	Q9Y5L4	0.00149	0.409379
248	HNRDL	Heterogeneous nuclear ribonucleoprotein D-like	O14979	8.43E-07	0.449263
249	RSMN	Small nuclear ribonucleoprotein-associated protein N	P63162	0.00044	0.488966
250	KHDR1	KH domain-containing, RNA-binding, signal transduction-associated protein 1	Q07666	0.00328	0.495463
251	ODO2	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	P36957	8.9E-06	0.39411
252	VWF	von Willebrand factor	P04275	2.85E-07	0.483379
253	TPP1	Tripeptidyl-peptidase 1	O14773	0.00084	0.305864
254	ATPB	ATP synthase subunit beta, mitochondrial	P06576	3.89E-05	0.245103
255	SMD1	Small nuclear ribonucleoprotein Sm D1	P62314	0.00085	0.145238
256	IMA1	Importin subunit alpha-1	P52292	0.00963	0.406813
257	TXND5	Thioredoxin domain-containing protein 5	Q8NBS9	1.07E-05	0.080149
258	SRSF7	Serine/arginine-rich splicing factor 7	Q16629	2.55E-05	0.508973
259	ATPA	ATP synthase subunit alpha, mitochondrial	P25705	0.00023	0.314086
260	CATC	Dipeptidyl peptidase 1	P53634	0.00028	0.279629
261	DHE3	Glutamate dehydrogenase 1, mitochondrial	P00367	7.02E-06	0.365557
262	EGLN	Endoglin	P17813	2.31E-07	0.424231
263	GNAQ	Guanine nucleotide-binding protein G(q) subunit alpha	P50148	0.00133	0.294205
264	PA1B3	Platelet-activating factor acetylhydrolase IB subunit gamma	Q15102	0.02462	0.322952
265	ATPG	ATP synthase subunit gamma, mitochondrial	P36542	0.0005	0.361723
266	H2B2E	Histone H2B type 2-E	Q16778	1.02E-05	0.397441
267	ICAM2	Intercellular adhesion molecule 2	P13598	7.28E-05	0.334323
268	NLTP	Non-specific lipid-transfer protein	P22307	3.4E-06	0.354413
269	H2AJ	Histone H2A.J	Q9BTM1	1.93E-07	0.240631
270	COX5A	Cytochrome c oxidase subunit 5A, mitochondrial	P20674	7.59E-08	0.324469
271	H4	Histone H4	P62805	1.94E-09	0.267303
272	SPB9	Serpin B9	P50453	0.025	0.647821
273	FBLN3	EGF-containing fibulin-like extracellular matrix protein 1	Q12805	0.00479	0.570135
274	NIBL1	Niban-like protein 1	Q96TA1	0.00657	0.563097
275	SYSC	Serine--tRNA ligase, cytoplasmic	P49591	0.00014	0.383472
276	IDHC	Isocitrate dehydrogenase [NADP] cytoplasmic	O75874	0.00487	0.335823

277	P4HA1	Prolyl 4-hydroxylase subunit alpha-1	P13674	7.33E-09	0.454328
278	DPYL2	Dihydropyrimidinase-related protein 2	Q16555	4.69E-08	0.448795
279	RL23A	60S ribosomal protein L23a	P62750	0.02933	0.426584
280	PRDX4	Peroxiredoxin-4	Q13162	3.06E-10	0.429889
281	FLNA	Filamin-A	P21333	8.99E-05	0.402438
282	AIMP2	Aminoacyl tRNA synthase complex-interacting multifunctional protein 2	Q13155	6.46E-09	0.26433
283	SEP11	Septin-11	Q9NVA2	7.15E-06	0.259319
284	CAVN1	Caveolae-associated protein 1	Q6NZI2	1.24E-09	0.198254

Appendix 3: Differentially abundant proteins after Homocys-albumin treatment

Sr. No.	Gene Symbol	Protein Name	Accession	*p*-value	Fold Change
1	LGMN	Legumain	Q99538	0.00216	1.982788
2	NQO1	NAD(P)H dehydrogenase [quinone] 1	P15559	0.00178	1.856427
3	UBE3D	E3 ubiquitin-protein ligase E3D	Q7Z6J8	0.00476	1.851472
4	MYG1	UPF0160 protein MYG1, mitochondrial	Q9HB07	0.01859	1.726781
5	TMED2	Transmembrane emp24 domain-containing protein 2	Q15363	0.0246	1.620446
6	ACTBL	Beta-actin-like protein 2	Q562R1	0.00168	1.610975
7	AIFM1	Apoptosis-inducing factor 1, mitochondrial	O95831	0.00895	1.58553
8	NDKA	Nucleoside diphosphate kinase A	P15531	0.001	1.550428
9	RS24	40S ribosomal protein S24	P62847	0.0002	1.537777
10	FKBP5	Peptidyl-prolyl cis-trans isomerase FKBP5	Q13451	0.00142	1.427206
11	UB2D3	Ubiquitin-conjugating enzyme E2 D3	P61077	0.00193	1.410992
12	UB2L3	Ubiquitin-conjugating enzyme E2 L3	P68036	0.02376	1.40136
13	MCM2	DNA replication licensing factor MCM2	P49736	0.00872	1.396831
14	RL35A	60S ribosomal protein L35a	P18077	0.00033	1.396308
15	HEBP2	Isoform 2 of Heme-binding protein 2	Q9Y5Z4-2	0.02239	1.389573
16	SCRN1	Secernin-1	Q12765	0.00224	1.375059
17	PXDN	Peroxidasin homolog	Q92626	0.00407	1.357282
18	BCAT2	Branched-chain-amino-acid aminotransferase, mitochondrial	O15382	0.04558	1.346837
19	TMX1	Thioredoxin-related transmembrane protein 1	Q9H3N1	0.01492	1.345042
20	KAD1	Adenylate kinase isoenzyme 1	P00568	0.02133	1.33605
21	CSN4	COP9 signalosome complex subunit 4	Q9BT78	0.04759	1.321038
22	FBRL	rRNA 2'-O-methyltransferase fibrillarin OS	P22087	0.03777	1.31167
23	H2A1C	Histone H2A type 1-C	Q93077	0.00745	0.75304
24	PPT1	Palmitoyl-protein thioesterase 1	P50897	0.04295	0.752088
25	PGBM	Basement membrane-specific heparan sulfate proteoglycan core protein	P98160	3.10E-08	0.750295

26	ITA5	Integrin alpha-5	P08648	2.40E-07	0.748844
27	LMNA	Prelamin-A/C	P02545	0.00159	0.745521
28	HIP1R	Huntingtin-interacting protein 1-related protein	O75146	7.72E-06	0.742906
29	H2B2D	Putative histone H2B type 2-D	Q6DRA6	0.00087	0.737643
30	VAMP5	Vesicle-associated membrane protein 5	O95183	0.02985	0.732768
31	JKIP3	Isoform 2 of Janus kinase and microtubule-interacting protein 3	Q5VZ66-2	0.0106	0.728006
32	EF1G	Elongation factor 1-gamma	P26641	1.30E-07	0.72478
33	PHB	Prohibitin	P35232	9.12E-06	0.720812
34	U2AF2	Splicing factor U2AF 65 kDa subunit	P26368	0.02179	0.714274
35	FA98A	Protein FAM98A	Q8NCA5	0.02075	0.702764
36	MMP14	Matrix metalloproteinase-14	P50281	2.77E-05	0.697769
37	H10	Histone H1.0	P07305	0.02993	0.695217
38	H2AJ	Histone H2A.J	Q9BTM1	0.01841	0.693493
39	SPB8	Serpin B8	P50452	0.05441	0.691129
40	MGN2	Protein mago nashi homolog 2	Q96A72	0.01236	0.683225
41	RAB13	Ras-related protein Rab-13	P51153	0.00262	0.67464
42	ADT2	ADP/ATP translocase 2	P05141	1.39E-05	0.670242
43	GORS2	Isoform 3 of Golgi reassembly-stacking protein 2	Q9H8Y8-3	0.03585	0.657719
44	CHP1	Calcineurin B homologous protein 1	Q99653	0.03718	0.635421
45	EF1B	Elongation factor 1-beta	P24534	1.40E-08	0.627833
46	H4	Histone H4	P62805	0.00015	0.61886
47	H32	Histone H3.2	Q71DI3	0.00951	0.607579
48	RALY	RNA-binding protein Raly	Q9UKM9	0.00017	0.60123
49	A4	Amyloid-beta A4 protein	P05067	0.0001	0.599094
50	IF1AX	Eukaryotic translation initiation factor 1A, X-chromosomal	P47813	0.01747	0.57837
51	TMF1	TATA element modulatory factor	P82094	0.00256	0.561988
52	FINC	Fibronectin	P02751	3.63E-11	0.557401
53	PEF1	Peflin	Q9UBV8	0.00225	0.268392