CHANGES IN PROTEOGLYCANS

IN ENDOTHELIAL CELLS

UNDER HYPERGLYCEMIC CONDITIONS

A Dissertation Submitted to the College of

Graduate Studies and Research

in Partial Fulfillment of the Requirements for

the Degree of Doctor of Philosophy

in the Graduate Program of Veterinary Biomedical Sciences

University of Saskatchewan

Saskatoon

Juying Han

PERMISSION TO USE

In presenting this dissertation in partial fulfilment of the requirements for a Doctor of Philosophy degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying this dissertation in any manner, in whole or in part, for scholarly purposes may be granted by Dr. L. Hiebert who supervised my thesis work or, in her absence, by the chair of the Graduate Program of Veterinary Biomedical Sciences. It is understood that any copying of publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and the University of Saskatchewan in any scholarly use which may be made of any material in this dissertation. Requests for permission to copy or to make use of other material in this dissertation in whole or in part should be addressed to:

Chair of the Graduate Program of Veterinary Biomedical Sciences

Western College of Veterinary Medicine

University of Saskatchewan

52 Campus Drive

Saskatoon, SK S7N 5B4

Canada

PREFACE

This dissertation has been organised as a series of manuscripts that was or will be submitted for publication in scientific journals. This is also a continuation of my Master's works. Some repetition of introductory and methodological material is unavoidable.

ABSTRACT

Heparan sulfate proteoglycan (HSPG) or heparan sulfate (HS) degradation may contribute to endothelial cell (EC) dysfunction in diabetes. HSPGs, syndecan and perlecan, contain a protein core with mainly HS glycosaminoglycans (GAGs) attached. HSPGs modulate growth factors and function in membrane filtering. Heparanase induction is likely responsible for diabetic HS degradation. Heparin protects endothelium and insulin regulates glucose metabolism. Our objectives were to observe HSPG changes by studying EC GAG content and gene expression of syndecan, perlecan and heparanase under hyperglycemic conditions with insulin and/or heparin treatment.

GAGs, including HS, were determined by the carbazole assay and visualized by agarose gel electrophoresis in porcine aortic EC cultures treated with high glucose (30 mM) and/or insulin (0.01 U/ml) for 24, 48 and 72 hours and/or heparin (0.5 μg/ml) for 72 hours. High glucose decreased cell GAGs and increased medium GAGs. GAGs increased with time in control cultures and in high glucose plus insulin treated medium. GAGs were decreased with insulin but increased with insulin or heparin plus high glucose.

Confluent cultured human aortic ECs were incubated with control medium, high glucose and/or insulin and/or heparin for 24 hours. Real time PCR determination showed that: high glucose increased heparanase, decreased syndecan and had no effect on perlecan mRNA; insulin or heparin with/without high glucose decreased and insulin and heparin with high glucose increased heparanase mRNA; heparin and insulin with high glucose increased but insulin decreased syndecan mRNA. Actinomycin D (10 μg/ml) inhibited heparanase and syndecan mRNA with high glucose plus insulin plus heparin and inhibited heparanase mRNA with high glucose compared to time 0 but not β-actin after addition for 0, 2, 4, 8 and 24 hours. Bioinformatic studies revealed that transcription factor Sp1 activates heparanase promoter by high glucose and may play a role in regulation of perlecan and syndecan promoters.

Insulin or heparin inhibited the reduction in EC GAGs and syndecan mRNA and induction in heparanase by high glucose, indicating their protective effect. Decreased GAGs by insulin may relate to the pathology of hyperinsulinemia. Transcriptional regulation by heparin and/or insulin may cause variation in gene expression of heparanase, syndecan and perlecan.

ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge the Heart and Stroke Foundation of Saskatchewan who provided partial funding for this project and the Department Graduate Fellowship from Veterinary Biomedical Sciences, Western College of Veterinary Medicine that financially supported me in order to complete my studies.

I would like to take this opportunity to sincerely thank my supervisor Dr. Linda Hiebert for her great guidance and encouragement on all aspects of my studies. I would also like to extend my appreciation to the other members of my advisory committee: Dr. George Forsyth, Dr. Bill Roesler, Dr. Lily Wu and the graduate chairs Dr. Baljit Singh and Dr. Gillian Muir. My gratitude also extends to Dr. Robert Linhardt, Dr. Fuming Zhang, and Dr. Jin Xie from Rensselaer Polytechnic Institute, Troy, NY. USA. for providing me with a great opportunity to learn GAG determination and HPLC analysis of HS disaccharides.

Last but not least, I would like to thank my parents, sisters, brother, husband and son for their unending love and emotional support; they were always there for me.

DEDICATION

This dissertation is written in dedication to my parents, Qinghua Han and Yunfeng Yang. They have always been very supportive all my life.

* *

The scientist is a practical man and his are practical (i.e., practically attainable) aims. He does not seek the ***ultimate*** *but the* ***proximate****. He does not speak of the last analysis but rather of the next approximation. His are not those beautiful structures so delicately designed that a single flaw may cause the collapse of the whole. The scientist builds slowly and with a gross but solid kind of masonry. If dissatisfied with any of his work, even if it be near the very foundations, he can replace that part without damage to the remainder. On the whole he is satisfied with his work, for while science may never be wholly right it certainly is never wholly wrong; and it seems to be improving from decade to decade.*

G. N. Lewis. Quoted in ***Stochiometry*** *by Leonard K. Nash. Addison-Wesley 1966. p. vii.).*

* *

TABLE OF CONTENTS

PERMISSION TO USE ……i

PREFACE ……..ii

ABSTRACT……iii

ACKNOWLEDGEMENTS……..v

DEDICATION……vi

TABLE OF CONTENTS……vii

LIST OF TABLES……xii

LIST OF FIGURES……xiii

LIST OF ABBREVIATIONS……..xv

1. LITERATURE REVIEW……1

1.1. Diabetes……..1

1.1.1. Definition of Diabetes……..1

1.1.2. Diabetic Cardiovascular Complications ……..3

1.1.3. Molecular Mechanism of Vascular Damage by Hyperglycemia ……8

1.1.4. Insulin……15

1.1.4.1. Insulin Functions and Structure……15

1.1.4.2. Biosynthesis of Insulin……16

1.1.4.3. Regulation of Insulin Secretion……..17

1.1.4.4. Insulin Action on Cells in the Vasculature……...19

1.2. Endothelial Dysfunction in Diabetes……22

1.2.1. Endothelium Properties……...22

1.2.2. Transportation of Glucose in Endothelial Cells……...24

1.2.3. The Concept of Endothelial Dysfunction……...25

1.2.4. Endothelial Dysfunction in Diabetes……27

1.3. Degradation of Heparan Sulfate Proteoglycan (HSPG) in Diabetes……29

1.3.1. Definition Proteoglycans……..29

1.3.2. Heparan Sulfate Proteoglycans (HSPGs)……30

1.3.3. HSPG Structures……..31

1.3.3.1. Syndecans……..31

1.3.3.2. Perlecan……...35

1.3.3.3. Heparin Serglycin……37

1.3.4. HSPG Biosynthesis……..38

1.3.5. HSPG Biophysiological Functions……..42

1.3.6. Analysis of Heparan Sulfate GAGs……47

1.3.7. HSPG in Vasculature……49

1.3.8. HSPG Degradation in Diabetes……..51

1.3.9. HSPG Degradation by Heparanase……..53

1.3.9.1. Heparanase Expression in Cells……..53

1.3.9.2. Heparanase Properties……..55

1.3.9.3. Heparanase Functions……56

1.3.9.4. Heparanase and Diabetes……..58

1.4. Regulation of HSPG Expression……..59

1.4.1. Heparan Sulfate Chain Regulation……59

1.4.2. Proteoglycan Core Protein Regulation ……..61

1.4.3. Insulin and HSPG Expression ……..64

1.4.4. Heparin and HSPG Expression……..65

1.5. Summary……66

2. OBJECTIVES ……71

3. Changes in Cultured Endothelial cell Glycosaminoglycans under Hyperglycemic Conditions and the Effect of Insulin and Heparin……73

3.1. Abstract……...73

3.2. Introduction ……..74

3.3. Materials and Methods……..77

3.3.1. Porcine Aortic Endothelial Cell (PAEC) Culture……77

3.3.2. Treatment of Cultures……78

3.3.3. Extraction of Total GAGs……...79

3.3.4. Determination of Total GAGs by the Carbazole Assay……..80

3.3.5. Gel Electrophoresis Analysis of GAGs……80

3.3.6. Determination of Total HS Disaccharides in Cells and Medium……..81

3.3.7. Statistic Analysis……82

3.4. Results……...83

3.4.1. Total GAGs in Cultures……..83

3.4.2. Comparison of Total GAGs in Cultures at Different Times……88

3.4.3. GAGs Analysis by Gel Electrophoresis……...88

3.4.4. Correlation of Total HS Disaccharides and Total GAGs……..92

3.5. Discussion ……..92

4. Characteristics of Heparan Sulfate Disaccharides in Endothelial Cells Treated with High Glucose……100

4.1. Abstract……100

4.2. Introduction……100

4.3. Methods……..……..101

4.4. Results……..102

4.4.1. Total GAGs……..102

4.4.2. Total HS Disaccharides……103

4.4.3. Characteristics of HS Derived from PAEC Cultures……103

4.4.4. HS Disaccharide in Control Cells and Medium……110

4.4.5. HS Disaccharides in Cells Treated with/without High Glucose……...110

4.4.6. HS Disaccharidess in Medium Treated with/without High Glucose……110

4.5. Discussion……115

5. Gene Alteration of Proteoglycans and Heparanase in Endothelial Cells under Hyperglycemic Conditions: The Effect of Insulin and Heparin……118

5.1. Abstract……118

5.2. Introduction……..120

5.3. Materials and Methods……...122

5.3.1. Human Aortic Endothelial Cell (HAEC) Culture……..122

5.3.2. Treatment of Cultures……123

5.3.3. Extraction of Total RNA……..123

5.3.4. Synthesis of cDNA……..124

5.3.5. Detection of Gene Expression by Real Time Quantitative PCR……124

5.3.6. Statistic Analysis……..125

5.4. Results……...126

5.4.1. Gene Expression……126

5.4.2. Mechanism of Gene Regulation……132

5.5. Discussion……134

6. Prediction of Promoter Regions and Possible Transcription Factors on the Genes of Heparanase, Perlecan and Syndecan……..150

7. GENERAL DISCUSSION……...154

8. REFERENCES……....163

LIST OF TABLES

4.1. Total GAGs Isolated from Cells and Medium……...104

4.2. Total Amount of HS Disaccharides Associated with Total GAGs in Control Cultures……..105

LIST OF FIGURES

1.1. Polyol Pathway.....10

1.2. The Aldose Reductase Pathway of Glucose Metabolism.....12

1.3. Formation of Advanced Glycosylation
End Products from Glucose.....14

1.4. The Process of Insulin Biosynthesis.....18

1.5. Major and Variable Sequences of Original and Fully
Sulfated Heparin and Heparan Sulfate.....39

1.6. The Process of Heparan Sulfate Biosynthesis.....43

1.7. Cleavage of HSPG by Heparanase.....54

1.8. Scheme of the Human Heparanase Gene and Protein.....57

1.9. Outline of the Hypothesis.....70

3.1. GAGs from Cultured Endothelial Cells
and Medium at 24 Hours.....84

3.2. GAGs from Cultured Endothelial Cells
and Medium at 48 Hours.....86

3.3. GAGs from Cultured Endothelial Cells
and Medium at 72 Hours.....87

3.4. Total GAGs Increased with Culture Time
in Both Cells and Medium.....90

3.5. Gel Electrophoresis Showing GAGs in Cells and Medium
Treated with Glucose and/or Insulin for 72 Hours.....91

3.6. Gel Electrophoresis Showing GAGs in Cells and Medium
Treated with Glucose and/or Heparin for 72 Hours.....93

3.7. Correlation of Total HS Disaccharides and
Total GAGs in Cultured Endothelial Cells.....94

LIST OF FIGURES CONTINUED

4.1. HS Disaccharide Peaks Shown in HPLC Analysis for Control and Glucose Treated Cells at 72 Hours……...107

4.2. HS Disaccharide Peaks Shown in HPLC Analysis for Control and Glucose Treated Medium at 72 Hours……109

4.3. HS Disaccharides in Control Cells and Medium at 24 Hours….....111

4.4. HS Disaccharides in Control Cells and Medium at 72 Hours….....……..112

4.5. HS Disaccharides in Cells Treated with/without Glucose at 24 Hours……113

4.6. HS Disaccharides in Cells Treated with/without Glucose for 72 Hours……114

4.7. HS Disaccharides in Medium Treated with/without Glucose for 72 Hours……116

5.1. Confirmation of Real Time PCR Products……127

5.2. Heparanase mRNA Expression in Endothelial Cells Treated with High Glucose and/or Insulin and/or Heparin……..129

5.3. Syndecan mRNA Expression in Endothelial Cells Treated with High Glucose and/or Insulin and/or Heparin……..130

5.4. Perlecan mRNA Expression in Endothelial Cells Treated with High Glucose and/or Insulin and/or Heparin……..131

5.5. Effect of Actinomycin D on Heparanase mRNA Expression Induced by High Glucose……133

5.6. Effect of Actinomycin D on Heparanase mRNA Expression Induced by Glucose plus Insulin plus Heparin.……..135

5.7. Effect of Actinomycin D on Syndecan mRNA Expression Induced by Glucose plus Insulin plus Heparin.……..136

LIST OF ABBREVIATIONS

AGE(s)	advanced glycosylation end-product(s)
BM	basement membrane
bFGF	basic fibroblast growth factor
CMF-DPBS	Ca^{2+}-Mg^{2+}-free Dulbecco's phosphate-buffered saline
CE–MS	capillary electrophoresis mass spectrometry
CS	chondroitin sulfate
CSPG(s)	chondroitin sulfate proteoglycan(s)
cGMP	cyclic 3'-5'- guanosine monophosphate
DS	dermatan sulphate
DSPG(s)	dermatan sulfate proteoglycan(s)
DM	diabetes mellitus
DAG	diacylglycerol
DD	double distilled
EC	endothelial cell
ECM	extracellular matrix
EGF	epidermal growth factor
EHS	Engelbreth-Holm-Swarm
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
FAS	fat acid synthase
GAG(s)	glycosaminoglycan(s)
Gal	galactose
GalN	galactosamine
GalNAc	N-acetylated galactosamine
GBM	glomerular basement membrane
GlcA	glucuronic acid
GlcN	glucosamine
GlcNAc	N-acetylated glucosamine
GlcNS	N-sulfated glucosamine
GPI	glycosyl-phosphatidylinositol
GRE	glucose response element
Glut-1	glucose transporter-1
Glut-2	glucose transporter-2
Glut-4	glucose transporter-4
HAEC(s)	human aortic endothelial cell(s)
HS	heparan sulfate
HSPG(s)	heparan sulphate proteoglycan(s)
HGF	hepatocyte growth factor
HPLC	high-performance liquid chromatography

LIST OF ABBREVIATIONS CONTINUED

ICAM-1	intracellular adhesion molecule-1
IdoA	iduronic acid
JNK	c-Jun N-terminal kinase
JUK/SAPK	Jun N-terminal kinase/stress activated protein kinase
K_{ATP}	ATP-sensitive K^+
KS	keratan sulfate
LDL	low-density lipoprotein
LMWH	low molecular weight heparin
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
MMP(s)	matrix metalloproteinase(s)
MMP-7	matrix metalloproteinases-7
MT-MMP-1	membrane-type matrix metalloproteinase-1
NADH	nicotinamide-adenine dinucleotide
NADPH	nicotinamide-adenine dinucleotide phosphate
N-CAM	neural cell adhesion molecule
NCP	neutrophil cationic protein
NDST	N-deacetylase/N-sulfotransferase
NO	nitric oxide
2OST	2-O-sulfotransferase
3OST(s)	3-O-sulfotransferase(s)
6OST(s)	6-O-sulfotransferase(s)
PAEC(s)	porcine aortic endothelial cell(s)
PAI-1	plasminogen activator inhibitor-1
PDGF	platelet derived growth factor
PGI_2	prostaglandin I_2
PG(s)	proteoglycan(s)
PKC	protein kinase C
PI	phosphoinositides
RAGE	receptor for AGE
ROS	reactive oxygen species
SE	standard error
TGF-β	transforming growth factor- β
TIMP-3	tissue inhibitor of metalloproteinase-3
tPA	tissue plasminogen activator
TNF-α	tumor necrosis factor-α
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial cell growth factor
VSMC(s)	vascular smooth muscle cell(s)
vWF	von Willebrand Factor
WHO	World Health Organization
Xyl	xylose

1. LITERATURE REVIEW

1.1. Diabetes

1.1.1. Definition of Diabetes

Diabetes is defined as a disorder of carbohydrate metabolism. The most common form of diabetes is diabetes mellitus (DM) in which there is an inability to oxidize carbohydrate due to disturbances in insulin function. Insulin is the principal hormone released by pancreatic β-cells that regulates uptake of glucose into cells (primarily muscle and fat cells) from the blood. Insulin makes it possible for most body tissues to remove glucose from the blood for use as fuel, for conversion to other needed molecules, or for storage, e.g. insulin is the principal control signal for conversion of glucose to glycogen for storage in liver and muscle cells. If the amount of insulin produced is insufficient, or cells respond poorly to the effects of insulin (insulin insensitivity or resistance), or if the insulin itself is defective, glucose is not handled properly by body cells nor stored appropriately in the liver and muscle. The net effect is persistent high levels of blood glucose, poor protein synthesis, and other metabolic derangements. Therefore, DM is considered to be a group of diseases characterized by hyperglycemia resulting from defects in insulin production and action, or both. DM is typically classified into

two main subtypes: type 1 and type 2. Hyperglycemia defines both types of diabetes and has adverse effects on endothelial function and vascular reactivity (Mather *et al*., 2001b). Type 1 diabetes develops when the pancreatic *β*-cells are destroyed by the body's autoimmune system and can not produce the insulin that regulates blood glucose. Type 2 diabetes usually begins as insulin resistance, a disorder in which the cells do not use insulin properly. As the need for insulin rises, the pancreas gradually loses its ability to produce or secrete insulin. Type 1 diabetes usually strikes children and young adults and may account for five to ten percent of all diagnoses of diabetes. Type 2 diabetes is associated with old age, obesity, family history of diabetes, prior history of gestational diabetes, impaired glucose tolerance, physical inactivity, and race/ethnicity and may account for 90 to 95 percent of all diagnosed cases of diabetes. Type 2 diabetes is increasingly being diagnosed in children and adolescents. The rate of new cases of type 2 was greater than the rate for type 1 diabetes at ages 10 to 19 years and a total of 1.6 million new cases of diabetes were diagnosed in people in United States aged 20 years or older in 2007 (National Diabetes Statistics 2007).

In 2000, according to the World Health Organization (WHO), at least 171 million people or 2.8% of the population worldwide suffer from diabetes (Wild *et al*., 2004). Its incidence is increasing rapidly, and it is estimated that by the year 2030, this number will almost double (Wild *et al*., 2004). DM occurs throughout the world, but is more common (especially type 2) in the more developed countries. The greatest increase in prevalence is, however,

expected to occur in Asia and Africa, where by 2030 most patients will likely be found probably due to urbanization and changes in lifestyle (Wild *et al*., 2004). For at least 20 years, diabetes rates in North America have been increasing substantially. According to “Diabetes in Canada: Highlights from the National Diabetes Surveillance System”, the number of people with diagnosed diabetes continues to grow, with almost 200,000 new cases of diabetes diagnosed in 2005-2006. Approximately 1.9 million Canadian men and women had been diagnosed with diabetes. This represents about 1 in 17 Canadians - 5.5 % of all women and 6.2 % of all men. In 2008 there were approximately 24 million people with diabetes in the United States alone. About 5.7 million people remain undiagnosed. Another 57 million people are estimated to have pre-diabetes (hhttp://www.cdc.gov/Features/diabetes factsheet/). The National Diabetes Information Clearinghouse estimated that diabetes costs were $174 billion in the United States in 2007 (National Diabetes Statistics 2007).

1.1.2. Diabetic Cardiovascular Complications

Cardiovascular diseases are the major DM complications. Among them are vascular complications, such as angiopathy characterized by microvascular pathology in the retina and renal glomerulus and changes typical of arterial disease in the macrovasculature. These result in nephropathy, retinopathy and neuropathy in microvessels and atherosclerosis in macrovessels. Both microvascular and macrovascular diseases contribute greatly to the morbidity and mortality in diabetes (Kassab

et al., 2001). Diabetes is associated with accelerated atherosclerotic macrovascular disease affecting arteries that supply the heart, brain and lower extremities. As a result, patients with diabetes have a much higher risk of myocardial infarction, stroke, and limb amputation. It is well established that chronic hyperglycemia results in microvascular complications in diabetes which is the leading cause of renal failure and blindness. Up to 80% of deaths in people with type 2 diabetes are attributed to cardiovascular disease and stroke (Hanefeld *et al*., 1996).

Diabetic microvascular disease affects the small vessels such as those present in the retina, near nerves and in the microvasculature of the kidneys. This systemic disease in diabetes clinically leads to retinopathy and glomerular dysfunction, and possibly contributes to neuropathy. A number of studies have suggested that hyperglycemia appears to be the central initiating factor for all types of diabetic microvascular diseases (Kamata *et al*., 1992; Ruderman *et al*., 1992). Some evidence derived from animal studies of experimental diabetes also showed lesions in the eye (Engerman and Kern, 1984) and kidney (Engerman and Kern, 1989; Rasch, 1981) similar to those in humans. Patients under better glycemic control developed fewer eye and/or renal complications in a clinical study (Pirart, 1977). Non-proliferation diabetic retinopathy is associated with pericyte loss, formation of microaneurysms, increased vascular permeability, and capillary closure that can lead to areas of non-perfusion and ischemia. The retina responds to this hypoxia by increased production of vascular endothelial cell growth factor

(VEGF), which promotes neovascularisation (Aiello *et al*., 1994). In diabetic nephropathy, an increase in both intraglomerular pressure and extracellular matrix (ECM) proteins results in basement membrane (BM) thickening, mesangial expansion, glomerular hypertrophy and narrowing of the lumen of the capillaries (Osterby *et al*., 1990). These changes reduce glomerular filtration area and function and impede blood flow. These alterations can progress to glomerulosclerosis (Mogensen *et al*., 1988), resulting in an increased albumin filtration rate and microalbuminuria (Deckert *et al*., 1989). Diabetic neuropathy refers to a group of diseases that affect the peripheral nerves which extend outside the brain and spinal cord and include three types: motor, sensory and autonomic nerves. The autonomic nerves, not consciously controlled, supply the heart, blood vessels, bladder and intestinal tract. Diabetic neuropathy is classified as either peripheral (including sensory and motor) or autonomic, with peripheral neuropathy being the most common manifestation. Diabetic neuropathy results from both chronic and acute elevations in blood glucose. Decreased nerve function by acute hyperglycemia and reduced nerve fibers and nerve fiber reproduction by chronic hyperglycemia, are believed to occur because of the formation of sorbitol and advanced glycosylation end-products (AGEs) (Clark, Jr. and Lee, 1995) which cause microangiopathy resulting in altered neuronal capillary flow (Koda-kimble and Carlisle, 1995).

Macrovascular complications in diabetes, including coronary artery disease, peripheral vascular disease and cerebrovascular disease, are the

result of an acceleration of atherosclerosis and increased thrombosis. Although the relationship between hyperglycemia and macrovascular disease in patients with diabetes is complicated by many other factors that influence atherogenesis, an association between asymptomatic hyperglycemia and coronary heart disease has been found in both middle-aged and elderly people (Jarrett *et al*., 1982; Mykkanen *et al*., 1990). Many factors, some of which may exist in the prediabetic state, particular in type 2 diabetes, are involved in the etiology of macrovascular disease. Hyperlipidaemia, hypertension, hyperinsulinaemia and decreased insulin sensitivity related to the insulin-resistance syndrome have been proposed to induce atherogenesis even before the clinical diagnosis of type 2 diabetes (Haffner *et al*., 1990). In addition, diabetes is associated with coagulopathy and endothelial dysfunction with impaired nitric oxide (NO) production, compounding the effects of hyperglycemia in promoting atherogenesis (Jokel and Colwell., 1997). A number of factors, such as hyperinsulinaemia, insulin precursors, and tumor necrosis factor-α (TNF-α) are thought to increase plasma plasminogen activator inhibitor-1 (PAI-1) concentrations. Local changes in vascular PAI-1 expression, which is increased in the neointima of atherosclerotic lesions, may also have an impact on the fibrinolytic balance in injured vessels. High PAI-1 levels inhibit fibrinolysis and facilitate the persistence of fibrin, which may damage the endothelium (Lupu *et al*., 1993). Endothelial dysfunction may reduce the antithrombotic properties of the endothelium by decreasing the synthesis of prostaglandin I_2 (PGI_2) and

diminishing the endothelial protective effects against platelet adhesion (Wu and Thiagarajan, 1996). The common features in diabetic vascular disease, due to development of chronic hyperglycemia, are exaggerated proliferation of endothelium and BM thickening (Fischer *et al*., 1979; Kefalides, 1981; Osterby *et al*., 1990) which result in narrowing of the blood vessel lumen causing thrombosis, ischemia, and ultimately infarction (Colwell and Lopes-Virella, 1988b).

Diabetic cardiomyopathy is characterized by cardiac contractile dysfunction and congestive heart failure due to myocellular hypertrophy and myocardial fibrosis (Hayat *et al*., 2004). This induces abnormal left ventricular filling with poor compliance or prolongation of left ventricular relaxation. The exact cause of this ventricular dysfunction is not known, but several mechanisms have been proposed, including metabolic abnormalities of glucose transport, increased ECM in the interstitium of the ventricular wall due to increased type IV collagen and a combination of nonenzymatic glycation and enzymatic O-linked glycosylation (Nishio *et al*., 1995), and abnormalities in fatty-acid metabolism and alteration of calcium uptake by the sarcoplasmic reticulum leading to cellular calcium overload. These changes can result in stiffer ventricular walls and impaired myocyte contractility (Mizushige *et al*., 2000; Tahiliani and McNeill, 1986). Macrovascular complications, leading to coronary heart disease may make a large contribution to heart failure in diabetic cardiomyopathy (Malmberg *et al*., 2000).

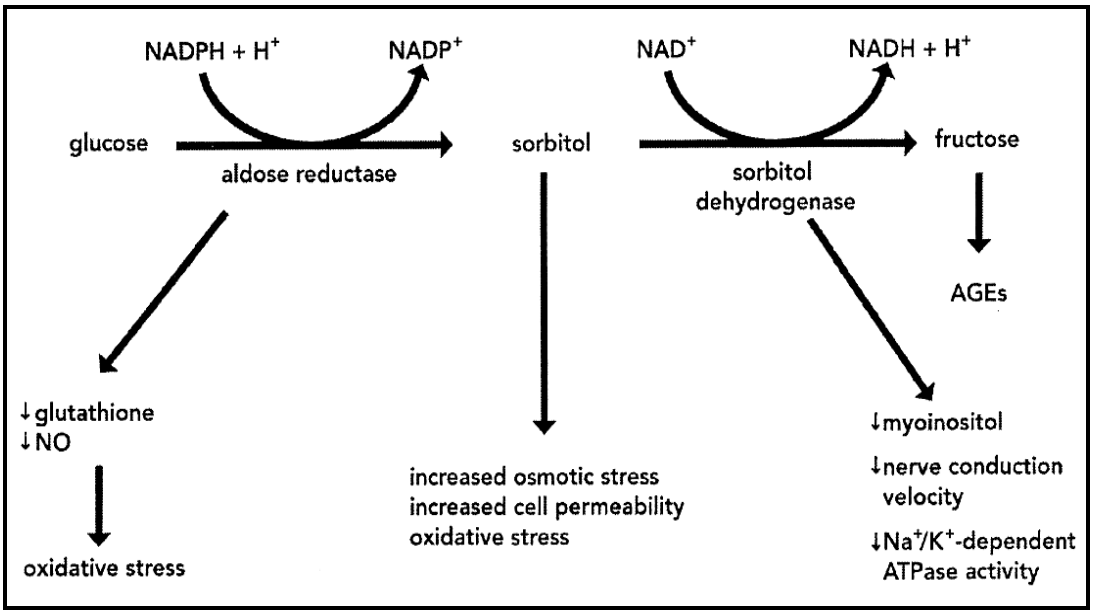
1.1.3. Molecular Mechanisms of Vascular Damage by Hyperglycemia

Hyperglycemia is considered to be one of the most important predisposing factors in the development of diabetic complications (Kamata *et al*., 1992; Ruderman *et al*., 1992). In clinical studies, patients under better glycemic control develop fewer eye and/or renal complications (Pirart, 1977). Hyperglycemia causes cell damage which ultimately leads to irreversible structural abnormalities characterized by loss of cells and progressive microvascular occlusion in the eye and kidney, and by intimal proliferation and occlusion in large vessels (Bresnick *et al*., 1977; Osterby *et al*., 1990; Steffes *et al*., 1989). Hyperglycemia-induced microvascular hypertension also contributes directly to an increase in vascular permeability, and the resultant extravasations of plasma proteins and growth factors, both in small and large vessels, may promote irreversible hypertension and vessel occlusion. Additional evidence is derived from studies showing that hyperglycemia associated with diabetes produce many of the metabolic, biochemical, and functional abnormalities seen in the vasculature. At least four different mechanisms have been postulated to contribute to these abnormalities.

The first mechanism which may contribute to diabetic complications is an increase in the formation of sorbitol (Bank *et al*., 1989; Pugliese *et al*., 1990; Tilton *et al*., 1989). In the hyperglycemic state, excess glucose

entering the polyol pathway can lead to the accumulation of sorbitol by aldose reductase coupled with oxidation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) to its oxidized form $NADP^+$. Sorbitol can be subsequently converted to fructose by sorbitol dehydrogenase coupled with the reduction of oxidized nicotinamide-adenine dinucleotide (NAD^+) to the reduced form NADH as shown in **Figure 1.1**. Aldose reductase is ubiquitous and therefore polyols accumulate in nearly all tissues of diabetic patients. Increased flux through this pathway due to hyperglycemia, increases intracellular sorbitol and fructose, increases osmotic pressure, decreases myoinositol concentration, and in combination with glycolysis, alters the intracellular redox balance. NADPH is required for NO regeneration and replenishes the antioxidant glutathione. Thus, the increased cytosolic $NADH/NAD^+$ ratio decreases NADPH, resulting in the formation of intracellular reactive oxygen species (ROS), leading to oxidative stress (Brownlee, 2001). Certain abnormalities in the microvasculature of diabetic rats are prevented by administration of an inhibitor of aldose reductase (Beyer-Mears *et al*., 1986; Cohen, 1986; Craven and DeRubertis, 1989b; Robison, Jr. *et al*., 1989).

The second mechanism is decreased myoinositol-dependent Na/K-ATPase as shown in **Figure 1.2**. Myoinositol is a normal constituent of the diet and is also synthesized by many cells (Greene *et al*., 1987). It plays an important role in signal transduction and the synthesis of phosphoinositides



(© Setter *et al*. 2003. Figure prepared by Dr. William Light on behalf of Medi Tech Media Ltd)

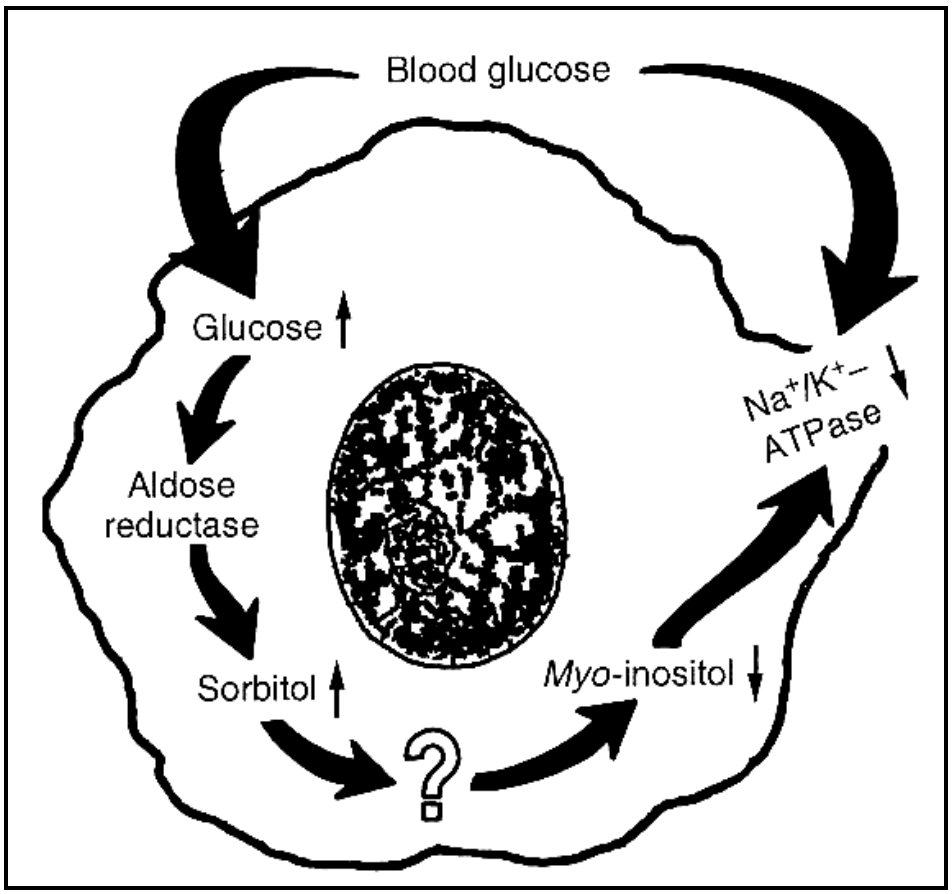
Figure 1. 1. Polyol Pathway

Activation of the polyol pathway results in a decrease of reduced $NADP^+$ and oxidized NAD+; these are necessary cofactors in redox reactions throughout the body. The reduction in these cofactors leads to decreased levels of reduced glutathione, NO and myoinositol.

(PI). Adenosine-mediated hydrolysis of PI leads to the release of a mediator that activates Na/K-ATPase and thereby regulates many cell functions. Depletion of the intracellular pool of PI caused by a sorbitol pathway-linked transport defect and a decrease in its response to adenosine both occur in diabetes, which can be prevented by aldose reductase inhibitors (Beyer-Mears *et al*., 1986; Robison, Jr. *et al*., 1989; Winegrad, 1987).

The third mechanism is increased synthesis of diacylglycerol (DAG) with the consequent activation of several isoforms of protein kinase C (PKC) (Craven and DeRubertis, 1989a; Lee *et al*., 1989b; Pugliese *et al*., 1990). Several studies have shown that hyperglycemia increases DAG concentration in several types of vascular cells and tissues and causes an activation of PKC, probably by its translocation from cytosol to the membrane (Lee *et al*., 1989a,b). PKC is probably activated by DAG synthesized by means of a stepwise acylation of glycerol 3-phosphate generated as a by-product of glycolysis. Increased DAG levels and activation of PKC have been linked to cell growth, permeability, contractility and synthesis of ECM proteins resulting in the abnormal vascular function associated with diabetes (Ruderman *et al*., 1992).

The fourth mechanism is covalent modification of protein by nonenzymatic glycosylation. Nonenzymatic glycosylation has been shown to result in qualitative and quantitative changes in extracellular components that can affect cell adhesion, growth, and matrix production



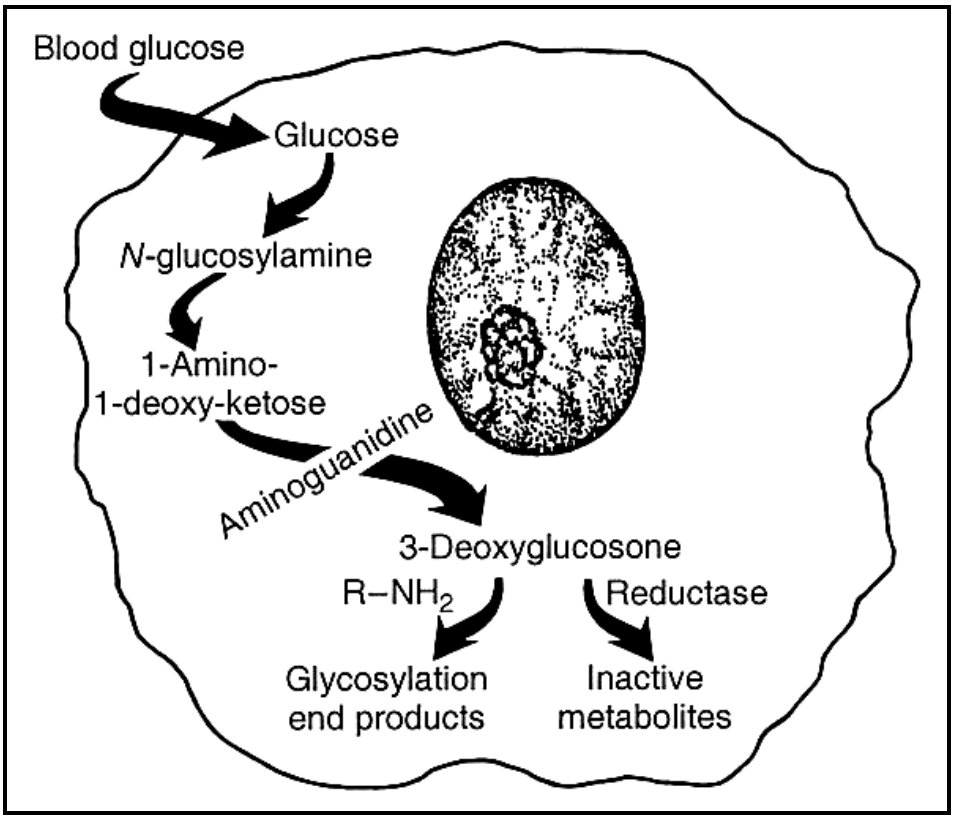
(Clark and Lee, 1995)

Figure 1.2. The Aldose Reductase Pathway of Glucose Metabolism

The aldose reductase pathway is activated by intracellular hyperglycemia, resulting in increased sorbitol formation. This, in turn (through an unknown mechanism, indicated by the question mark), results in decreased myo-inositol formation and ultimately in decreased cellular activity of Na^+K^+-ATPase. Hyperglycemia also directly inhibits ATPase activity. The vertical arrows indicate increases (↑) and decreases (↓) in the substances in question.

(Charonis *et al*., 1990; Sensi *et al*., 1989; Tsilibary *et al*., 1988). Many of these effects are mediated by AGEs, which form from Amadori products (1-amino-1-deoxyketose) through a complex series of dehydration, rearrangements and redox reactions (Monnier and Cerami, 1983) as described in **Figure 1.3**. Matrix accumulation of AGEs may further accelerate diabetic vascular occlusion by blunting the effect of the vasodilatory factor NO, which is quenched by AGEs in a dose-dependent fashion (Bucala *et al*., 1991). AGEs may exert their biological effects through the receptor for AGE (RAGE) coupled signaling pathway (Schmidt *et al*., 2000). Interaction of AGEs with endothelial RAGE may activate cellular events such as upregulation of transcription factor NF-kappaB, and activation of NAD(P)H-oxidase and p38 MAP/ERK1/2 MAP kinase cascades (Bierhaus *et al*., 2005; Wautier *et al*., 2001). Transcriptional increase in RAGEs activate a number of genes relevant to atherogenesis including vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), tissue factor, ET-1, and additional proinflammatory cytokines (Goldin *et al*., 2006). AGE/RAGE-mediated gene expression induced intracellular ROS formation (Basta *et al*., 2002). The production of ROS, AGE, RAGE, cytokines, and other gene products are involved in the development of diabetic complications and atherogenesis.

Alterations in four such interrelated hyperglycemia-driven pathways have been linked to the early microvascular changes associated with



(Clark and Lee, 1995)

Figure 1.3. Formation of Advanced Glycosylation End Products from Glucose

The formation of advanced glycosylation end-products (AGEs) from glucose occurs through the nonenzymatic formation of early glycosylation products (N-glucosylamine) that then undergo acid-base catalysis to form Amadori products (1-amino-1-deoxyketose). AGEs result from the degradation of the Amadori products into reactive carbonyl compounds that react with free amino groups ($R\text{-}NH_2$). The formation of AGEs *in vivo* is retarded by reductase.

diabetes in animal and human tissues (Walker and Viberti, 1991; Williamson *et al*., 1988). Hyperglycemia can also contribute directly to microvascular occlusion by stimulating ECM synthesis (Brownlee and Spiro, 1979; Moran *et al*., 1991). Various abnormalities, such as increased AGEs, PKC and cytokines such as transforming growth factor-*β* (TGF-*β*), and increased oxidative stress in diabetes contribute to synthesis of ECM proteins, i.e. type IV collagen, laminin and fibronectin. In addition, loss of cells may indirectly contribute to further microvascular occlusion by stimulating excessive production of ECM (McNeil *et al*., 1989; Okuda *et al*., 1990; Saito *et al*., 1988).

1.1.4. Insulin

1.1.4.1. Insulin Functions and Structure

Insulin, a circulatory hormone produced in response to elevated glucose concentration in the blood, regulates the metabolism of glucose. Glucose is liberated from dietary carbohydrate such as starch or sucrose by hydrolysis within the small intestine, and is then absorbed into the blood. Elevated concentrations of glucose in blood stimulate synthesis and secretion of insulin from pancreatic *β*-cells. Insulin acts on cells throughout the body to stimulate uptake, utilization and storage of glucose. Besides the role in regulating glucose metabolism, insulin stimulates lipogenesis, diminishes lipolysis, increases amino acid transport into cells, and modulates transcription, altering cell content of numerous mRNAs. Therefore, insulin is

able to stimulate cell growth, DNA synthesis, and cell replication, effects that it holds in common with insulin-like growth factors (Bornfeldt *et al*., 1992; Sowers, 1997).

Insulin is a rather small peptide consisting of two straight chains with a molecular weight of about 6 KDa. The A chain, containing 21 amino acids, and the B chain, containing 30 amino acids, are held together by two disulfide bonds. In addition, the A chain contains an intrachain disulfide ring **(Figure 1.4)**. Although the amino acid sequence of insulin varies among species, the positions of the three disulfide bonds, the N-terminal and C-terminal amino acid of the A chain, and the hydrophobic character of the amino acid at the C-terminal of B chain are highly conserved. These similarities in the amino acid sequence of insulin lead to a three dimensional conformation of insulin that is very similar among species and critical to biological activity. Indeed, pig insulin has been widely used to treat human patients. Insulin molecules have a tendency to form dimers, three of which in the presence of zinc form a crystalline hexameric unit with a three-fold axis passing through two zinc atoms. Crystalline zinc insulin is the basic pharmaceutical preparation of greatest importance in therapy (Dodson and Steiner, 1998).

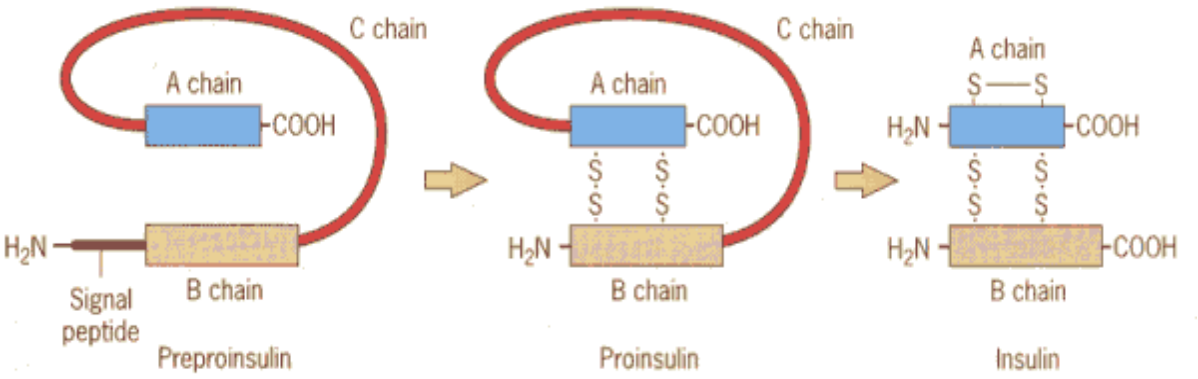
1.1.4.2. Biosynthesis of Insulin

Insulin is synthesized in significant quantities only in *β*-cells of the islets of Langerhans in the pancreas. The insulin gene on human

chromosome 11 is the ancestral member of a superfamily coding for a variety of related growth factors. The gene is composed of three exons and two introns. The insulin mRNA is translated as a single chain precursor called preproinsulin, molecular weight 11.5 KDa, containing four sequential peptides. After removal of its signal peptide during insertion into the endoplasmic reticulum, proinsulin is generated with three other peptides: an amino-terminal B chain, a carboxy-terminal A chain and a connecting peptide in the middle known as the C peptide. Within the endoplasmic reticulum, proinsulin is exposed to several specific endopeptidases which excise the C peptide, thereby generating the mature form of insulin as described in **Figure 1.4.** Insulin and free C peptide are packaged in the Golgi into secretory granules which accumulate in the cytoplasm. When the *β*-cells are appropriately stimulated, insulin is secreted from the cells by exocytosis and diffuses into the islet capillary blood. The C peptide is also secreted into blood, but has no known biological activity (Genuth, 1993).

1.1.4.3. Regulation of Insulin Secretion

Insulin is secreted primarily in response to elevated blood glucose concentrations. Elevated glucose concentrations in extracellular fluid lead to glucose transport into the *β*-cells by facilitated diffusion through a glucose transporter-2 (Glut-2) (Meglasson and Matschinsky, 1986). Elevated glucose within the *β*-cell causes an increase in the ratio of ATP to ADP that ultimately leads to depolarization of the plasma membrane via inhibition of ATP-sensitive K^+ (K_{ATP}) channels. The subsequent activation of calcium channels



(Snustad *et al.*, 1997)

Figure 1. 4. The Process of Insulin Biosynthesis

The preproinsulin contains four sequential peptides including a signal peptide and A, B and C chains. After removal of its signal peptide, proinsulin is generated with three other peptides: an amino-terminal B chain, a carboxy-terminal A chain and a connecting C chain. A chain and B chain are connected by two disulfide bonds. Mature insulin is formed by excising the C peptide from proinsulin through specific endopeptidases in the endoplasmic reticulum.

results in influx of extracellular calcium. The resulting increase in intracellular calcium is thought to be one of the primary triggers for exocytosis of insulin-containing secretory granules (Deeney *et al*., 2000; Henquin, 1990). Elevated glucose not only stimulates insulin secretion, but also transcription of the insulin gene and translation of its mRNA further increases insulin production (Evans-Molina *et al*., 2007). The normal fasting blood glucose concentration in humans and most mammals is 80 to 90 mg/100 ml (4 to 5 mM), associated with very low levels of insulin secretion. Some neural stimuli (e.g. sight and taste of food) and increased blood concentrations of other fuel molecules, including amino acids and fatty acids, also promote insulin secretion. Stimulation of insulin release is readily observed in animals or people (Berthoud *et al*., 1981; Teff *et al*., 1991).

1.1.4.4. Insulin Action on Cells in the Vasculature

Insulin initiates its cellular responses by binding to its cellular receptor, a transmembrane, multisubunit glycoprotein that contains insulin-stimulated tyrosine kinase activity (Kido *et al*., 2001). The insulin receptor cDNA was cloned (Ebina *et al*., 1985; Ullrich *et al*., 1985) and a crystal structure of the receptor protein was determined (Hubbard *et al*., 1994). The cellular content of insulin receptors is variable, with the highest level of expression in cells that are most responsive to insulin for glucose, lipid and protein metabolism, specificially adipose, skeletal muscle and liver. The abundance of receptor mRNA and protein are upregulated with differentiation of adipocyte and muscle precursor cells as they obtain an insulin-sensitive phenotype (Sibley

et al., 1989). In BC3H1 mouse muscle cell line, exposure to insulin reduces receptor mRNA abundance, which may play a role in regulation of receptor number *in vivo* (Mamula *et al*., 1990). In rare cases of severe insulin resistance, due to mutations in the receptor gene, extreme reduction in receptor abundance has been shown. However, altered receptor abundance is not believed to play a major role in common forms of insulin resistance in obesity or type 2 diabetes (Imano *et al*., 1991).

The vasculature is an insulin-responsive tissue. The activation of insulin receptors by insulin binding results in diverse signalling processes that mediate the many actions of insulin in vascular cells. Glucose uptake and metabolism are insulin independent in human micro- and macrovascular ECs. The major glucose transporting protein of ECs, however, is the glucose transporter-1 (Glut-1) which is not influenced by insulin (Gosmanov *et al*., 2006; Pekala *et al*., 1990). The main physiological role of insulin receptor expression in ECs appears to be the rapid and unidirectional transport of intact insulin across the endothelial monolayer to insulin-sensitive tissues (Bertelsen *et al*., 2001). Another important effect of insulin in ECs is to evoke vasorelaxation through activation and/or increased expression of endothelial nitric oxide synthase (eNOS) (Fisslthaler *et al*., 2003; Wang *et al*., 2006). As a result, elevated blood flow augments delivery of glucose and insulin to metabolic insulin target tissues, where both substances directly promote glucose uptake (Kim *et al*., 2006). Actions of insulin include the regulation of cell growth, gene expression, and protein synthesis. Studies of cultured

vascular smooth muscle cell (VSMC) in the presence of insulin showed increasing cell mitogenosis, protein synthesis and production of matrix proteins. These changes can result in atherogenesis through VSMC hypertrophy and hyperplasia, and ECM protein synthesis (Feener and King, 1997; Howard *et al*., 1996). However, endothelial-derived NO can reduce the progression of atherosclerosis through inhibiting proliferation of VSMC and platelet adhesiveness (Mollace *et al*., 1991; Moncada *et al*., 1988; Radomski *et al*., 1987). The ability of insulin to increase production of NO has been shown in cultured ECs (Zeng and Quon, 1996). NO is an important vasodilator and plays a critical role in the regulation of vascular tone and blood pressure (Vallance *et al*., 1989). In contrast, it has been shown that a high dose of insulin increases ET-1 levels in cultured ECs (Ferri *et al*., 1995b; Mandal *et al*., 2000). Increased ET-1 levels are also found in type 2 diabetic subjects in response to hyperinsulinemia (Ferri *et al*., 1995a; Ferri *et al*., 1995b). ET-1 exerts a potent vasoconstrictor effect on the vasculature and produces an elevation of systemic blood pressure in anesthetized animals (Miller *et al*., 1989; Yokokawa *et al*., 1989). Some studies showed that hypertensive patients have high ET-1 levels in their plasma (Kohno *et al*., 1990; Shichiri *et al*., 1990) and patients with endothelin dependent malignant hemangioendothelioma have hypertension induced by an elevated level of plasma ET-1 (Yokokawa *et al*., 1991). The actions of insulin appear to be contrary regarding the production of both NO and ET-1. Reports show the ability of insulin to induce vasodilatation is low in insulin resistance and

diabetes (Steinberg *et al*., 1996) which could be due to inactivation of NO or the inability of ECs to produce NO (Ting *et al*., 1996). Thus, insulin may have antihypertensive and anti-atherogenic actions at an appropriate physiological dose or in the non-diabetic state.

1.2. Endothelial Dysfunction in Diabetes

1.2.1. Endothelium Properties

Endothelial cells had long been viewed as passive inert vascular lining cells with their primary essential function being the maintenance of vessel wall permeability. It is now known however that they play important roles in the regulation of vascular tone, coagulation and fibrinolysis, cellular growth and differentiation, and immune and inflammatory responses. The endothelium, a distributed organ consisting of a monolayer of metabolically active ECs lining all blood and lymphatic vessels, is currently viewed as a dynamic, heterogeneous, disseminated organ that possesses vital secretory, synthetic, metabolic and immunologic functions and plays an important role in the structural integrity and normal function of the circulatory system (Aird, 2004). ECs derived from diverse tissues or vascular sites exert variation in the appearance, physiological functions and response to pathological stimuli. ECs participate in a large number of physiological processes including the control of vasomotor tone, the trafficking of cells and nutrients, the regulation of permeability, and the maintenance of blood fluidity. In addition, the endothelium mediates new blood vessel formation, contributes to the balance

of pro- and anti-inflammatory mediators, and may play a role in antigen presentation. In accomplishing these tasks, the endothelium exhibits a remarkable "division of labor". For example, arterial endothelium is primarily responsible for mediating vasomotor tone; endothelium in postcapillary venules regulates leukocyte trafficking; capillary ECs display organ-specific barrier properties (e.g. blood brain barrier versus fenestrated, discontinuous endothelium in hepatic sinusoids); and ECs from different vascular beds balance local hemostasis via the expression of site-specific patterns of anticoagulants and procoagulants (Aird, 2001). In recent years, *in vivo* phage display and direct proteome mapping of the intact vasculature have revealed a rich diversity in EC surface markers (Arap *et al*., 2002; Oh *et al*., 2004).

The vascular endothelium has extensive regulatory capacities and senses changes in hemodynamic forces and blood borne signals to release a number of vasoactive substances which are critical to maintain vascular homeostasis. Endothelium acts as a selective permeability barrier for substances including H_2O, CO_2, ions, small lipid molecules, and plasma proteins for exchange between the blood and extravascular tissue, maintaining the homeostatic balance between the intra- and extra- cellular environment. Endothelium plays a role in metabolism by displaying enzymes responsible for a number of important biochemical reactions including angiotensin-converting enzyme (ACE) which is an ectoenzyme found on the luminal surface of the endothelium. One function of ACE is to convert angiotensin I to angiotensin II, a vasopressor (Webb and Cockcroft, 1990).

ECs normally inhibit platelet adhesion and aggregation by producing PGI_2 and NO. ECs limit activation of the coagulation cascade by the thrombomodulin-protein C and the antithrombin III pathway, and regulate fibrinolysis by producing tissue plasminogen activator (tPA) and its inhibitor PAI-1, and thus maintain blood fluidity (Borsum, 1991; Pearson, 1991). ECs secrete multiple vasoactive substances critical in the regulation of vascular tone as well as a wide array of cytokines and growth factors such as TGF-*β* and angiotensin II. Endothelium affects VSMC and mensangial cell function by producing mediators such as NO and ET-1. ET-1 stimulates contraction and proliferation of VSMC and mesangial cells while NO has opposite effects (Stehouwer, 1998).

1.2.2. Transportation of Glucose in Endothelial Cells

Vascular ECs have been described as “glucose-blind”, due to their inability to down-regulate the rate of glucose transport when exposed to high glucose levels for 24 hours (Kaiser *et al*., 1993; Sasson *et al*., 1996). These observations suggested that vascular ECs are unique in their inability to modify glucose transport when exposed to extracellular high glucose concentrations. An unregulated influx of high glucose causes overproduction of ROS, which consequently impair vascular EC functions (Creager *et al*., 2003; Guzik *et al*., 2002; Hammes *et al*., 2003; Milstien and Katusic, 1999). In addition, the uptake of D-glucose in ECs occurs via the glucose transporter-1(Glut-1), which is not influenced by insulin, in contrast to glucose transporter-4 (Glut-4) in muscle cells (Gosmanov *et al*., 2006). Therefore,

glucose taken up in ECs reflects the glucose level in the blood independently of insulin sensitivity (Bertelsen *et al*., 2001). In contrast, vascular ECs in response to changes in ambient glucose showed reversible regulation of Glut-1 expression and their hexose transport system. This suggested that the down-regulatory response may protect ECs against deleterious effects of increased glucose influx during long-term or chronic hyperglycemic conditions (Alpert *et al*., 2005). Various types of vascular ECs, such as macro- and micro-vascular ECs, may respond differently to hyperglycaemic conditions. For example, bovine retinal ECs maintained a constant level of glucose uptake throughout a 72 hour incubation period at either 5 or 30 mM glucose, while bovine brain and rat heart ECs down-regulated glucose transport within 24 hours of exposure to 30 mM glucose (Milstien and Katusic, 1999). Acute high glucose challenges are harmful to bovine aortic ECs due to the inability to down-regulate glucose influx (Alpert *et al*., 2005). The human or porcine aortic ECs treated with high glucose used in our present study may be similar to bovine aortic ECs. At 24 hours, human or porcine aortic ECs may be impaired by high glucose and could be used as an *in vitro* model for EC injury by hyperglycemia.

1.2.3. The Concept of Endothelial Dysfunction

The vascular endothelium is not a mere barrier between intravascular and interstitial compartments, but a widely spread organ, which is responsible for the regulation of hemodynamics, angiogenic vascular remodeling, and metabolic, synthetic, inflammatory, antithrombogenic, and

prothrombogenic processes. As any other organ, the vascular endothelium is a subject for dysregulation, dysfunction, insufficiency, and failure. Endothelial dysfunction describes disturbances in the barrier function of the vascular endothelium; impaired antithrombogenic properties; perturbed angiogenic capacity; inappropriate regulation of vascular smooth muscle tonicity, proliferative capacity, and migratory properties; perturbed synthetic functions; and prevention of neutrophils and monocytes from diapedesis. The endothelium is involved in both the physiological regulation of vascular tone and the structural transformation of the vessel under pathological conditions. Endothelial dysfunction has been implicated in the pathogenesis and clinical course of all known cardiovascular diseases and is associated with future risk of adverse cardiovascular events (Schachinger *et al*., 2000; Suwaidi *et al*., 2000). Under physiological conditions, ECs continuously secrete NO, which relaxes VSMCs and ensures vessel patency. Damaged or excessively activated ECs can also secrete vasoconstrictor factors, the best known of which is ET- l, as well as factors that affect the differentiation and growth of VSMCs. ET-1 and other factors are released from ECs into the bloodstream, where their chemotactic action can induce leukocytes and platelets to migrate to the endothelial wall. The disturbed permeability of the endothelium leads to an increased influx of substances including adhesion molecules and inflammatory factors from the circulation into the vessel wall. ECs induce adhesion by expressing specific surface adhesion molecules such as selectins, integrins and a supergene family of immunoglobulins that can

interact with ligands on leukocytes and platelets (Bombeli *et al*., 1998; Joris *et al*., 1983). The expression of adhesion molecules is increased in ECs chronically exposed to risk factors for atherosclerosis. In addition, EC dysfunction can lead to accelerated intravessel blood coagulation. It is evident that the endothelium plays a central role in many of the early pathophysiological processes involved in atherosclerosis.

1.2.4. Endothelial Dysfunction in Diabetes

The vascular endothelium is the initial target site of injury in diabetes. The proximate causes of endothelial injury are not known but likely include hyperglycemia, exposure to AGEs, activation of PKC, increased expression of TGF-*β* and VEGF, oxidative stress and other components of the insulin resistance syndrome which are hyperinsulinaemia, hypertension and dyslipidaemia (Stehouwer *et al*., 1997). These factors probably contribute to endothelial dysfunction in human diabetic complications and include the following: the endothelium loses its antithrombotic and profibrinolytic nature and may instead acquire prothrombotic and antifibrinolytic properties, a transition marked by high plasma levels of thrombomodulin and PAI-1. Platelet adhesion and aggregation are no longer inhibited but may actually be stimulated by prethrombotic factors released from injured ECs, such as tissue factor and von Willebrand Factor (vWF) (Stehouwer, 1998). Endothelial dysfunction in diabetes mellitus may result from a decreased bioavailablity of NO coupled with an exaggerated production of ET-1 (Mather *et al*., 2001a,b). Endothelium-dependent, NO-mediated vasodilatation

decreases and VSMC contraction is enhanced so hypertension ensues (Elliott *et al*., 1993). One of the most important forms of endothelial dysfunction is BM thickening and an increase in vascular permeability which may result from loss of heparan sulfate proteoglycan (HSPG) (Kanwar *et al*., 1980). Releasing of bioactive factors from heparan sulfate (HS) chains due to HSPG degradation may also be an important mechanism for endothelial dysfunction (Kanwar *et al*., 1980).

In diabetes, BM is thickened and altered in composition, likely due to enhanced synthesis of matrix proteins by TGF-*β* activity and inadequate counter-regulation of matrix protein synthesis by the defective matrix itself (Chen *et al*., 2001; Wolf *et al*., 2005). Chondroitin sulfate proteoglycans (CSPGs) and dermatan sulfate proteoglycans (DSPGs) are increased while HSPGs are markedly reduced in diabetes (Heickendorff *et al*., 1994). The thickness of the glycocalyx, the glycoprotein–polysaccharide, which contains large amounts of HSPGs is markedly reduced (Nieuwdorp *et al*., 2006a,b). Loss of the glycocalyx leads to a wide spectrum of vascular abnormalities, which include the adhesion of mononuclear cells and platelets to the endothelial surface, attenuated NO availability which may cause a moderately increased leakage of macromolecules through the endothelium in hyperglycemia and diabetes. This phenomenon might be the basis of the Steno Hypothesis (Deckert *et al*., 1989), which proposes that microalbuminuria in diabetes reflects a systemic leakage of albumin and

atherogenic lipoproteins through the endothelium, thus reflecting an enhanced risk for atherothrombosis and cardiovascular disease.

1.3. Degradation of Heparan Sulfate Proteoglycan (HSPG) in Diabetes

1.3.1. Definition of Proteoglycans

Proteoglycans (PGs) are ubiquitous macromolecules produced by most eukaryotic cells, and are found predominantly on the cell surface, in the ECM and intracellular granules (Kjellen and Lindahl, 1991). The structure of PGs includes a core protein onto which are bound numerous sulfated and/or acetylated glycosaminoglycans (GAGs). The core protein is the backbone of the PG structure and contains a few or many GAG binding sites. Most biological activities attributed to PGs depend on the presence of the core protein. The core protein may provide a scaffold for the appropriate immobilization and spacing of the attached GAG chains (Brennan *et al*., 1983; Hautanen *et al*., 1989). The core protein assumes an anchoring function for attaching the PG core protein to the cell surface, which may be essential for the appropriate positioning of a GAG-bound ligand (Marcum *et al*., 1986; Wagner *et al*., 1989). Anchoring of PGs to the cell surface may be a prerequisite for their ability to influence the adhesion of cells to the ECM e.g. binding of HS chains to fibronectin exposes the cell binding site of this protein and thus promotes the interaction with its integrin receptor (Johansson and Hook, 1984; Woods and Couchman, 1988). The core

proteins interact with the cell and mediate cell responses such as reorganization of the actin cytoskeleton and the formation of focal cell adhesions (Bernfield and Sanderson, 1990; Bourin and Lindahl, 1993).

GAG chains made up of repeating acetylated and sulfated hexosamine and hexuronic acid subunits are organized in patterns which impart localized regions of high anionic charge alternating with neutral segments (Petitou *et al*., 1986). The relative arrangement of these charged and uncharged areas provides the molecule with additional information carrying ability. The unique structural features of PGs endow them with properties that may influence a wide range of biological processes. Several structurally related GAGs, other than HS, including dermatan sulphate (DS), chondroitin sulfate (CS), and hyaluronic acid have been identified in association with cell membranes and in the ECM (Hook *et al*., 1984). HSPGs mainly carry HS GAG chains on their core proteins. They can be classified as those present on the cell surface, such as syndecans, those anchored to the cells surface by glycosyl-phosphatidylinositol (GPI) such as glypican, and those present in BM, including perlecan, agrin and collagen type XVIII. Two types of HSPG mainly found in vasculature are syndecans expressed on EC surface and perlecan in ECM or BM.

1.3.2. Heparan Sulfate Proteoglycans (HSPGs)

HSPGs play an important role in cell to cell and cell to ECM or BM interaction in blood vessels. They are concentrated mostly in the intima,

where ECs lie on their BM, and in the inner media which contains ECM and several elastic laminae in large vessels. In capillaries, HSPGs are found mainly in the subendothelial BM, where they support proliferation and migrating ECs and stabilize the structure of the capillary wall. The BM contains type IV collagen associated with laminins, entactin and HSPGs. The major HSPGs in the cardiovascular system belong to the syndecans, glypican, and perlecan core protein families. Syndecans and glypican represent the majority of HSPG gene families and are present on the surface of mammalian cells. Syndecan core protein is a transmembrane protein and carries both HS and CS side chains, whereas the glypican core protein is covalently linked to membrane lipids via GPI anchors and mainly carries HS chains (David *et al*., 1990). Perlecan is the most complicated HSPG and carries most of the HS GAG chains. It has a large core protein with five domains to which three HS chains are usually attached. It is mainly secreted into all BMs and many ECMs and interacts with collagens, laminin, and other components (Murdoch *et al*., 1992). Therefore, HS is the most common GAG found on the EC surface and in the ECM and a prominent component of blood vessels.

1.3.3. HSPG Structures

1.3.3.1. Syndecans

The syndecan gene family has been classified into four members in mammals using molecular biology techniques of cloning of the syndecan

core protein and sequencing cDNA-derived amino acid (Bernfield *et al*., 1992). The four characterized family members are syndecan-1 (syndecan) (Jalkanen *et al*., 1987), syndecan-2 (fibroglycan) (Marynen *et al*., 1989), sydecan-3 (N-syndecan) (Carey *et al*., 1992) and syndecan-4 (amphiglycan) (David *et al*., 1992). Syndecan-1 and -3 and syndecan-2 and -4 can be considered to form two subfamilies, based on structural comparison of their core proteins such as size, GAG attachment site and sequences (Bernfield *et al*., 1992). Virtually all adhesive cells express at least one syndecan, and most express multiple syndecans (Kim *et al*., 1994).

Syndecan family core proteins exhibit very specific distribution among different cells, tissues, and at different developmental stages (Kim *et al*., 1994). Syndecan-1, -2, and -4 are mainly distributed on ECs and VSMCs at vascular basolateral surfaces (Cizmeci-Smith *et al*., 1992; Kojima *et al*., 1992). Syndecan-3 has been characterized as the neural representative of the syndecan family, but is also expressed in some stratified epithelia and differentiating cartilage cells (Gould *et al*., 1992).

All syndecan core proteins contain different amino acid sequences in the extracellular domain (ectodomain) and conserved transmembrane and cytoplasmic domains (Bernfield *et al*., 1999). Syndecans are anchored to plasma membranes via a 24-25 amino acid long hydrophobic transmembrane domain (Stipp *et al*., 1994). The conservation of amino acids within the transmembrane domain is striking and appears to be the conservation of a unique and defined structure with a role in the dimerization

or oligomerization of syndecan with itself or possibly with other membrane proteins. These domains contain regions for interactions with other membrane proteins and for localization to distinct membrane compartments and may be sites of signalling or internalization (Lebakken and Rapraeger, 1996). The cytoplasmic domains are relatively short containing 28-34 amino acids. A membrane proximal common region, containing a serine and a tyrosine, is strictly conserved among all the family members suggesting a sequence-specific function (Rapraeger and Ott, 1998). The common region may be the site for formation of cytoplasmic signalling complexes (Fanning and Anderson, 1996). The cytoplasmic domain likely interacts with evolutionarily stable components such as cytoskeletal actin and tubulins, and regulatory proteins calmodulin (Doolittle, 1991). Syndecan ectodomain sequences show only limited amino acid sequence similarity. The ectodomains are among the most rapidly diverging vertebrate proteins with the exception of their regions for GAG attachment, cell interaction, proteolytic cleavage, and oligomerization. The ectodomains contain two regions for GAG attachment. In syndecan-1 and -3, the GAG attachment sites occur in two distinct clusters; one is near the N-terminus and the other near the membrane-attachment site, separated by a proline- and threonine-rich sequence in the case of syndecan-3. The existence of GAG binding sites in syndecan-2 and -4 is only at the distal part (Kokenyesi and Bernfield, 1994). One interesting feature common to syndecans is the presence of dibasic sequences surrounded by acidic amino acids at the border of the

transmembrane domain and ectodomain or within the proximal ectodomain. These dibasic sequences are potential protease cleavage sites. The selective cleavage of the core protein, known as shedding, is regulated by tyrosine kinase, PKC, serine proteases and other signaling pathways (Fitzgerald *et al*., 2000; Jalkanen *et al*., 1987). Shedding removes the ectodomains and their attached polysaccharides as intact PGs from the cell surface and releases them into the pericellular or extracellular space. Therefore, shedding regulates the amount of HS not only on the cell surface but also in the ECM. The intact PGs still retain their ability to bind ECM ligands such as basic fibroblast growth factor (bFGF) (Kato *et al*., 1998). The shedding of syndecan-1 and -4 from epithelial cells or ECs is enhanced by specific proteases and growth factors such as plasmin, thrombin and members of the epidermal growth factor (EGF) family and by cellular stress (mechanical, heat shock, hyperosmolarity) acting through the Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) pathway (Jalkanen *et al*., 1987; Subramanian *et al*., 1997). Thrombin and plasmin may cleave at mono- or dibasic-sites adjacent to the plasma membrane in the vertebrate syndecans (Subramanian *et al*., 1997). In the case of syndecan-1, the shedding of ectodomains from the surfaces of cultured cells is part of normal turnover and involves release of the soluble ectodomain (Bernfield *et al*., 1992; Jalkanen *et al*., 1987; Saunders *et al*., 1989). Shedding of syndecan-1 was originally shown to be sensitive to tissue inhibitor of metalloproteinase-3 (TIMP-3) (Fitzgerald *et al*., 2000; Subramanian *et al*., 1997) and can be

mediated by matrix metalloproteinase-7 (MMP-7) (matrilysin) and membrane-type matrix metalloproteinase-1 (MT-MMP-1) (Endo *et al*., 2003; Li *et al*., 2002).

1.3.3.2. Perlecan

Perlecan is one of the most heavily studied PGs and has been found in all BMs and many ECMs. Its name is derived from the pearl-like structure in rotary-shadowing indicating a variable number of globules separated by a thin segment. The sequence of perlecan is known in human and mouse (Murdoch *et al*., 1992; Noonan *et al*., 1991). The human perlecan gene resides on the telomeric region of chromosome 1 and has been localized to the 1p36.1 band (Iozzo *et al*., 1997). The core protein is about 80 nm long and contains 5-7 variable-length globular domains (Olsen, 1999). Its precursor protein is about 400-470 KDa, and the mature core protein appears to have a similar size (Ledbetter *et al*., 1985). Mammalian perlecan core protein contains five domains: one unique domain at the N-terminal region and four other domains containing motifs present in other proteins. Mammalian perlecan contains a 21 amino acid signal peptide sequence, followed by a unique region of domain I. This unique module contains an acidic region followed by three Ser-Gly-Asp sequences located in close proximity to one another which are the primary HS GAG chain binding sites for perlecan. Domain II is homologous to the low-density lipoprotein (LDL) receptor and contains a four times repetitive element consisting of six cysteine residues spaced over a 40 amino acid region with conserved acidic

and hydrophobic amino acids (Noonan *et al*., 1991). It has been proposed that this domain would direct the PG secretion to the basolateral side of the BM. Domain III is comprised of modules with homology to the laminin EGF-like domain and the laminin B domain. One EGF-like module followed by a single laminin B domain and three EGF-like modules comprise a structure that is repeated three times. The laminin EGF-like modules are rich in cysteine residues while the laminin B domain lacks cysteine and has a globular structure. Domain III contains a potential GAG binding site, but it is unsubstituted (Dolan *et al*., 1997). It possibly has interactions with cell surfaces and/or other components of the BM. Domain IV is comprised solely of immunoglobulin IgG repeats. These repeats align with themselves as well as with the immunoglobulin repeats of neural cell adhesion molecule (N-CAM) (Noonan *et al*., 1991). N-CAM is involved in cell-cell adhesion and can bind HS. Domain IV may enable perlecan to form dimers or intracellular looping (Inoue *et al*., 1989). Domain V is the C-terminal of perlecan and contains three laminin G domain like modules spaced by two EGF-like modules. Domain V has the potential to support GAG attachment due to sequences of Ser-Gly-Xxx-Gly and Ser-Gly-Asp/Glu scattered throughout this domain (Timpl, 1993). The different perlecan clones have similar sequences, however, the sizes of HSPGs in different BMs and cell lines differ remarkably. Whether these changes are due to alternative splicing or proteolysis in the tissue is unclear at the moment (Klein *et al*., 1988). Usually three HS side chains attach to the N-terminal end of the molecule (Noonan *et*

al., 1991). Occasionally, it may contain both HS and CS chains (Danielson *et al*., 1992). Perlecan derived from ECs contained HS and no CS which is the most common forms of this molecule (Iozzo *et al*., 1994).

1.3.3.3. Heparin Serglycin

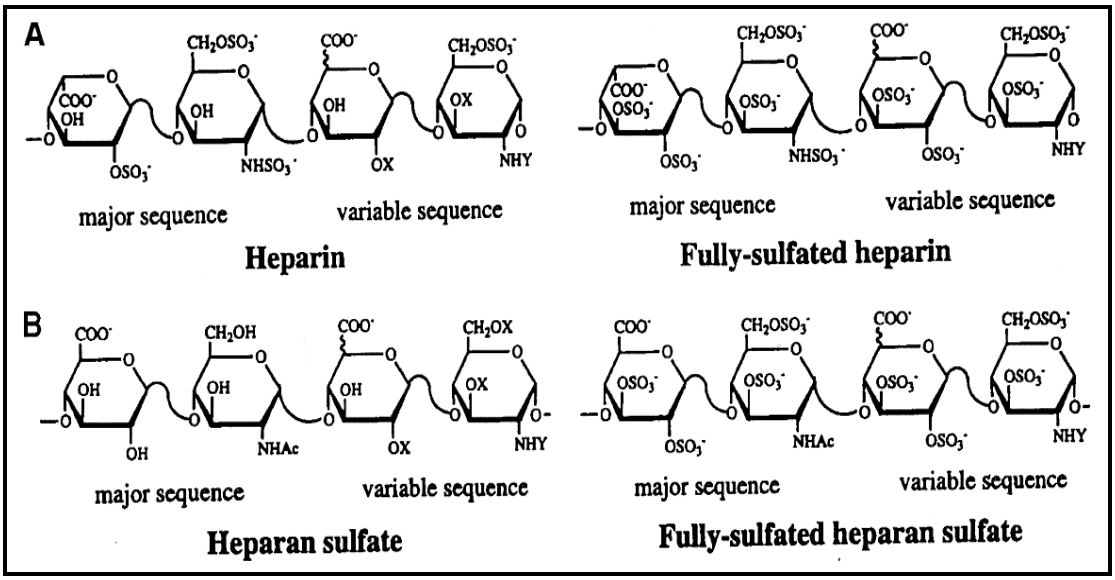
Heparin is expressed in the “basophilic” granules of mast cells which are located in connective tissue, near the capillaries and in the walls of blood vessels. It is biosynthesized in mast cells as heparin PG containing a unique core protein serglycin. The multiple heparin polysaccharide chains are covalently attached to the core protein (Robinson *et al*., 1978). The heparin chains are cleaved at random points along the chains to give polydisperse mixtures of smaller heparin polysaccharide. The polysaccharides are stored in the cytoplasmic secretory granules of mast cells as non-covalent complexes with basic proteases and are released as GAGs. Heparin is generally described as an anionic polysaccharide or a sulfated GAG with irregular sequences. Heparin has a characteristic, anticoagulant property and is synthesized in various tissues, especially in the liver, lung, and gut. In the kidney, it has been shown that glomerular epithelial cells and ECs have the ability to produce heparin-like GAGs (Castellot, Jr. *et al*., 1985; Castellot, Jr. *et al*., 1986).

Although heparin is generally considered a product of mast cells and HS is the product of other mammalian cells, heparin and HS have very similar disaccharide repeating units and are constructed from the same

monosaccharide building blocks. The uronic acid moiety is primarily iduronic acid in heparin versus glucouronic acid in HS. The components of heparin are more highly sulfated and therefore heparin has a high negative charge density, the result of sulfate and carboxylate residues present in the structure (Harpel *et al*., 1996) as described in **Figure 1.5**. These negative charges on the luminal surface of endothelium are thought to play an important role in controlling vascular permeability by acting as a "charge barrier" to the transvascular movement of the largely anionic plasma proteins such as albumin (Hardebo and Kahrstrom, 1985). The disaccharide from heparin contains 2.7 sulfate groups on average, compared to 0.6-1 sulfate groups per disaccharide from HS (Hileman *et al*., 1998). There are no distinct characteristics to separate heparin and HS based upon their structure.

1.3.4. HSPG Biosynthesis

During biosynthesis, a series of cytoplasmic enzymes are needed to catalyze nucleotide sugars UDP- xylose (Xyl), UDP-Galactose (Gal), UDP-Glucuronic acid (GlcA), N-acetyl glucosamine (GlcNAc) to form polysaccharide chains (Hirschberg *et al*., 1998). The structure of GAG chains depends on the regulated expression and action of multiple glycosyltransferases, sulfotransferases, and an epimerase, which are arrayed in the lumen of the Golgi apparatus. As shown in **Figure 1.6,** the initial step in the formation of the linkage region is the transfer of Xyl from UDP-Xyl to the hydroxyl group of specific serine residues on the core protein catalyzed by xylosyltransferase. Then two UDP-Gal and one UDP-GlcA are



(Garg *et al*., 2000)

Figure 1.5. Major and Variable Sequences of Original and Fully Sulfated Heparin (A) and Heparan Sulfate (B).

There are more sulfated groups in heparin than heparan sulfate. There are more negative charges in the sequences of fully sulfated versus original heparin and heparan sulfates

added sequentially to the non-reducing end of the growing chain catalyzedby their corresponding transferase (Esko and Zhang, 1996). After assembly of the linkage region, the GlcNAc and GlcA residues are added alternatively to the linkage region tetrasaccharide by GlcNAc/GlcA transferases to form the polysaccharide backbone (Duncan *et al*., 2001). The individual saccharide unit is subjected to a series of sequential enzymatic modification reactions in which the products of one reaction are substrates for the next. As the HS chain is polymerized, it undergoes a series of modifications that include GlcNAc N-deacetylation and N-sulfation, C5 epimerization of GlcA to iduronic acid (IdoA), and variable O-sulfation at C2 of IdoA and GlcA, at C6 of GlcNAc and N-sulf glucosamine (GlcNS) units, and occasionally at C3 of glucosamine (GlcN) residue. These modifications involve the enzymes GlcNAc N-deacetylase/N-sulfotransferase (NDST), and glucosaminyl 6-O-sulfotransferases (6OST), and the glucosaminyl 3-O-sulfotransferases (3OST) (Liu *et al*., 1999a; Rosenberg *et al*., 1997).

The majority of GAG chains added to syndecan and perlecan core proteins is of the HS type although syndecan-1 and -4 have been shown to be modified by CS chains as well (Rapraeger *et al*., 1985; Shworak *et al*., 1994). For syndecan-1 the membrane proximal sites are modified mostly by CS and the N-terminal sites are modified predominantly by HS (Kokenyesi and Bernfield, 1994). The addition of HS or CS to syndecan-4 is not dependent on the specific sites (Shworak *et al*., 1994). The consensus sequence for GAG attachment on the core protein is the amino acid serine

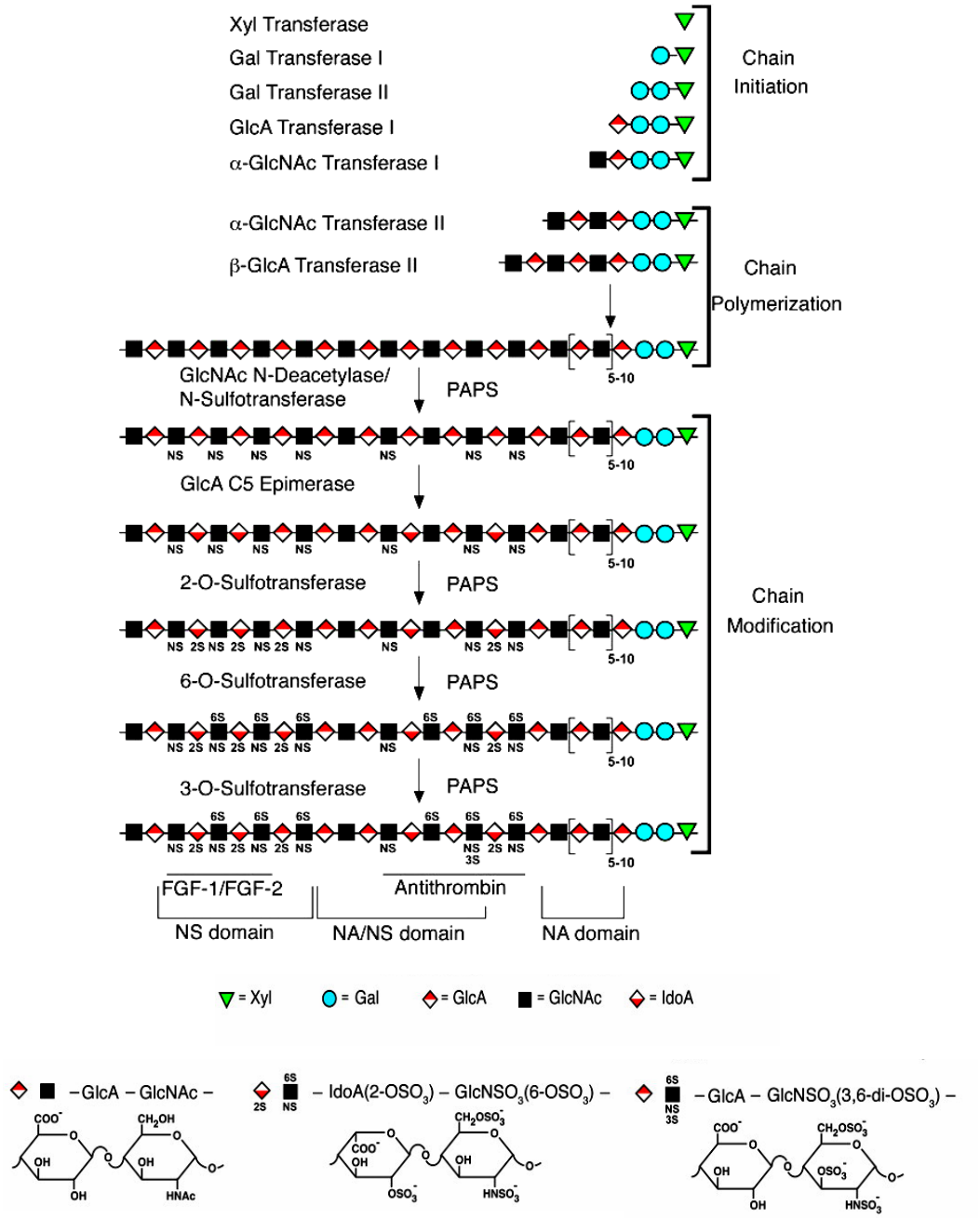
followed by a glycine. The structures of the linkage tetrasaccharides connecting HS or CS are identical. The GAG-substituted portion of the core protein is linked to serine units by a sequence of GlcA-Gal-Gal-Xyl tetrasaccharide sequence. Alternatively addition of GlcA or IdoA and GlcNAc with various sulfation forms the HS GAG chain while the CS chain is formed by adding GlcA or IdoA and N-acetyl galactosamine (GalNAc) with various sulfation. The linkage of GlcN and GlcA/IdoA in HS is α1, 4 linkage, while the linkage of galactosamine (GalN) and GlcA/IdoA in CS is *β*1, 3 linkage.

The polysaccharide chains of heparin are attached to their core protein serglycin *via* the same GlcA-Gal-Gal-Xyl tetrasaccharide linker as in HSPG (Sugahara and Kitagawa, 2000). Biosynthesis of the heparin chains is by the same synthesis/modification reactions as HS, but the modifications of the [GlcA-GlcNAc]*n* polymer chains are more localized in HS than in heparin (Lindahl, 1989). Transformation of the [GlcA-GlcNAc]*n* polymer chains to heparin begins with replacement of the N-acetyl groups of GlcNAc residues with sulfate groups. N-deacetylation and N-sulfation is initiated at random sites on the polymer chains, and then continues along the chain from those sites until isolated GlcNAc are encountered (Conrad, 1998). The presence of isolated GlcNAc residues in heparin is interpreted to indicate they can not be a substrate for N-deacetylase. No unsubstituted GlcN residues are found in heparin, which suggests that N-deacetylation and N-sulfation is performed *in vivo*. All subsequent modifications depend on N-sulfate groups for substrate recognition, thus N-deacetylation and N-sulfation play a key role in

determining the fine structure of heparin. Heparin contains less GlcNAc than HS, the GlcNAc to GlcNS ratio is approximately 1:4 in heparin *vs* 1:1 in HS. The GlcNAc-containing disaccharides occur in predominantly unmodified NA domains (without sulfation), the GlcNS-containing disaccharides in NS domains (with sulfation) (see **Figure 1.6**), with mixed sequences of N-acetyl and N-sulf disaccharides separating the two domains. The mixed sequences may be functionally significant, as illustrated by the antithrombin-binding pentasaccharide, which contains both GlcNAc and GlcNS (**Figure 1.6**) (Esko and Lindahl, 2001).

1.3.5. HSPG Biophysiological Functions

The core proteins serve as the anchors for polysaccharides on the surface of cells determining when and where HS is in a specific tissue. Core proteins are also directly involved in HSPG biological functions. Several tyrosine and serine phosphorylation sites were identified on the cytoplasmic domain of the core protein of syndecan-1 and syndecan-4, suggesting that the syndecan core proteins participate in cellular signal transduction (Horowitz and Simons, 1998; Ott and Rapraeger, 1998; Volk *et al*., 1999). The structure of the polysaccharides determines the PG ligand binding to exhibit biological functions. The extent and sequence of the HS chain modifications provide the chain with short stretches having specific binding properties such as the pentasaccharide sequence of heparin binding antithrombin-III (Lindahl *et al*., 1984) . Anchoring of HSPGs to the cell



(Esko and Lindahl, 2001)

Figure 1.6. The Process of Heparan Sulfate Biosynthesis

Three steps are involved in the heparan sulfate biosynthesis the chain initiation, polymerization and modification. The symbols used are defined by the structures shown below the scheme. Structural domains (NA, NA/NS, and NS) are defined with regard to the distribution of GlcN N-substituents as indicated. Also shown are regions that have been implicated in binding of specific ligands, such as FGF-1/FGF-2 and antithrombin.

surface may be a prerequisite for their ability to influence the adhesion of cells to the ECM or to other cells. For example, binding of HS to fibronectin exposes the site allowing fibronectin to interact with its integrin receptor. HSPG has a similar effect on N-CAM of nerve cells (Johansson and Hook, 1984; Woods and Couchman, 1988). Syndecans on the cell surface are believed to be the endogenous receptor for circulating growth factors i.e., bFGF, TGF-*β*, VEGF, hepatocyte growth factor (HGF) and chemokines that regulate cell proliferation, differentiation and migration (Linhardt and Toida, 1997).

Perlecan core protein contains individual domains homologous to molecules involved in cell proliferation, lipoprotein uptake and cell adhension. Each of these domains has exhibited one or more binding sites for a number of ligands including BM components (Brown *et al*., 1997; Hopf *et al*., 1999; Whitelock *et al*., 1999), cell adhension molecules and growth factors (Hayashi *et al*., 1992; Mongiat *et al*., 2000; Sharma *et al*., 1998). LDL domains could potentially bind to lipoproteins and may contribute to the retention of lipoproteins at the earliest stage of atherosclerosis (Paka *et al*., 1999). Interactions between HS chains and laminin may be important for its binding in the BM. Perlecan may participate in cell adhesion through fibronectin, the appearance of perlecan in the vicinity of adhesion sites in cultured fibroblasts supporting this possibility (Singer *et al*., 1987). It may affect differentiation and gene expression through many receptors, in this respect resembling laminin, a molecule controlling cell behaviour. Perlecan

expression also can be regulated by cytokines and growth factors that are responsible for regulating a wide variety of cellular functions including embryogenesis, differentiation, migration, proliferation and tumorigenesis. Modulation of growth factors, membrane filtering, serving as receptors, and cell signalling are some recognized functions of perlecan (Arikawa-Hirasawa *et al*., 1999; Bernfield *et al*., 1999; Olsen, 1999).

The ability of the HS side chains to interact with enzymes such as coagulation factors, mast cell proteases, lipoprotein lipase and cytokines such as granulocyte macrophage-colony stimulating factor, interferon, interleukins also suggests an involvement in the control of normal and pathologic processes, such as wound healing, tumor growth and vessel formation (Vlodavsky *et al*., 1993). Hence, HSPGs play important roles in physiological processes of embryonic development, tissue repair, and regulation of blood coagulation, cartilage function and glomerular filtration (Bernfield *et al*., 1992; Kjellen *et al*., 1983; Salmivirta and Jalkanen, 1995). Several disease conditions such as cardiovascular diseases, cancer, inflammation, diabetes and amyloidosis are associated with changes in the expression of PGs as well as with structural and functional alterations of their GAG components (Iozzo and Cohen, 1993; Jackson, 1997; Rosenberg *et al*., 1997; van der Woude and van Det, 1997). The majority of cell surface HSPGs are endocytosed and undergo unique prelysosomal and classic lysosomal degradations. Thus, most mammalian cells contain substantial amounts of HS degradation intermediates (Yanagishita and Hascall, 1992).

The processes involved in the metabolic turnover of HSPGs in ECM are currently largely unknown. Considering the biological roles of HSPGs in this location, i.e., integration of matrix structure, modulation of growth factor activities, etc., elucidation of their metabolic regulation poses a very interesting question.

Heparin is most well known as an anticoagulant and/or antithrombotic agent to maintain blood flow in the vasculature through the binding and activation of antithrombin III and other mechanisms (Harpel *et al*., 1996). Exogenous heparin is also capable of ameliorating increased vascular permeability caused by various polycationic substances including protamine, poly-L-lysine or neutrophil cationic protein (NCP) (Fairman *et al*., 1987; Peterson *et al*., 1987). Heparin is considered as a potent vasodilator that most likely lowers elevated blood pressure in hypertensive animal models by interacting with vascular endothelial cyclic 3'-5'- guanosine monophosphate (cGMP) or releasing endothelial derived NO (Mandal *et al*., 1995). NO may subsequently suppress ET-1 production by a cGMP-dependent pathway (Boulanger and Luscher, 1990), decrease VSMC contractility and increase vascular wall refractoriness to other vasoconstrictor substances (Mandal *et al*., 1995). The therapeutic action of heparin is ascribed to its anti-inflammatory properties, including inhibition of blood coagulation, complement inactivation, and suppression of leukocyte function (Wardle, 1996). For example, heparin inhibits leukocyte rolling and adhesion on the endothelium (Bazzoni *et al*., 1993; Ley *et al*., 1991), and aggregation,

degranulation and superoxide anion generation by activated neutrophils (Laghi *et al*., 1984). Heparin also protects endothelial cells from injury due to ROS (Hiebert and Liu, 1990).

1.3.6. Analysis of Heparan Sulfate GAGs

Unlike other biopolymers such as polynucleotides or polypeptides, HS GAGs cannot be amplified because of their structural diversity and complex biosynthesis. Since polysaccharides are only available in very small amounts, there is no opportunity to sample or study actual cell-surface HS GAG sequences that a given protein encounters and binds to *in vivo*. In addition, the heterogeneity and complexity of HS GAGs create significant challenges to their purification and characterization of structure–function relationships. These limitations have led to the recent development of sensitive analytical tools for characterizing small amounts of material. HS can be analyzed in terms of disaccharide composition. The disaccharides are distinguished by the presence of variable sulfated or nonsulfated GlcA/IdoA and GlcN residues. Generally, the basic structural disaccharides of HS appears to be quite ancient and the same set of disaccharides exists in most tissues, but their relative content varies quantitatively in different species or tissues, or ones with different biological activity (Linhardt and Toida, 1997). For example, ECs predominantly express the disaccharides of GlcA-GlcNS3S as in the connective tissue mast cells and are a critical substructure binding antithrombin (Bourin and Lindahl, 1993; Rosenberg *et al*., 1997), whereas kidney HS contains a large amount of IdoA2S-GlcNS3S (Edge and Spiro,

1990). Increasing the sensitivity of detection of HS GAG oligosaccharides has been addressed by introducing UV chromophores, and covalent derivatization using fluorescent or radioactive tags (Kinoshita and Sugahara, 1999; Linhardt and Toida, 1997; Vives *et al*., 1999). Recent advances in mass spectrometric methods have enabled detection of picograms to femtograms of material without any covalent tags (Juhasz and Biemann, 1994). The use of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has made it possible to determine the mass of sulfated oligosaccharides non-covalently complexed with basic peptides to great accuracy (Rhomberg *et al*., 1998a; Venkataraman *et al*., 1999). Other powerful approaches include combining state-of-the-art separation methodology with mass spectrometric technology such as capillary electrophoresis mass spectrometry (CE–MS) (Linhardt and Toida, 1999) high-performance liquid chromatography (HPLC) (Vives *et al*., 1999) and nanoelectrospray MS (Pope *et al*., 2001). These analytical tools have been applied to dissect the HS GAG oligosaccharide into smaller fragments using a series of depolymerizing enzymes and other chemical methods and determining the sequence of the oligosaccharides based on specific properties of the smaller fragments (Kreuger *et al*., 2001). Selective enzymes and analytical methods that exploit bacterial lyases and lysosomal exoenzymes have become available for structural and sequence analysis of HS GAGs (Ernst *et al*., 1998; Rhomberg *et al*., 1998a; 1998b). Although these methods provide excellent sensitivity and facilitate direct identification

of the disaccharide on the basis of mass, they do not provide quantitative results. There are two significant approaches to sequencing HS GAG polysaccharides. In the first methodology, the purified oligosaccharide is treated with an exoenzyme that specifically removes a monosaccharide (or a sulfate group) from the non-reducing end and the resulting product is analyzed by polyacrylamide gel electrophoresis (Turnbull *et al*., 1999), or HPLC (Merry *et al*., 1999; Vives *et al*., 1999). The product is subsequently treated with other exoenzymes and analyzed in an iterative fashion to read the sequence of the HS GAG from the non-reducing end. Although this method works very well for most sequences, the lack of exoenzymes to remove certain modifications causes problems. For instance, N-sulf hexosamines at the non-reducing end cannot be removed in a single step, but instead require *N*-desulfation, followed by chemical reacetylation and cleavage by the *N*-acetyl glucosamidase enzyme. Alternatively, chemical methods that clip at the N-sulf glucosamine can be used (such as treatment with nitrous acid). Chemical treatments do not specifically remove the terminal monosaccharide, but instead attack all such residues randomly, resulting in a ladder of multiple products. Unlike protein sequencing, the tools for the analysis of HS GAGs are just coming of age and require further in depth studies.

1.3.7. HSPGs in Vasculature

HSPGs are prominent components of blood vessels. In the vascular endothelium, HSPG are found both on the cell surface facing the lumen and

the underlying BM (Saku and Furthmayr, 1989). In large vessels, they are concentrated mostly in the intima, where ECs lie on their BM, and in the inner media which contains ECM including several elastic laminae. In capillaries, they are found mainly in the subendothelial BM, where they support proliferation and migrating ECs and stabilize the structure of the capillary wall.

The ECM is a meshwork-like substance within the extracellular space and includes the cell BM. It is composed of a complex mixture of proteins many of which are glycoproteins and PGs having carbohydrate chains attached to them including HSPGs. These glycoproteins include a wide variety of collagens, laminins, fibronectin, thrombospondins and elastins. Elastins provide flexibility to arteries, lungs, and skin, while collagen type 1 and 3, fibronectin and thrombospondins function as scaffolding proteins and are involved in matrix signaling by interacting with integrin family of proteins and triggering growth-promoting signals. Fibronectin interacting with PGs, fibrin/fibrinogen and collagens are important for cell adhesion, growth and migration. ECM not only provides structural support to cells and tissues, but also plays important roles in regulating the behaviour of cells in multicellular organisms. ECM displays a very dynamic equilibrium where there is constant synthesis, degradation and reorganization. HSPGs in the BM of the ECM are associated with other matrix proteins such as collagen type IV, laminins, antactin and fibronectin (Iozzo, 1998; Rops *et al*., 2004). The PGs promote adhesion of the cell to ECM through binding molecules such as fibronectin and laminin. HSPGs play an important role in the interaction between cell to

cell and cell to ECM or BM. ECs synthesize the constituents of the ECM and control vascular permeability to macromolecules by modulating the biochemical and biophysical properties of the ECM (Borsum, 1991; Chon *et al*., 1997). HSPGs facing the lumen on the cell surface have been shown to be important for extravasation of immune cells during inflammatory reactions (Rops *et al*., 2004; Wang *et al*., 2005). Reduced HSPG perlecan expression was associated with plaque accumulation in human carotid atherosclerotic lesions (Tran *et al*., 2007).

1.3.8. HSPG Degradation in Diabetes

Changes in arterial PG are seen in both atherosclerosis and diabetes (Stevens *et al*., 1976; Wasty *et al*., 1993). In both conditions, HS GAG was decreased, with the greatest decrease in diabetic patients with atherosclerosis (Wasty *et al*., 1993). The association between hyperglycemia, HS, and impaired organ function has been well-studied in kidney and other organs, both in patients with diabetes and in animal models, where HSPGs are important regulators of glomerular filtration. Perlecan has an important role in the maintenance of the glomerular filtration barrier. Reduction of HSPG synthesis had been found in glomerular basement membrane of both diabetic human and rats and in cultured glomerular epithelial cells exposed to high glucose (Brown *et al*., 1982; Parthasarathy and Spiro, 1982; van Det *et al*., 1996b). Heparinase degradation of kidney HS resulted in impaired glomerular filtration of ferritin and albumin (Kanwar *et al*., 1980; Rosenzweig and Kanwar, 1982). It has been shown that the

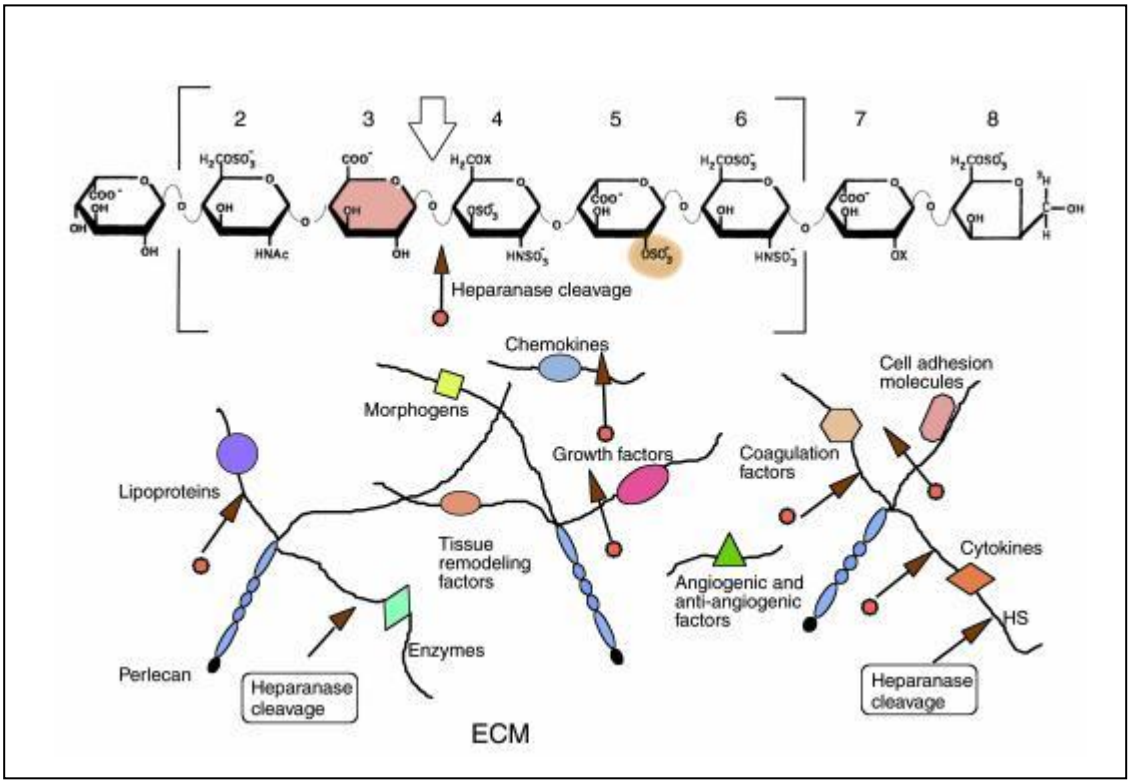
biosynthesis of HSPG and HS chain sulfation is decreased in the hyperglycemic state. Further studies revealed that the decrease occurs specifically in the HS GAG component (Jensen, 1997; Kjellen *et al*., 1983). HS GAGs were decreased in arteries of diabetic patients (Wasty *et al*., 1993). HS GAG but not core protein was decreased in cultured ECs and kidney cells induced by high glucose (van Det *et al*., 1996b; Vogl-Willis and Edwards, 2004b). Immunofluorescence studies on renal biopsies also showed a decrease in staining of HS GAG without changes in core protein staining (Tamsma *et al*., 1994). The content of arterial HS was negatively correlated with plasma glucose concentration in diabetic monkeys (Edwards *et al*., 2004). In addition, cultured mesangial and kidney visceral epithelial cells have shown a decrease in HSPG when cultured in high glucose (30 mM) versus control (5 mM glucose) media (van Det *et al*., 1996b). However, it is controversial whether the decrease in GAGs is accompanied by or independent of, a decrease in the core protein under hyperglycemic conditions in vascular ECs. Changes in GAG content or composition may affect the structure and organization of other matrix components. Moreover, integrin-linked signalling events may be perturbed, leading to altered cell protein expression (Wu and Dedhar, 2001). It has been identified that perlecan polymorphism in the GAG-attachment region of domain I is associated with diabetic nephropathy (Hansen *et al*., 1997). The same polymorphism in a nondiabetic population was associated with a reduced atherogenic lipid risk profile but no decrease in cardiovascular disease

suggesting the favourable effect on lipids was counteracted by adverse perlecan mediated interactions in the arterial wall (Cai *et al*., 2000; Vogl-Willis and Edwards, 2004a).

1.3.9. HSPG Degradation by Heparanase

1.3.9.1. Heparanase Expression in Cells

Heparanase is an endo-*β*-D-glucuronidase capable of cleaving glycosidic bonds of HS side chains at a limited number of sites by a hydrolase mechanism. The degraded HS fragments are still an appreciable size (5-7 KDa) (Freeman and Parish, 1998; Vlodavsky and Friedmann, 2001). This suggests substrate specificity of the enzyme recognizing a particular and relatively rare HS structure (Bourin and Lindahl, 1993). A 2-O sulfate group on a hexuronic acid residue located two monosaccharide units away from the cleavage site appears to be essential for substrate recognition by heparanase (Conrad, 1998) as shown in **Figure 1.7**. Platelets, cytotrophoblasts, mast cells, neutrophils, macrophages, and T and B lymphocytes express heparanase under normal physiological conditions. Platelets have the highest levels of heparanase (Freeman *et al*., 1999; Freeman and Parish, 1998). In fact, serum heparanase is mainly derived from activated platelets (Ihrcke *et al*., 1998). Among various organs, the kidney is second to the placenta in heparanase content. Furthermore, heparanase is only rarely expressed in human or bovine aortic ECs exposed to various physiological activators (Godder *et al*., 1991; Nakajima *et al*.,



(Vlodavsky and Friedmann, 2001)

Figure 1.7. Cleavage of HSPG by Heparanase

Heparanase cleaves HSPGs (arrows) and releases a variety of physiologically and pathologically important molecules. Inset shows the heparanase recognition and cleavage site. X in sugar unit 4 represents hydrogen or SO_3^-.

1986). Controversially, ECs are able to bind, internalize, and degrade high-M_r heparin, and since HS is similar to heparin, this suggests that ECs may contain an endoglucosidase that degrades HSPG intracellularly (Barzu *et al*., 1987; Godder *et al*., 1991). Heparanase is also localized to tertiary granules of neutrophils and mast cells and is released upon tumor necrosis factor (TNF-α) and calcium ionophore treatment (Matzner *et al*., 1985; Mollinedo *et al*., 1997; Bashkin *et al*., 1990). Heparanase was released by diapedesis and extravasation of a number of immune cells, including neutrophils, macrophages, and lymphocytes (Bartlett *et al*., 1995; Parish *et al*., 1998; Vlodavsky *et al*., 1992), while heparanase inhibitors exhibited anti-inflammatory activity (Parish *et al*., 1998). Cleavage of HS side chains by degranulated heparanase during inflammation may facilitate the passage of blood-borne normal and malignant cells into tissues by altering the composition and structural integrity of the subendothelial ECM (Bartlett *et al*., 1995; Vlodavsky *et al*., 1992; Vlodavsky and Friedmann, 2001).

1.3.9.2. Heparanase Properties

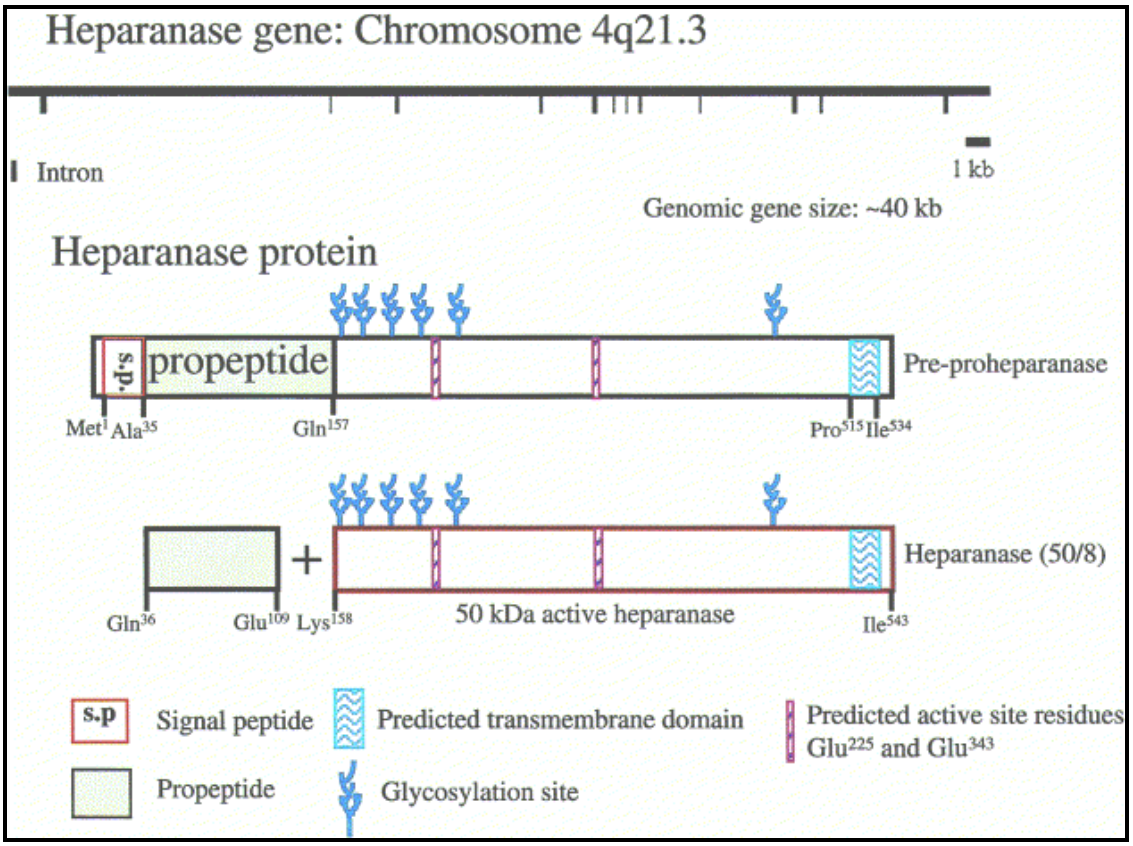
Recently, partial sequencing of heparanase purified from human placenta, platelets, and hepatoma cells led to the cloning of a cDNA and gene encoding the heparanase protein (Hulett *et al*., 1999; Toyoshima and Nakajima, 1999; Vlodavsky *et al*., 1999). The heparanase gene is located on human chromosome 4q21.3. The heparanase cDNA contains an open reading frame of 1629 bp encoding a 61.2 KDa polypeptide of 543 amino

acids. The mature active 50 KDa enzyme has its N-terminus 157 amino acids downstream from the initiation codon (Fairbanks *et al*., 1999; Toyoshima and Nakajima, 1999; Vlodavsky *et al*., 1999) suggesting post-translational processing of the heparanase polypeptide at an unusual cleavage site as shown in **Figure 1.8**.

Processing and activation occur during incubation of the full-length 65 KDa recombinant enzyme with several normal and transformed cells and to a less extent, with their conditioned medium (Vlodavsky *et al*., 1999). The heparanase precursor may bind to the cell surface, most likely to HS, and is then converted to its highly active 50 KDa form in a process accompanied by endocytosis of the processed form. This is demonstrated by the observation that heparanase activity is readily obtained after transfection of mammalian cells with cDNA encoding the entire heparanase precursor (Dempsey *et al*., 2000; Vlodavsky *et al*., 1999).

1.3.9.3. Heparanase Functions

Heparanase may facilitate the release of a multitude of HS bound growth factors, cytokines and chemokines that would enhance the immune reaction or activate vascular endothelium (Elkin *et al*., 2001; Vaday and Lider, 2000). HSPGs play a key role in the self-assembly and integrity of the multi-molecular architecture of BM and ECMs, hence, their cleavage is likely environment. Enzymatic degradation of HS is, therefore, involved in diverse fundamental biological and pathological phenomena associated with cell



(Vlodavsky *et al*., 2001)

Figure 1.8. Scheme of the Human Heparanase Gene and Protein.

migration, including embryonic morphogenesis, angiogenesis, inflammation, neurite outgrowth, tissue repair and cancer metastasis (Bandtlow and Zimmermann, 2000; Nakajima *et al*., 1988; Parish *et al*., 1998; Vlodavsky *et al*., 1992; Wight *et al*., 1992). Since HSPGs are important constituents of blood vessels and the BM, cleavage of HS is expected to facilitate extravasation of blood-borne tumor cells, as well as sprouting of angiogenic ECs by modulating growth factor activity and bioavailability. Cancer invasion and metastasis involves degradation of ECM components, including collagens, laminins, fibronectin and HSPGs. The malignant cell is able to accomplish this task through the concerted sequential action of enzymes such as matrix metalloproteinases (MMPs), serine and cysteine proteases, and endoglucosidases. Expression of heparanase is associated with the metastatic potential of tumor cells (Hulett *et al*., 1999; Nakajima *et al*., 1988; Parish *et al*., 1998; Vlodavsky *et al*., 1994; Vlodavsky *et al*., 1999) and increased levels of heparanase have been found in sera of animals and human cancer patients bearing metastatic tumors (Nakajima *et al*., 1988). Increased heparanase mRNA and heparanase activity has been found in high glucose-treated ECs (Han *et al*., 2007).

1.3.9.4. Heparanase and Diabetes

Heparanase activity was found in the urine of some diabetic patients associated with poor glycemic control (Katz *et al*., 2002). Although heparanase protein was located in both the glomerular mesangial and

epithelial cell lysates, there was little or no detectable activity in intact cells and their conditioned medium (Katz *et al*., 2002). This result was confirmed by immunohistochemical staining showing the presence of the heparanase protein in both the glomeruli and the tubules of normal human and rat kidney (Levidiotis *et al*., 2001). A decreased intensity of HS staining correlated with proteinuria expressed as a function of creatinine clearance (Tamsma *et al*., 1994) suggesting alteration of HSPG side chains relevant in the pathogenesis of diabetic nephropathy. The abnormalities in HSPG metabolism were also observed in the aortic intima of diabetic patients suggesting changes in HSPG could occur in both the macro- and micro-vasculature. Heparanase cleaves HS at specific interchain sites and induces loss of the anionic barrier on the luminal surface of endothelium resulting in a widespread rise in vascular permeability and vasculopathy. This result was determined by observing an increased GBM permeability after perfusion with bacterial heparinase (Rosenzweig and Kanwar, 1982).

1.4. Regulation of HSPG Expression

1.4.1. Heparan Sulfate Chain Regulation

The regulatory mechanisms for biosynthesis of complex HS GAGs differ significantly from those of protein molecules. The latter can be regulated primarily by controlling gene transcription and translation mechanisms. The regulation of HS GAG biosynthesis comprises many cellular functions including the regulation of core protein biosynthesis,

multiple glycosyltransferases, sulfotransferases, and epimerase. Each regulation requires separate biosynthetic regulation. The localization of carbohydrate-synthesizing enzymes in the Golgi apparatus is an important factor that would certainly affect the biosynthetic regulation of HSPG (Uhlin-Hansen and Yanagishita, 1993). Most of the enzymes involved in modifying the chain have now been purified and molecularly cloned. The GlcNAc NDST, the 6OSTs, and the 3OSTs each represent a gene family whose members appear to be expressed in a tissue-specific and developmentally regulated pattern. In recent years, there has been a virtual explosion in the number of enzyme isoforms identified. There are five 3OSTs, four NDSTs, and three 6OSTs identified so far (Aikawa and Esko, 1999; Habuchi *et al*., 2000; Kusche-Gullberg *et al*., 1998; Liu *et al*., 1999b). Substrate specificity studies performed *in vitro* indicate that the individual members of each enzyme subfamily catalyze the same reaction, but in different chemical contexts. For example, 3OST-1 is the only 3OST isozyme that can form the antithrombin binding sequence (i.e., domains containing GlcA-GlcNS3S). In contrast, 3OST-2 transfers sulfate to GlcA2S-GlcNS and IdoA2S-GlcNS, whereas 3OST-3A transfers sulfate to IdoA2S-GlcN, where the GlcN has an unsubstituted amino group, thus generating the binding site for the viral gD glycoprotein (Liu *et al*., 1999a; Shukla *et al*., 1999). The three 6OSTs add sulfate to the C6 of GlcN units, but the preferred location of the target relative to GlcA and IdoA varies (Habuchi *et al*., 2000). The four NDST isozymes show variation in relative ratios of N-deacetylase and N-sulfotransferase

activity. Modeling studies of the NDSTs against the crystal structure of the sulfotransferase domain of NDST1 (Kakuta *et al*., 1999) suggest that modulations of the binding cleft for the sugar chain may confer different substrate specificities for the enzymes (Aikawa *et al*., 2001). In contrast to these sulfotransferases, only one 2-O-sulfotransferase (2OST) (Bai and Esko, 1996; Rong *et al*., 2001) and one epimerase (Crawford *et al*., 2001; Li *et al*., 2001) appear to exist in vertebrates. A survey of lower organisms has shown only single isozymes for the other sulfotransferases, suggesting that the ancestral forms of these enzymes perform all the basic reactions of HS biosynthesis required to generate the diversity of structure necessary for the various biological activities essential to the organisms. A mutation in the gene for the IdoA 2OST by insertional mutagenesis causes renal agenesis and neonatal lethality in the mutant mice (Rosenberg *et al*., 1997). Analysis of tissues from these animals shows a complete loss of 2-O-sulfated IdoA containing disaccharides, consistent with the idea that only one 2OST exists (Wilson *et al*., 2002).

1.4.2. Proteoglycan Core Protein Regulation

The genetic regulation of core protein and multiple carbohydrate-synthesizing enzymes is key in the regulation of HSPG biosynthesis. This regulation can be primarily controlled by gene transcription and translation mechanisms. The regulation of syndecan expression is one of the fundamental mechanisms that allow syndecans to perform specific functions

in a temporal and spatial manner, and these features may enable these HSPGs to function specifically *in vivo*. Syndecan-1 is expressed on a variety of cell types and has been proposed to be regulated at both the mRNA and protein levels (Bernfield *et al*., 1992). For instance, syndecan-1 expression in mesenchymal cells is induced at the transcriptional level by platelet derived growth factor (PDGF) and angiotensin II (Cizmeci-Smith *et al*., 1993), the antimicrobial peptide PR-39 (Gallo *et al*., 1994)), and bFGF (Elenius *et al*., 1992). Syndecan-1 expression can also be upregulated post-transcriptionally in stratifying keratinocytes (Sanderson *et al*., 1992) and in mesenchymal cells during kidney formation (Vainio *et al*., 1992), and in resident peritoneal macrophages (Yeaman and Rapraeger, 1993). Macrophages responding to an inflammatory signal in the peritoneal cavity express abundant syndecan-1 mRNA compared to resident peritoneal macrophages and blood monocytes (Hayashida *et al*., 2006). Despite the expression of abundant syndecan-1 mRNA, the PG is not detected in elicited macrophages suggesting that this transcript is not utilized and the presence of a post-transcriptional block (Hayashida *et al*., 2006). PKA increased exocytosis of intracellular syndecan-1 and reduced endocytosis of cell surface syndecan-1 which could indicate that cell surface syndecan-1 levels are increased post-translationally (Hayashida *et al*., 2006), since PKA has been shown to enhance exocytosis (Bouchard *et al*., 2004; Katsura *et al*., 1997) and inhibit endocytosis of cell surface proteins and increase their cell surface levels (Bradbury and Bridges, 1992; Salazar and Gonzalez, 2002). Therefore, the molecular mechanisms

responsible for syndecan-1 regulation in cells described above may reflect a widespread process controlling the expression of this molecule in a variety of cell and tissue types.

Regulation of perlecan expression has also been examined at the transcriptional and translational levels, suggesting a wide range of potential mechanisms that can be used to control the presence of perlecan in a particular tissue during development or in disease. The SV-40 Large T oncogene was found to inhibit transcription of the perlecan gene while stimulating the transcription of the actin gene in a SV-40 transformed renal cell line (Piedagnel *et al*., 1994). cAMP and prostaglandins E1 and E2 which are known to increase cAMP concentrations, caused a rapid decrease in perlecan mRNA and protein levels in rat glomerular epithelial cells while they caused an increase in perlecan mRNA in embryonal F9 carcinoma cells which are used to study the effects of cell differentiation (Chakravarti *et al*., 1993; Ko *et al*., 1996). These results confirmed that a control mechanism of perlecan mRNA regulation is directly responsive to cAMP and a study on perlecan's promoter proposed a putative cAMP transduction element (Iozzo *et al*., 1997). Furthermore, these results also emphasized that the same mediator can act on perlecan expression differently. TGF-*β* was found to increase perlecan expression and core protein production in human mesangial and synovial cells (Dodge *et al*., 1995; van Det *et al*., 1996a). An increase in perlecan expression in colon carcinoma cells by TGF-*β* appeared to be at the posttranscriptional level possibly by enhancing perlecan mRNA

stability (Dodge *et al*., 1990). In addition, perlecan may be subject to translational regulation through an estrogen-stimulated pathway which is a critical step in the attachment of competent blastocysts to the epithelium of the uterus (Smith *et al*., 1997). The studies of glucose regulating perlecan expression indicated that the effect of glucose on perlecan occurs at the transcriptional and posttranscriptional level (Kasinath *et al*., 1996; Templeton and Fan, 1996).

1.4.3. Insulin and HSPG Expression

Increased insulin concentration and decreased vasculature sensitivity to insulin have been identified as independent risk factors for cardiovascular disease in insulin-resistance syndromes and type 2 diabetes (Despres *et al*., 1996; Howard *et al*., 1996). Binding of insulin to insulin receptors results in diverse signalling processes that mediate many actions of insulin in vascular cells including modulating transcription, altering cell content of numerous mRNAs and stimulating cell growth, DNA synthesis, and replication (Bornfeldt *et al*., 1992; Sowers, 1997).

Syndecans, the major HSPGs on EC plasma membranes, are believed to interact with many bioactive factors including insulin that affect cellular physiological and pathological processes. The sequence alignments demonstrate a remarkable similarity at the nucleotide level between the mouse syndecan and human insulin receptor cDNA sequences (Ebina *et al*.,

1985). The similar region between these cDNAs is at the 5'- and 3'-untranslated region suggesting that posttranscriptional controls are shared by these two molecules (Ratner *et al*., 1987; Saunders *et al*., 1989). HSPGs are known to bind growth factors such as bFGF on ECs and TGF-*β* on a variety of cell types (Saksela *et al*., 1988; Segarini and Seyedin, 1988). Since insulin is a known growth factor, the sequence similarity between syndecan and the insulin receptor cDNA, suggest that syndecans may be the binding site or receptor for insulin or, syndecan may compete with the insulin receptor to bind insulin. Therefore, the binding function of insulin to HSPG on the cell surface may affect cellular behaviour and synthesis of both HS chains and core proteins of HSPG.

1.4.4. Heparin and HSPG Expression

Heparin protects endothelium by increasing its concentration in endothelium hundreds to thousands-fold more than in plasma (Hiebert *et al*., 1993) and by increasing HS amounts on EC surfaces (Nader *et al*., 1991). Heparin stimulated ECs to synthesize HS with increased sulfated disaccharides (Nader *et al*., 1989). Heparin is protective of cultured ECs damaged by exposure to free radicals (Hiebert and Liu, 1990) and able to protect HS from degradation by inhibiting heparanase upregulation (Han *et al*., 2007). In addition to EC protection, heparin blunted enhanced actin expression in cultured VSMCs treated with high glucose (Mandal *et al*., 2000). Heparin inhibits mitogenesis and migration of cultured mesangial cells

(Castellot, Jr. *et al*., 1985) and proliferation of glomerular epithelial cells (Adler, 1991; Karlsson and Marklund, 1987). Heparin inhibits induction of immediate early genes (Hofer *et al*., 1996; Miralem *et al*., 1996) and attenuates the expression of potentially injurious mediators in mesangial cells (Kitamura *et al*., 1994). Moreover, heparin and HSPG inhibit apoptosis of glomerular mesangial and epithelial cells suggesting that heparin may function as a general inhibitor of apoptosis in a wide range of cell types (Ishikawa and Kitamura, 1999).

1.5. Summary

Diabetes is a chronic and progressive disease induced by defective production or function of insulin that impacts the quality of people's lives worldwide. The cardiovascular complications of diabetes are the leading cause of death in people with diabetes. One of the most essential characteristics of diabetic complications is EC dysfunction or injury caused by hyperglycemia. Since ECs are a unique organ distributed in the inner layer of all vessels that regulate vascular bioactivity and function, in diabetes EC dysfunction or injury is responsible for retinopathy and nephropathy in micro-vessels and atherosclerosis in macro-vessels. The "steno hypothesis" raised by Deckert *et al*., in 1989 suggested that genetic dysregulation of HS/GAG production was associated with diabetic cardiovascular complications. Based on their hypothesis, HSPG degradation has received considerable focus among several proposed mechanisms of endothelial

dysfunction in diabetes, particularly in the kidney. Studies on the alteration of HSPG derived from ECs will illustrate the mechanism of EC dysfunction or injury, and therefore, help us understand the pathological consequences of diabetic cardiovascular complications. HSPGs are macromolecules containing a core protein with highly negatively charged HS chains attached. Two main types of HSPG, synthesized by ECs in the vasculature, are syndecans presented on the cell surface and perlecan in the ECM. Syndecans are believed to be part of the endogenous receptor for circulating growth factors and chemokines that regulate cell proliferation, differentiation and migration (Linhardt and Toida, 1997) and may be a prerequisite for the ability of ECs to influence the adhesion of cells to the ECM or to other cells. The multi-domain perlecan also possesses functions such as modulation of growth factors, membrane filtering, serving as a receptor and influences cell signalling (Arikawa-Hirasawa *et al*., 1999; Bernfield *et al*., 1999; Olsen, 1999). Therefore, degradation of HSPG may cause increased vascular permeability and damage to the vascular wall due to loss of negative charges, disturbance of the functions of bioactive factors and adhesion molecules, and detachment of cells from the BM. Evidence from studies of both humans and animals demonstrated that the content of HS and HSPG were reduced in glomerular and skin BM and in aortic intimas of diabetic patients. HSPG synthesis was also reduced in organs of diabetic rats. However, studies on changes in core proteins and HS chains of HSPG are not always consistent and remain controversial in diabetes. The effect of high glucose on HSPG core protein or

HS GAGs is still unknown, particularly, the alteration of HS GAGs in ECs under hyperglycemic conditions at the levels of both degradation and synthesis. HS chains are believed to be degraded by heparanase (Vlodavsky and Friedmann, 2001) which is observed in tissues of diabetic models and various cultured cells. Previous studies localized heparanase protein in both glomeruli capillaries and tubular epithelium and detected heparanase activity in the urine of diabetic patients (Katz *et al*., 2002; Shafat *et al*., 2006). Expression of heparanase mRNA and activity was detected in high glucose-treated ECs (Han *et al*., 2007). The studies of heparanase expression in diabetes have been restricted to diabetic nephropathy. There has been little study of heparanase levels associated with other diabetic cardiovascular complications or in vascular endothelium injured by hyperglycemia. Although induction of heparanase by high glucose is at least partly responsible for HS degradation, it is still unknown if changes in core proteins of syndecan or perlecan by high glucose are also correlated with heparanase induction.

The antithrombotic drug heparin is considered as a potent vasodilator (Mandal *et al*., 1995) and lowers blood pressure in spontaneously hypertensive rats (Yokokawa *et al*., 1994). It has been shown that heparin protects endothelium by increasing HS synthesis and reducing ROS production. Insulin regulates uptake of glucose into cells and lower blood glucose concentration which may reduce toxicity of high glucose in the vasculature. The protective effect of heparin and/or insulin on ECs injured by high glucose has shown reduced EC intercellular gaps and the inhibition of

heparanase upregulation (Mandal *et al*., 2000; Han *et al*., 2007). Heparin and/or insulin are shown to prevent ECs from heparinase I injury (Han *et al*., 2005). Although both heparin and insulin affect the expression of heparanase in ECs treated with high glucose, the influence of these two compounds on core proteins of syndecans and perlecan in ECs under hyperglycemic condition is still a mystery. Heparin and insulin influence HSPGs by different mechanisms, but they may collectively or synergistically activate or inactivate the gene expression of syndecan, perlecan and heparanase under hyperglycemic conditions.

Our ***hypothesis*** for this study as outlined in **Figure 1.9** is that **"Gene expression and GAG contents of syndecan and perlecan are altered by high glucose. The induction of the heparanase gene by high glucose is correlated with these alterations. Insulin and heparin will prevent these alterations"**.

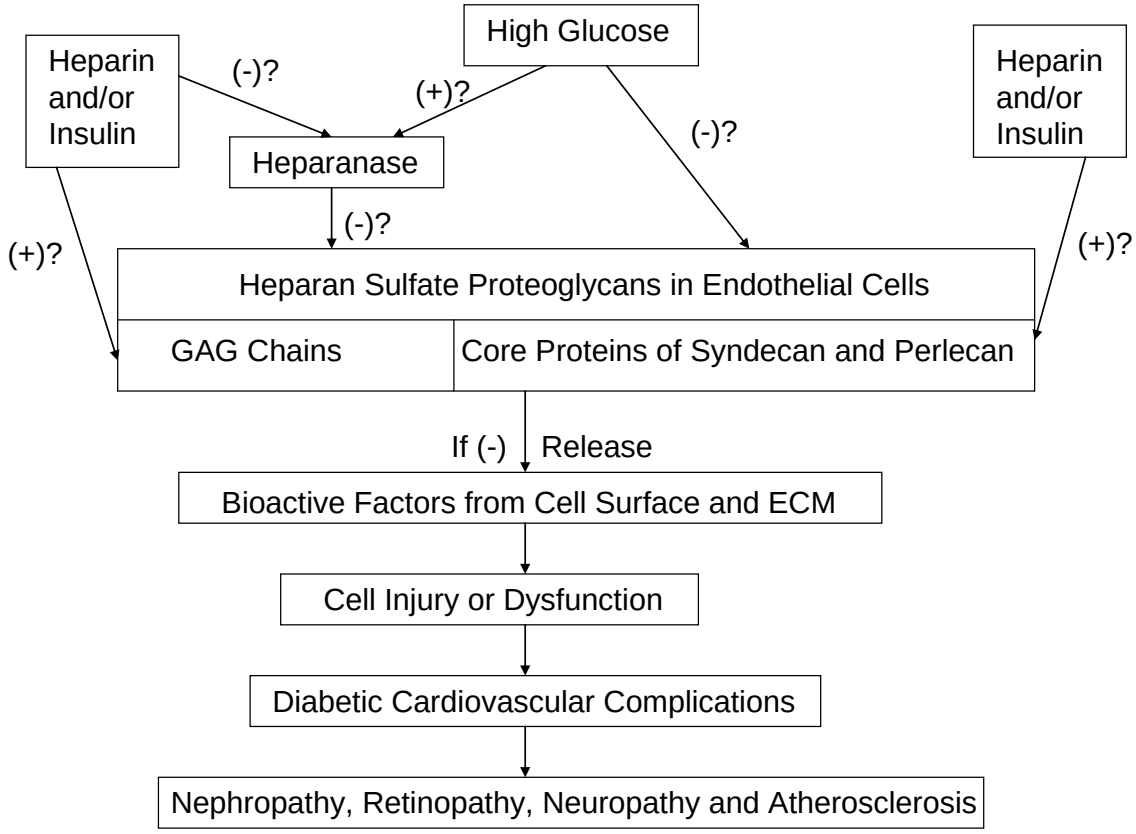


Figure 1.9. Outline of the Hypothesis

2. OBJECTIVES

The purpose of this study was to determine if total GAGs and HS disaccharides are altered in cultured ECs and medium under hyperglycaemic conditions and the effect of insulin and heparin on the GAG alterations. An additional goal was to elucidate the gene expression of core proteins of HSPG syndecan and perlecan as well as heparanase in cultured ECs under hyperglycaemic conditions and the effect of insulin and/or heparin. In addition, the regulatory mechanisms of gene expression for syndecan and heparanase was explored.

The specific objectives are:

- To determine changes in GAG content in cultured porcine aortic endothelial cells (PAECs) and their medium treated with high glucose and/or insulin and/or heparin

- To determine changes in HS disaccharides in PAECs treated with high glucose at 24, 48 and 72 hours

- To determine gene expression in heparanase, syndecan and perlecan in human aortic endothelial cells (HAECs) treated with high glucose and/or insulin and/or heparin

- To determine the mechanism of gene expression of heparanase and syndecan treated with high glucose or high glucose plus insulin plus heparin

3. Changes in Cultured Endothelial Cell Glycosaminoglycans under Hyperglycemic Conditions and the Effect of Insulin and Heparin*

3.1. Abstract

Background: Heparan sulfate proteoglycans (HSPGs) contain glycosaminoglycan (GAG) chains made primarily of heparan sulfate (HS). Hyperglycemia in diabetes leads to endothelial injury and nephropathy, retinopathy and atherosclerosis. Decreased HSPG may contribute to diabetic endothelial injury. Decreased tissue HS in diabetes has been reported, however, endothelial HS changes are poorly studied.

Objective: To determine total GAGs, including HS, in endothelium under hyperglycemic conditions and the protective effect of insulin and heparin.

Methods: Confluent primary porcine aortic endothelial cells (PAECs) were divided into control, glucose (30 mM), insulin (0.01 unit/ml) and glucose plus insulin treatment groups for 24, 48 and 72 hours. Additionally, PAECs were treated with glucose, heparin (0.5 μg/ml) and glucose plus heparin for 72 hours. GAGs were isolated from cells and medium. GAG concentrations were determined by the carbazole assay and agarose gel electrophoresis.

Results: GAGs were significantly increased only in control and glucose plus insulin groups at 72 versus 24 hours. Glucose decreased cell GAGs and increased medium GAGs, and insulin alone decreased cell GAGs at all times compared to control. In the glucose plus insulin group, cell GAGs were less than control at 24 hours, and greater than glucose or insulin alone at 48 and 72 hours while GAGs in medium were greater than control at all times and glucose at 72 hours. Heparin increased GAGs in glucose-treated cells and medium.

Conclusions: High glucose and insulin alone reduces endothelial GAGs. In hyperglycemic conditions, heparin or insulin preserves GAGs which may protect cells from injury. Insulin is an effective diabetic therapy since it not only lowers blood glucose, but protects endothelium.

***** Published in *Cardiovascular Diabetology* 2009 8:46.

3.2. Introduction

A GAG is a linear, highly negatively charged, heteropolysaccharide macromolecule possessing a characteristic disaccharide repeat sequence which usually comprises an amino sugar with D-glucosamine or galactosamine and a uronic acid residue of either D-glucuronic acid or iduronic acid. Based on the composition of disaccharides, GAG structures are categorized into six common types including CS, DS, HS, keratan sulfate (KS), hyaluronic acid, and heparin. The GAG chain is covalently attached to a core protein through an O-link to serine or an N-link to an asparagine

residue and this structure is called a proteoglycan. Proteoglycans are expressed by all mammalian cells and are found on the cell surface, in the ECM and intracellular granules (Kjellen and Lindahl, 1991).

HS is a prominent component of blood vessels and the most common GAG found on the EC surface and in the ECM. HSPGs are believed to be the endogenous receptors for circulating growth factors and chemokines that regulate cell proliferation, differentiation and migration (Linhardt and Toida, 1997). The interaction of HS side chains with bioactive factors controls physiological processes of embryonic development, tissue repair, blood coagulation, cartilage function and glomerular filtration (Bernfield *et al*., 1992; Kjellen and Lindahl, 1991; Salmivirta and Jalkanen, 1995), and pathological processes of wound healing, vessel formation, and tumor cell growth, adhesion, invasiveness and metastasis (Vlodavsky *et al*., 1993). HS degradation is considered to be a major cause of endothelial dysfunction resulting in disturbance of vascular integrity and barrier properties, due to decreased negative charge and increased permeability, and release of bioactive substances such as cytokines, enzymes and growth factors bFGF and TGF-*β*. Changes in the expression of proteoglycans, as well as structural and functional alterations of their GAG components, are associated with cardiovascular disease, cancer, inflammation, amyloidosis and diabetes (Iozzo and Cohen, 1993; Jackson, 1997; Rosenberg *et al*., 1997; van der Woude and van Det, 1997).

Several studies suggest that HS degradation is associated with diabetic nephropathy (van den Hoven *et al*., 2006; Vernier *et al*., 1983). An increased glomerular albumin filtration rate was observed in microalbuminuric patients with overt diabetes (Deckert *et al*., 1989). Increased glomerular basement membrane (GBM) permeability was associated with diminished GBM HS content (Vernier *et al*., 1983). Similar changes in HS content were observed in aortic intimas of diabetic patients (Wasty *et al*., 1993). HS in skin basement membrane in patients with diabetic nephropathy was also significantly reduced compared to diabetic patients without nephropathy. Similarly, reduced ^{35}S-labeled HSPG synthesis was observed in aorta, liver and intestinal epithelium of diabetic rats (Brown *et al*., 1982; Kjellen *et al*., 1983; Levy *et al*., 1984). This evidence suggests that changes in HS metabolism could occur not only in the kidney, but also in any tissue or organ in the diabetic condition, indicating a link between HS abnormalities and vascular complications both in the micro and macro vasculature. In addition, heparanase, an endoglucuronidase produced by ECs induced by high glucose, could be responsible for the cleavage of HS chains (Han *et al*., 2007). Heparanase activity was increased in the urine of diabetic patients (Katz *et al*., 2002) and reduction of HS moieties in the glomeruli of patients with overt diabetic nephropathy in type 2 diabetes was correlated with heparanase upregulation (van den Hoven *et al*., 2006). However, specific changes in GAGs from ECs under hyperglycemic conditions are not well studied. The purpose of this study was to use primary

porcine aortic ECs (PAECs) under high glucose conditions, as an *in vitro* model to determine if changes in GAGs are seen with hyperglycemia. Since the hormone insulin regulates glucose metabolism and promotes glucose uptake and utilization to reduce glucose concentration in hyperglycemia, we also wished to determine the effect of insulin on GAG alteration. Since our previous studies showed that heparin also protected ECs from high glucose injury (Han *et al*., 2005), the effect of heparin was also studied.

3.3. Materials and Methods

3.3.1. Porcine Aortic Endothelial Cell (PAEC) Culture

Primary PAECs were cultured by the method of Gotlieb and Spector (Gotlieb and Spector, 1981). Porcine aortic segments obtained from local abattoirs were washed three times in Ca^{2+}-Mg^{2+}-free Dulbecco's phosphate-buffered saline (CMF-DPBS) and connective tissue was trimmed from the adventitial surface. The aorta was held upright by clamping one end with hemostats. The aortic lumen was rinsed three times with CMF-DPBS and then filled with collagenase (Type IV, SIGMA, St. Louis, MO, USA; 1 mg/ml in CMF-DPBS) for six minutes. After removing the collagenase, the lumen was gently rinsed with medium (M199 containing 5.5 mM D-glucose, without heparin or insulin, GibcoBRL, Life Technologies, Inc., Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS, GibcoBRL), 50 μg/ml penicillin (SIGMA) and 10 μg/ml streptomycin (SIGMA). The medium with cells was plated onto 60 mm culture dishes which were then incubated at

37°C with 5% CO_2/95% air in a humidified environment. PAECs were identified by their morphological appearance of cobblestone-like flattened cells, and the presence of von Willebrand factor (vWF) in initial cultures. Non-endothelial-like cells, such as smooth muscle cells and fibroblasts were destroyed cell by cell using a pasteur pipette and mechanical suction before the first passage. To pass cells, confluent cultures were washed twice with CMF-DPBS and cells were detached by trypsin (0.025% with EDTA in CMF-DPBS) for two or three minutes at room temperature. The cells were resuspended in medium and transferred to 60 mm dishes for further passage. For experiments, the cells were grown in 60 or 100 mm dishes at passage four.

3.3.2. Treatment of Cultures

PAECs were grown in 100 mm culture dishes (surface area 78.5 cm^2 /dish) with an estimated 6 million cells/dish at confluence. Cells were incubated with four different treatments (3 dishes/group) including control (serum free medium), high glucose (30 mM), insulin (0.01 unit/ml), or high glucose plus insulin in serum-free medium for 24, 48 and 72 hours. In a separate experiment ECs in 100 mm dishes were treated with high glucose (30 mM), and/or heparin (0.5 μg/ml), or serum free medium (control) with one dish/group for 72 hours. Additionally, PAECs were grown in 60 mm dishes and treated as controls or with high glucose for 24, 48 and 72 hours with 3 dishes/group. After the specified time interval, cell medium was collected into 15 ml centrifuge tubes. Cells were scraped from the dish using a cell scraper,

the dish was washed with DPBS, and cells and washings were collected into a 1.5 ml tube and centrifuged to pellet cells. The cell pellets and medium were frozen at - 80 °C. Samples were freeze-dried before they were analyzed.

3.3.3. Extraction of Total GAGs

Medium and cell pellets were digested by pronase (0.2 mg/ml in 0.1 M Tris buffer containing 0.1 M $CaCl_2$, pH 8.0) for 24 hours at 37 °C. Then the digested samples were freeze-dried a second time. Total GAG extraction was performed using Vivapure Ion Exchange Mini Spin Columns (Vivascience, Germany). Urea buffer (8 M urea with 2% w/v CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate), 500 µl, was added to the samples which were then triturated with a pipette to insure homogenization. The samples were then centrifuged at 5000 x g for 5 minutes to remove the precipitate. The supernatant was added into the Spin Columns which had previously been equilibrated with 500 µl urea buffer followed by centrifugation. The columns with supernatant were centrifuged at 5000 x g for 10 minutes to remove the flow-through. Columns were washed once with 500 µl of urea buffer. Then columns were washed five times with 500 µl of 200 mM NaCl, after which total GAGs were eluted with 3 x 100 µl of 16% NaCl. To precipitate the total GAGs, 1.2 ml of methanol was added to the sample, making the final methanol concentration 80% of volume, and chilled overnight at 4 °C. The total GAG fraction was collected by

centrifugation at 2500 x g for 15 minutes. The resulting precipitate was dissolved in 100 μl of distilled water.

3.3.4. Determination of total GAGs by the Carbazole Assay

The carbazole assay was used to examine the total GAGs isolated from cultured PAECs and medium in experiments where cultures were treated with high glucose and/or insulin. Briefly, 25 μl of the extracted GAG was added to 150 μl of reagent A (25 mM $Na_2B_4O_7$ in concentrated H_2SO_4) which was heated at 100 °C for 10 minutes. After the reaction was cooled on ice, 5 μl of reagent B (carbazole 1.25 g in 1 L 100% ethanol) was added to the reaction which was heated at 100 °C for another 15 minutes. The absorbance was read by spectrometry at 525 nm using a 96-well micro titer plate. The concentrations of GAGs were calculated by comparing to a standard curve made with heparan sulfate from porcine intestinal mucosa (SIGMA).

3.3.5. Gel Electrophoresis Analysis of GAGs

Agarose gel electrophoresis described by Jaques (Jaques *et al*., 1990) was used to identify GAGs recovered from control ECs and ECs treated with glucose, insulin and glucose plus insulin for 72 hours, and with glucose, heparin and glucose plus heparin for 24 hours. Total GAGs were extracted from cells and medium as describe above. Then 3-5 μl distilled water was added to each dried GAG sample and 2 μl of the dissolved GAG was added to the lanes on the agarose (1%) gel electrophoresis slide. Slides

were run in barbital buffer (pH 8.6) for 20 minutes. Slides were then fixed in 0.01% (w/v) cetavlon for at least 2 hours. After drying, slides were stained with 0.04% (w/v) Toluidine Blue in 80% acetone and background colour was removed with 1% acetic acid. HS was identified and visualized by comparing to reference HS from porcine intestinal mucosa (0.1 μg/μl, SIGMA).

3.3.6. Determination of Total HS Disaccharides in Cells and Medium

Additional experiments were performed for determination of HS disaccharides in cells and medium. Total GAGs from three 60 mm dishes were combined in order to obtain enough material for HS disaccharide analysis by HPLC. Freeze-dried total GAG extracts were dissolved in 20 μl of 0.2 M Tris-HCl buffer (pH 8.0), then 2 μl of Chondroitinase ACII (0.05 unit/μl) and 10 μl of Chondroitinase ABC (0.01 unit/μl) were added to the samples which were incubated at 37 °C overnight. Microcon YM-3, 3000 NMWL Centrifugal Filter Devices (Millipore, USA) were used to obtain HS disaccharides. First, the device was washed with 0.1 M NaOH once followed by washing with 50 μl H_2O four to five times until the pH of the flow-through was neutral. Second, the chondroitinase digested samples were transferred to the devices and centrifuged at 10,000 x g for 25 minutes to remove chondroitin sulfates. After the devices were washed with double distilled (DD) H_2O three to four times, the inserts were removed and put into a new collecting tube. A mixture of 10 μl of heparinase I (0.3 unit), heparinase II (0.1 unit) and heparinase III (0.1 unit) in 6 mM NaCl and 3 mM Na_3PO_4 buffer (pH

7.1) were added to the column. After incubation at 37 °C for 24 hours to digest HS, the samples were centrifuged at 10,000 x g for 10 minutes to remove the buffer. Then HS disaccharides were eluted by three to four additions of 50 μl DD H_2O. The elution was freeze-dried and then dissolved in 15 μl DD H_2O. Then 10 μl of the sample was analyzed by HPLC. The analysis was performed on a post-column fluorescence RPIP-HPLC using Shimazu LC-10Ai system equipped with a RF535 fluorescence HPLC monitor and an analytical C18 column (5 μm, 4.6 × 250 mm). The mobile phase was A: 8% acetonitrile, 3 mM tributylamine acetate, pH 5.0; B: 8% acetonitrile, 3 mM tributylamine acetate, 0.2 M NaCl, pH 5.0 with a step linear gradient 0-15 min, B 0-30%; 15-30 min, B 30-100%; 30-50 min, B 100% at a flow rate of 1.0 ml/min. Column temperature was 55 °C. Post-column reaction reagents were C: 1% 2-cyanoacetamite and D: 0.75 M NaOH with a flow rate at 0.2 ml/min. The reaction temperature was 120 °C and detected with Ex 346 nm, Em 410 nm.

3.3.7. Statistical Analysis

Data for total GAGs analysed by the carbazole assay are expressed as mean +/- standard error (SE) from three dishes per group. A one-way ANOVA was used to determine significant differences between groups. Values of $P<0.05$ were considered to be statistically significant. A one-tailed t-test was used to determine significant differences between total GAGs at 24 and 72 hour.

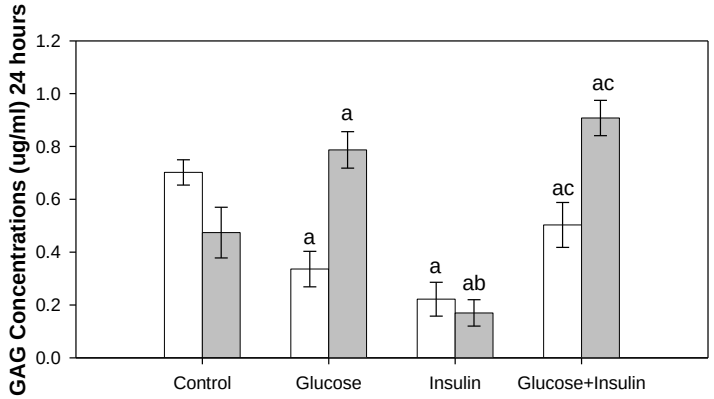
3.4. Results

3.4.1. Total GAGs in Cultures

Total GAGs were extracted from cells and medium grown in 100 mm dishes after 24, 48 and 72 hour treatment with glucose (30 mM) and/or insulin (0.01 unit/ml).

24 hours: The GAG concentration was significantly decreased in high glucose or insulin treated cells compared to control (**Figure 3.1.A)**. When high glucose was present, insulin significantly increased GAG concentrations in cells compared to insulin alone, although concentrations were still significantly less than control cells. In medium from the same cultures, GAG concentrations were significantly increased in high glucose and decreased with insulin treatment compared to control. GAG concentrations in medium were significantly increased by glucose plus insulin compared to control and insulin alone treated cultures. When the ratio of GAGs in cells and medium was expressed as a percentage, there were more GAGs in cells than medium in control and insulin alone treated cultures, while in high glucose-treated cultures with and without insulin the ratio was reversed **(Figure 3.1.B)**. The percentage of total GAGs in glucose-treated cells was significantly lower than control and with insulin alone. The percentage of total GAGs in high glucose-treated medium with and without insulin was significantly increased compared to control and insulin alone.

A



B

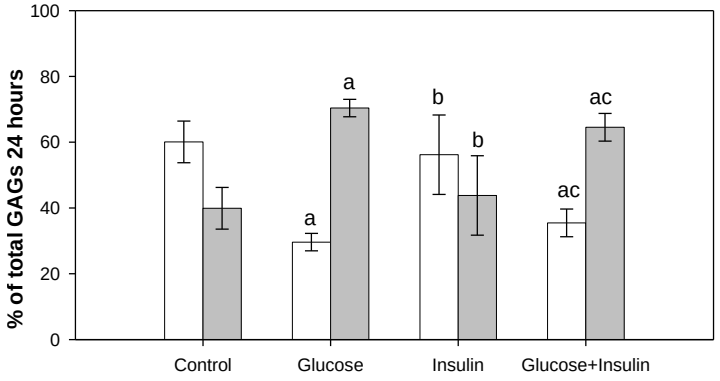


Figure 3.1. GAGs from Cultured Endothelial Cells and Medium at 24 Hours

Cells were treated with high glucose (30 mM) and/or insulin (0.01 unit/ml) for 24 hours. Total GAGs were isolated from cells (white bar) and medium (grey bar) and determined by the carbazole assay (3 dishes/group). Data were analyzed by a one-way ANOVA. Significantly different than: a, Control; b, Glucose; c, Insulin. $p<0.05$

48 hours: GAG concentrations in cells were significantly decreased in high glucose or insulin treated compared to control cultures. Total GAGs in insulin treated cells when high glucose was present was significantly greater than high glucose or insulin alone and similar to control cells (**Figure 3.2.A)**. In the same cultures GAG concentration in medium was significantly increased in high glucose and high glucose plus insulin treated compared to control cultures. When GAG percentages were calculated more GAGs were found in medium than cells for all groups. The percentage of total GAGs in cells was decreased in all treatments compared to controls and increased in insulin plus high glucose compared to high glucose or insulin alone (**Figure 3.2.B)**. The percentages of total GAGs in medium were increased in all treatment groups compared to control and decreased in high glucose plus insulin treated cultures compared to high glucose and insulin alone.

72 hours: Relative GAG concentrations in cells were similar to those seen at 24 hour and significantly less than control for all treatment groups. High glucose plus insulin significantly increased GAG concentrations compared to high glucose or insulin alone **(Figure 3.3.A)**. The GAG concentrations in medium were increased in glucose plus insulin treated cultures compared to the other three groups. When GAG percentages in cells and medium were calculated more GAGs were found in medium than cells for all groups while in controls the cells contained most of the GAGs. The percentage of total GAGs significantly decreased in cells and increased in medium for all treatments compared to control **(Figure 3.3.B)**.

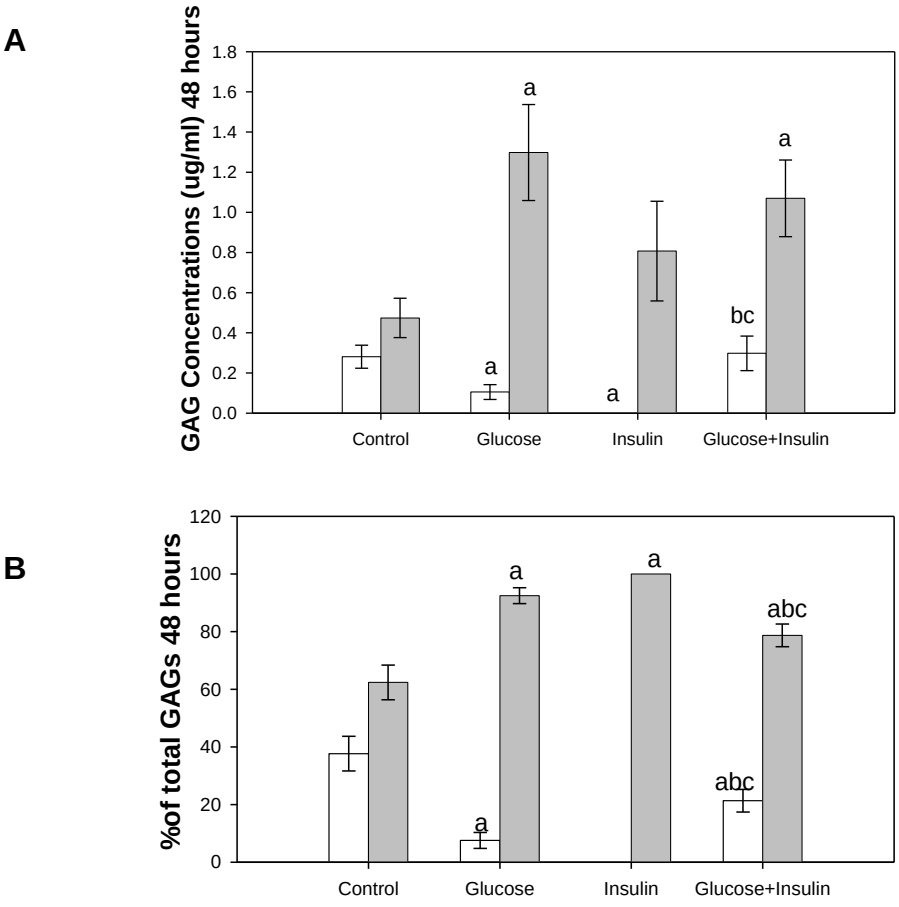


Figure 3.2. GAGs from Cultured Endothelial Cells and Medium at 48 Hours

Cells were treated with high glucose (30 mM) and/or insulin (0.01 unit/ml) for 48 hours. Total GAGs were isolated from cells (white bar) and medium (grey bar) and determined by the carbazole assay (3 dishes/group). Data were analyzed by a one-way ANOVA. Significantly different than: a, Control; b, Glucose; c, Insulin. $p<0.05$

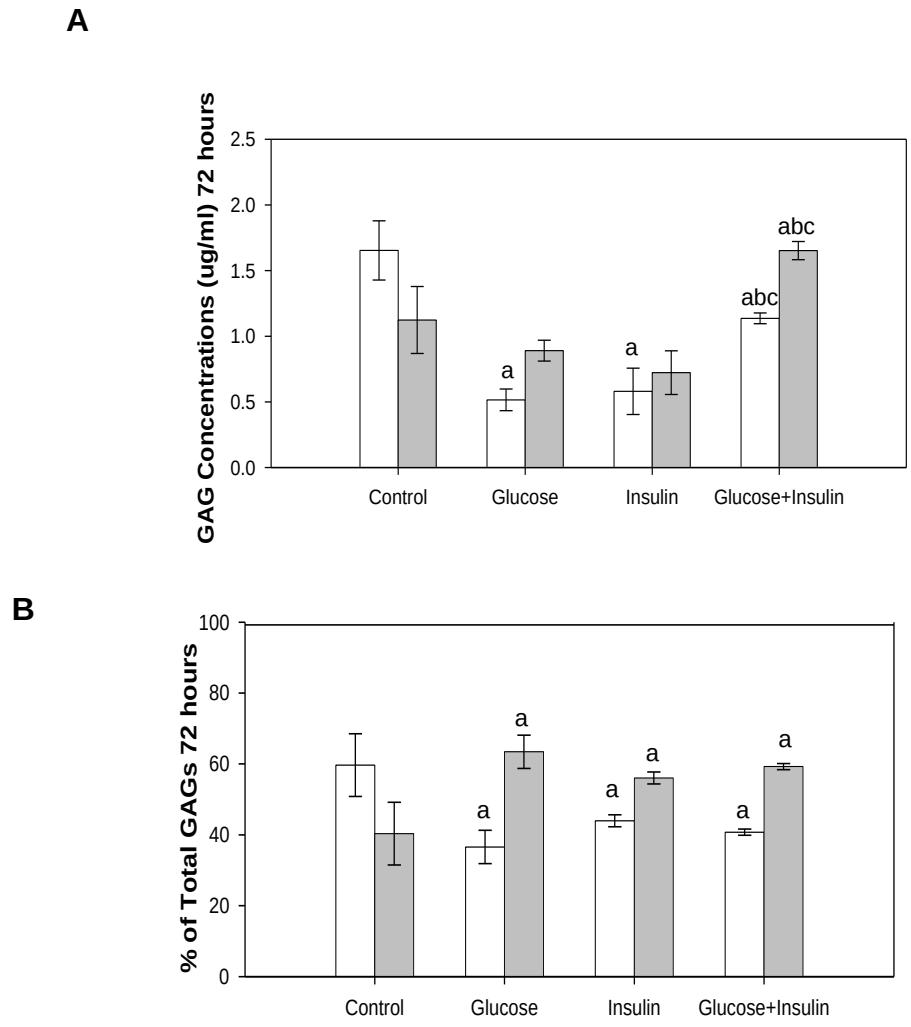


Figure 3.3. GAGs from Cultured Endothelial Cells and Medium at 72 Hours

Cells were treated with high glucose (30 mM) and/or insulin (0.01 unit/ml) for 72 hours. Total GAGs were isolated from cells (white bar) and medium (grey bar) and determined by the carbazole assay (3 dishes/group). Data were analyzed by a one-way ANOVA. Significantly different than: a, Control; b, Glucose; c, Insulin. $p<0.05$

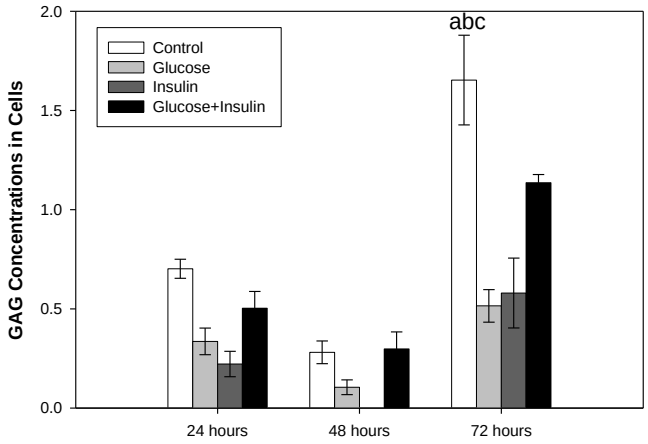
3.4.2. Comparison of Total GAGs in Cultures at Different Times

GAG concentrations were significantly increased in control cells and in control and glucose plus insulin treated medium at 72 compared to 24 hours. GAG concentrations in control cells at 72 hours were greater than high glucose and insulin alone treated cells at 72 hours. GAG concentrations in glucose plus insulin treated medium were greater than all other treatment groups at 72 hours **(Figure 3.4.A and B)**. Significantly more GAGs were found in cells plus medium in control and high glucose plus insulin treated cultures at 72 compared to 24 hours and to other groups at 72 hours **(Figure 3.4.C).** However, cell GAGs in control and glucose and/or insulin treatment at 48 hours were decreased compared to both 24 and 72 hours.

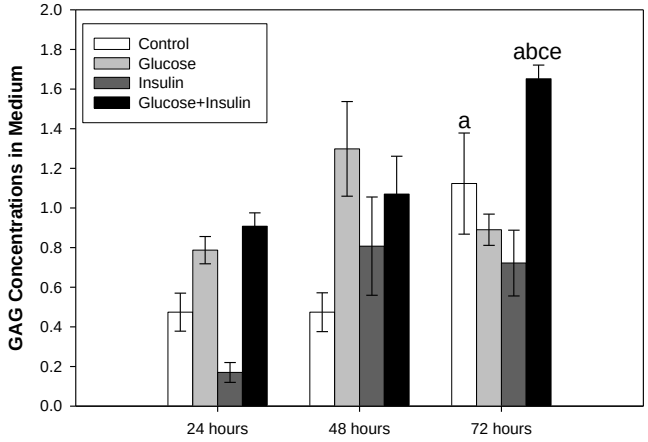
3.4.3. GAG Analysis by Gel Electrophoresis

In order to better define GAGs extracted from cells treated with high glucose and/or insulin for 72 hours, GAGs were exposed to agarose gel electrophoresis, as shown in **Figure 3.5**. In agreement with the carbazole assay at 72 hours, control cells showed a higher GAG content then medium, while with glucose treatment there were less GAGs in cells then medium. Glucose plus insulin treatment showed a greater GAG content than insulin treatment alone both in cells and medium. In insulin alone treated cultures, the faster migrating component was reduced in cells compared to control and high glucose-treated cells. Since heparin interferes with HS determination in HPLC and the carbazole assay, GAGs extracted from cells treated with high

A


GAG Concentrations in Cells
2.0
1.5
1.0
0.5
0.0
Control
Glucose
Insulin
Glucose+Insulin
abc
24 hours
48 hours
72 hours

B


GAG Concentrations in Medium
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Glucose
Insulin
Glucose+Insulin
a
abce
24 hours
48 hours
72 hours

C

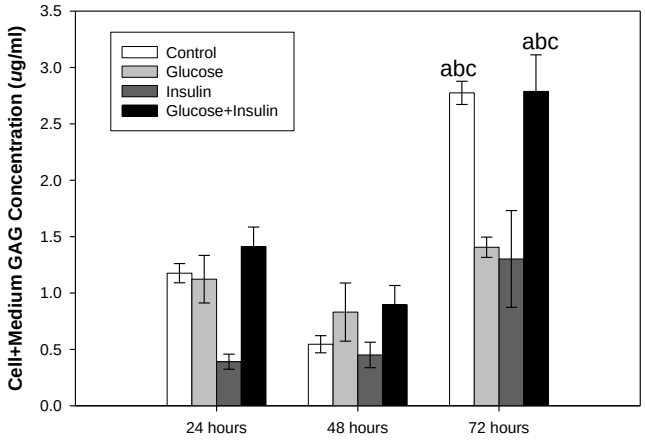

Cell+Medium GAG Concentration (ug/ml)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Glucose
Insulin
Glucose+Insulin
abc
abc
24 hours
48 hours
72 hours

Figure 3.4. Total GAGs Increased with Culture Time in Both Cells and Medium

Cells were treated with high glucose (30 mM), insulin (0.01 unit/ml), glucose+insulin and control medium for 24, 48, 72 hours (3 dishes/group). GAGs isolated from cells (A), medium (B) and cells+medium (C) and determined by the carbazole assay are shown. A one-tailed t-test was used to determine significant differences between treatment at 24 and 72 hour. Significantly greater than: a, same group at 24 hours; b, glucose at 72 hours; c, insulin at 72 hours; d, glucose+insulin at 72 hours; e, control at 72 hours. $p<0.05$

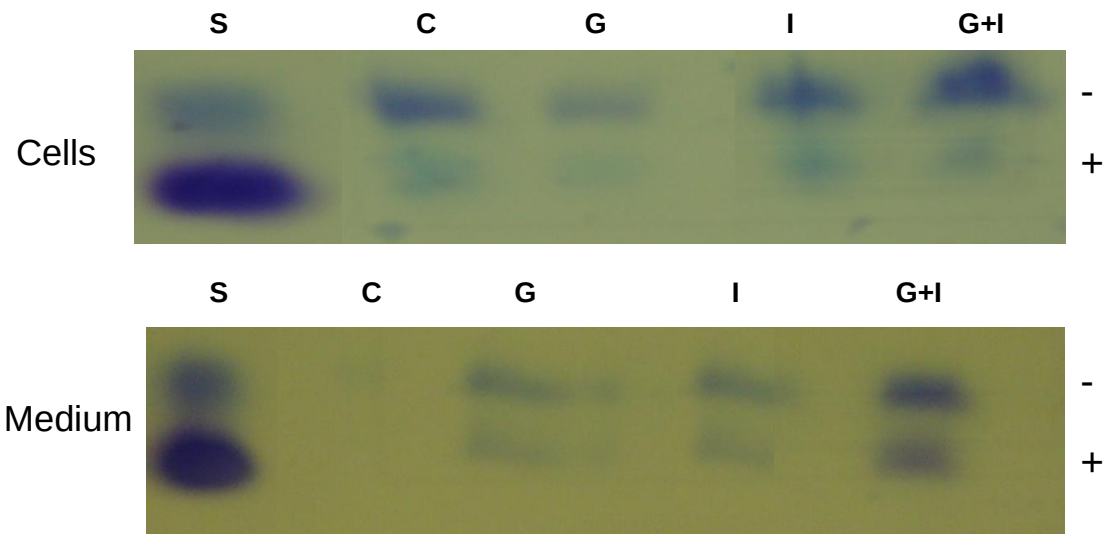


Figure 3.5. Gel Electrophoresis Showing GAGs in Cells and Medium Treated with Glucose and/or Insulin for 72 Hours

Cells were treated with high glucose (30 mM) and/or insulin (0.01 unit/ml) for 72 hours. Total GAGs were isolated from cells and medium and visulized by agarose gel electrophoresis (3 dishes combined/group). +/- indicates charge direction of migration of samples.

Lanes: S, standard, heparan sulfate from porcine intestinal mucosa; C, control; G, glucose; I, insulin; G+I, glucose+insulin

glucose and/or heparin for 72 hours were only determined and distinguished on gel electrophoresis, as shown in **Figure 3.6**. Control and glucose-treated cells showed results similar to those mentioned above for glucose and/or insulin treatment. Glucose plus heparin had more GAG content in cells compared to glucose or heparin alone and in medium compared to heparin alone. In medium the fast migrating band was greatest for heparin plus glucose treatment while the slower migrating bands were greater for the treated group than control.

3.4.4. Correlation of Total HS Disaccharides and Total GAGs

Control cells and cells treated with high glucose (30 mM) (three 60 mm dishes/group) were cultured for 24, 48 and 72 hours. Total GAGs and HS disaccharides were obtained from the same samples. There was a good correlation between total HS disaccharides and total GAGs obtained **(Figure 3.7)**.

3.5. Discussion

Syndecans and perlecan are major HSPGs synthesized and secreted by ECs. Syndecans are mainly expressed on the cell surface and perlecan is presented in the ECM (Kaji *et al*., 2000). HS chains derived from HSPG are the main GAGs found in ECs (Gharagozlian *et al*., 2006). In the present study, we found that total HS determined by HPLC correlated well with total GAGs determined by the carbazole assay **(Figure 3.7)**. Therefore, total

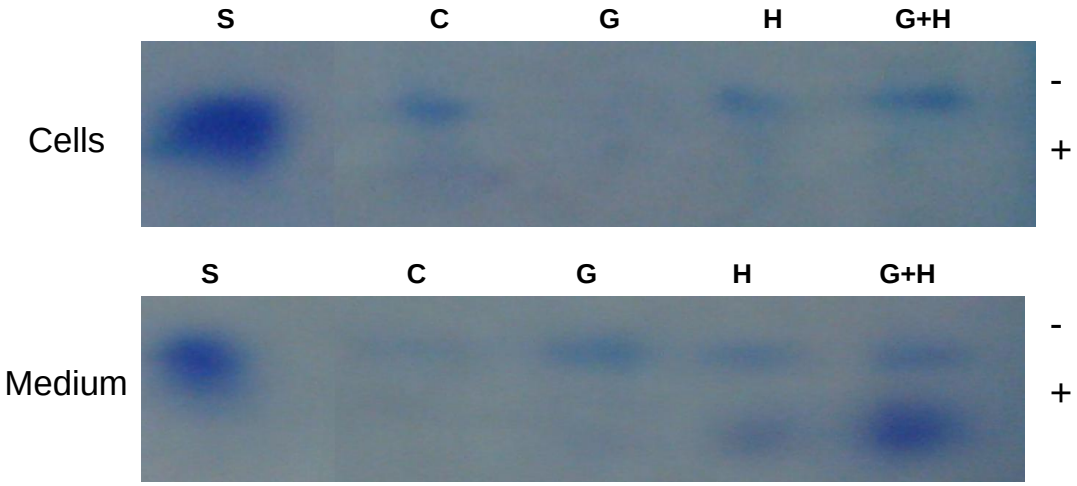


Figure 3. 6. Gel Electrophoresis Showing GAGs in Cells and Medium Treated with Glucose and/or Heparin for 72 Hours

Cells were treated with high glucose (30 mM) and/or heparin (0.5 μg/ml) for 72 hours. Total GAGs were isolated from cells and medium and visulized by agarose gel electrophoresis (1 dish/group). +/- indicates charge direction of migration of samples. Lanes: S, standard, heparan sulfate from porcine intestinal mucosa; C, control; G, glucose; H, heparin; G+I, glucose+heparin

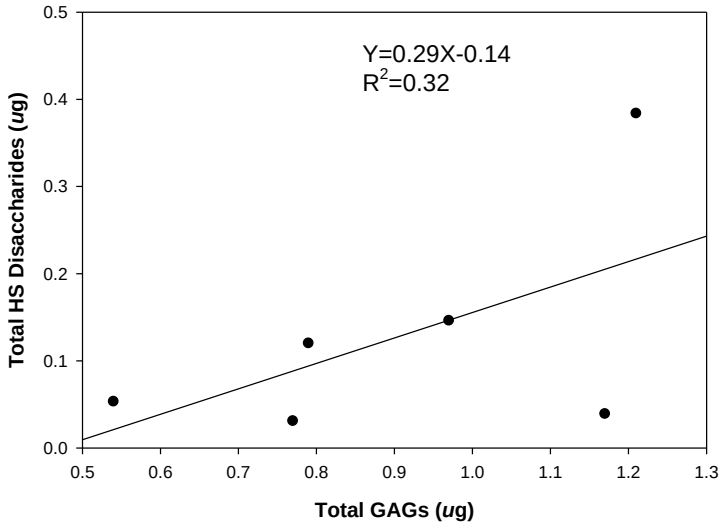


Figure 3. 7. Correlation of Total HS Disaccharides and Total GAGs in Cultured Endothelial Cells

Control cells and cells treated with high glucose (30 mM) were cultured for 24, 48 and 72 hours (3 dishes/group). Total GAGs were isolated, after combining the cells and medium separately from the three dishes, and were determined by the carbazole assay. HS disaccharides were obtained by heparinase digestion and determined by RPIP-HPLC systems

GAGs likely reflect total HS present within endothelium. The GAGs were extracted from the entire cultured endothelial monolayer including mainly syndecans and perlecan.

Total GAGs from the cells and medium were determined when cultured ECs were exposed to high glucose and/or insulin. At the earliest observed time (24 hours), cells treated with high glucose showed a reduction in total GAGs **(Figure 3.1)**. The observation that cell total GAGs decreased and medium GAGs increased up to 72 hours with high glucose treatment **(Figure 3.3, 3.4, and 3.5)** are consistent with the finding that GAG contents from syndecan-1 and perlecan were decreased in cultured primary ECs treated with high glucose for two and five days (Vogl-Willis and Edwards, 2004a; Vogl-Willis and Edwards, 2004b). Decreased cell GAGs suggest hyperglycemia could induce HSPG degradation or inhibit HSPG synthesis in ECs. The possible consequences of decreased GAGs are reduced interaction of several biofactors such as growth factors, coagulation factors, chemokines, adhesion molecules, and lipoprotein lipase with HSPG in the vasculature (Nugent and Iozzo, 2000). Loss of these biofactors is likely to induce endothelial injury. We previously observed endothelial injury following addition of high glucose in the same culture model (Han *et al*., 2005). Although our culture model uses aortic ECs, and ECs in macro- and micro-vessels have different properties, both are characterized by the same pathological features in diabetes mellitus. Endothelial injury likely contributes to diabetic cardiovascular complications both in micro- and macro-vessels

such as nephropathy, retinopathy and atherosclerosis (Colwell and Lopes-Virella, 1988a; Richardson *et al*., 1980).

Increased total GAGs in medium with high glucose treatment suggest that GAGs are released from cell proteoglycan core proteins. The core protein may remain on the cell surface. This was indirectly confirmed by immunoprecipitation assays where the core protein of syndecan-1 remained on ECs and was expressed at lower intensity in medium from high glucose-treated ECs (Gharagozlian *et al*., 2006). Similarly, immunostaining studies showed that reduction in HS GAGs in the GBM under diabetic conditions was not accompanied by a reduction in the HSPG core protein (Tamsma *et al*., 1994). As well, decreased HSPG synthesis by human aortic ECs in high glucose conditions was not the result of a decrease in GAG size, further suggesting that entire GAG chains were released into culture medium (Vogl-Willis and Edwards, 2004a). It is unlikely that GAGs are further degraded in medium.

Insulin controls blood glucose utilization and influences the metabolism of fat and protein. Insulin also has effects on the expression of numerous genes. Insulin alone decreased GAG concentration in cells at all time points in our study. In a study of regulation of HSPG metabolism and hepatocyte growth by insulin and phosphatidylinositol, insulin markedly stimulated the rate of internalization of matrix HSPG and phospholipase C and therefore may control cell surface HSPG turnover (Ishihara *et al*., 1987). Thus, insulin may regulate enzymes involved in metabolism of proteoglycans.

Insulin promoted shedding of syndecan ectodomains from 3T3-L1 adipocytes, by an unknown mechanism (Reizes *et al*., 2006). However, it is unknown whether these changes are coordinated by phosphatidylinositol, the second messenger in the action of binding insulin to its receptor, and whether shedding of syndecans are seen in ECs in response to insulin. In our studies there was a trend that insulin alone increased GAGs in culture medium as culturing time increased **(Figure 3.4)** which may indicate HSPG turnover or shedding of syndecan into medium, however, GAGs were significantly reduced in 72 hour cultures compared to control suggesting that GAG synthesis may also be inhibited. These limited observations cannot define the precise mechanisms involved in insulin reduction of GAGs in cultured ECs, and further investigation is required. Considerable evidence indicates that vascular endothelium is a physiological target of insulin and a potential link between insulin resistance and atherosclerosis (Hsueh and Law, 1999; Mather *et al*., 2001a). Endothelial dysfunction is one of the earliest detectable signs in insulin resistance (Zeng *et al*., 2000). Our present study shows that decreased GAG content in insulin alone treated cells may lead to endothelial dysfunction caused by GAG degradation and/or inhibition of GAG synthesis.

The evidence for insulin influencing different cultured cells under high glucose conditions is varied and controversial. Studies on cultured mesangial cells treated with glucose and insulin showed insulin did not influence HSPG content independent of the ambient glucose levels (Olgemoller *et al*., 1992). Insulin was unable to correct the 30 mM glucose induced reduction in the

HSPG synthesis of rat glomerular epithelial cell layers that resemble the GBM (Kasinath, 1995). However, reduction in chondroitin sulfate proteoglycan synthesis found in articular cartilage in diabetic rats could be completely restored by administration of insulin (Unger *et al*., 1991). Administration of large doses of insulin restored HSPG synthesis in BM following reduction of HSPG by implantation of Engelbreth-Holm-Swarm (EHS) tumor cells in diabetic mice (Rohrbach *et al*., 1983). Our current studies showed that: insulin increased GAGs in high glucose-treated cells compared to insulin alone at all times and to high glucose alone at 48 and 72 hours; insulin increased medium GAGs at all times compared to control, at 24 and 72 hours compared to insulin alone; insulin was able to maintain high glucose-treated cell plus medium GAGs at control levels at 72 hours **(Figure 3.4.C)**. Previous studies noted that insulin alone increased ET-1 levels (Mandal *et al*., 2000). Perhaps insulin maintains HSPGs under hyperglycemic conditions through its effects on ET-1. Although these studies indicate that insulin has the potential to improve hyperglycemia-induced alteration of HSPG in ECs, the effect is time dependent. It is possible that insulin modulation of proteoglycan metabolism under hyperglycemic conditions is cell or tissue-specific and specific for individual species of proteoglycans.

Heparin, used as an antithrombotic drug, is also considered a potent vasodilator (Mandal *et al*., 1995), and lowers blood pressure (Yokokawa *et al*., 1994). Heparin has the ability to inhibit heparanase upregulation induced

by high glucose in cultured ECs (Han *et al*., 2007). Our results suggest that heparin increases GAG content in cells treated with high glucose suggesting the protective effect of heparin on ECs. Taken together with evidence that heparin stimulates HS synthesis and modification of HS in ECs (Nader *et al*., 1989; Nader *et al*., 1991), this further suggests that heparin's protective action may occur due to an increase in GAG synthesis or inhibition of heparanase production.

In summary, ECs are targets for hyperglycemia in diabetes mellitus, resulting in cardiovascular complications. Alteration of GAGs synthesized by cells is an important pathological mechanism, which can be correlated with cell injury by hyperglycemia. Insulin or heparin undoubtedly protects cell GAGs from degradation and/or increases GAG synthesis which may protect cells from high glucose injury. These results confirm the efficacy of insulin as a therapeutic drug for the diabetic patient since it not only lowers blood glucose levels, but also protects the vasculature. The effectiveness of heparin on the protection of ECs through maintaining GAG content was also confirmed. Elevated insulin alone without hyperglycemia may have a damaging effect. The exact mechanism of insulin influence on EC HSPG metabolism needs to be further elucidated at the levels of gene expression, and cell signaling pathways.

4. Characteristics of Heparan Sulfate Disaccharides in Endothelial Cells Treated with High Glucose

4.1. Abstract

HS is a sulfated polysaccharide that is found on the surface of most cells and is present in the ECM. The changes in HS disaccharide content in ECs under high glucose conditions are unknown. Our objectives were to isolate and determine total amount of GAGs in cells and medium of control and high glucose-treated EC cultures and to characterize HS disaccharides present in the recovered GAGs. Total GAGs were determined by the carbazole assay and HS disaccharides were determined by HPLC. The characterization of disaccharides recovered differ in medium but not in cells between control and glucose-treated cultures. Both total GAGs and HS disaccharides were decreased in ECs and increased in medium with high glucose treatment.

4.2. Introduction

HSPGs are found on the surface of most cells and are part of the ECM. HS GAGs are part of the proteoglycans and consist of repeating units of disaccharides that are highly variable in structure. HS has been shown to bind and regulate the activities of many proteins, including enzymes, growth

factors, ECM proteins, and the cell surface proteins of pathogens (Bernfield *et al*., 1999; Rostand and Esko, 1997). Genetic studies have recently shown that HS interaction with proteins reflects its ability to regulate biological processes, such as cell signaling and morphogenesis *in vivo* (Lander and Selleck, 2000; Perrimon and Bernfield, 2000). Several disease conditions such as cardiovascular diseases, cancer, inflammation, diabetes and amyloidosis are associated with changes in structural and functional alterations of GAG components (Iozzo and Cohen, 1993; Jackson, 1997; Rosenberg *et al*., 1997; van der Woude and van Det, 1997). HS degradation has been found in diabetic nephropathy, skin BM and other organs. However, the changes in HS disaccharide content in ECs under high glucose conditions are unknown. The present study was to determine the effect of high glucose on changes in GAG content and HS disaccharide composition in cultured ECs and medium. Total GAGs were determined by the carbazole assay and HS disaccharides were determined by HPLC.

4.3. Methods

Treatment of Cells: Six 60 mm dishes of porcine aortic ECs (Passage 4) were cultured in serum-free medium. Three dishes were used as control (M199) and three were treated with high glucose (30 mM) for 24, 48 and 72 hours.

Collection of Samples: Medium was collected from each dish into 15 ml centrifuge tubes. Cells were washed once with calcium magnesium

free-DPBS (CMF-DPBS), scraped from the dish using a cell scraper, and collected into 1.5 ml tubes using 1 ml CMF-DPBS. Then cell pellets were collected by centrifugation. All samples were stored at - 80 °C.

Extraction and Determination of total GAGs: Similar methods were used to extract and determine total GAGs as described in the methods in **Chapter 3.**

Obtaining HS Disaccharides: After freeze-drying, total GAGs were digested by chondroitinases A, B, C and ACII. Samples were passed through the Microcon YM-3 Centrifugal Filter Devices (Millipore, USA) to remove degraded chondroitin sulfates. Heparan sulfate stayed on the membrane in the devices. Heparinase I, II and III were added to the membrane and HS was digested into HS disaccharides. The detailed method is described in **Chapter 3.2.6**.

Analysis of HS Disaccharides: Freeze-dried digested HS, dissolved in 10 μl DD water was run on HPLC to determine the amount and characteristics of HS disaccharides. The detailed method is described in **Chapter 3.2.6**.

4.4. Results

4.4.1. Total GAGs

When GAGs were determined by the carbazole assay, the amount of GAGs increased with time in both control cells and medium. Data are shown

in **Table 4.1**, the amount of GAGs is higher in cultured cells than in medium, both in control and high glucose-treated cultures at all times.

4.4.2. Total HS Disaccharides

The total amount of HS disaccharides as determined by HPLC was increased both in control cells and medium when 72 hour was compared to 24 hour. This is also correlated with that for total GAGs as shown in **Table 4.2**.

4.4.3. Characteristics of HS Derived from PAEC Cultures

As shown in **Figure 4.1 and 4.2**, eight disaccharides were detected, by HPLC, in the HS standard. The eight disaccharides are: **1:** ΔUA-GlcNAc, **2:** ΔUA-GlcNS, **3:** ΔUA-GlcNAc6S, **4:** ΔUA2S-GlcNAc, **5:** ΔUA-GlcNS6S, **6:** ΔUA2S-GlcNS, **7:** ΔUA2S-GlcNAc6S, **8:** ΔUA2S-GlcNS6S. Two HS disaccharides #4 and #5 were absent in cultured PAECs compared to standard as shown in **Figure 4.1.B**. There was no difference between control and high glucose-treated cells in terms of types of disaccharide, but the amount of disaccharides obtained from control cells was more than glucose-treated cells shown by comparing the values on the Y-axis for control versus high glucose **(Figure 4.1.B and C)**. Disaccharides #4, 6 and 8 were not detectable in control medium **(Figure 4.2.B)**. There was no diference in the amount of disaccharides from control versus glucose medium, but an additional disaccharide #5 along with #4, 6 and 8 was not detectable in high glucose-treated medium **(Figure 4.2.C)**.

Table 4.1. Total GAGs Isolated from Cells and Medium

Sample	Cell						Medium					
	Control			Glucose			Control			Glucose		
Hours	24	48	72	24	48	72	24	48	72	24	48	72
Conc. (μg/ml)	9.7	11.7	12.1	11.7	8.9	11.3	5.4	7.7	7.9	5.1	5.5	6.2
Total (μg)	0.97	1.17	1.21	1.17	0.89	1.13	0.54	0.77	0.79	0.51	0.55	0.62

Control cells and cells treated with high glucose (30 mM) were cultured for 24, 48, 72 hours (1 sample/group). Then GAGs were isolated from cells and medium and determined by the carbazole assay.

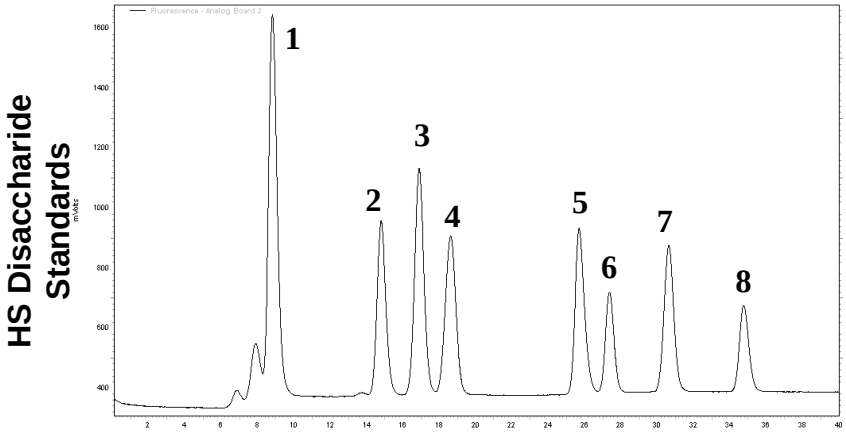
Table 4.2. Total Amount of HS Disaccharides Associated with Total GAGs in Control Cultures

Sample	Cell			Medium		
Hours	**24**	**48**	**72**	**24**	**48**	**72**
Total HS* (ng)	145.9	39.0	383.6	53.1	30.8	119.9
Total GAGs (ug)	0.97	1.17	1.21	0.54	0.77	0.79

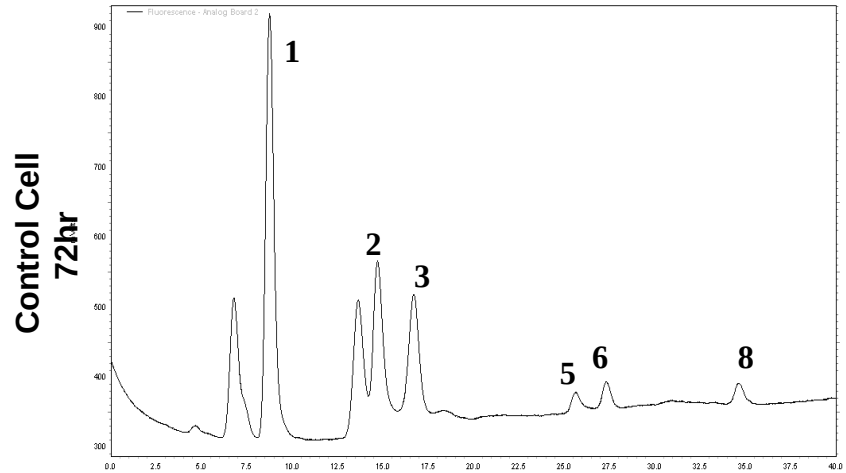
Control cells and cells treated with high glucose (30 mM) were cultured for 24, 48, 72 hours (1 sample/group). Then GAGs were isolated from cells and medium and determined by the carbazole assay. HS disaccharides were determined by HPLC.

* Total HS refers to the combination of total amount of each individual HS disaccharide.

A:


HS Disaccharide Standards
1
2
3
4
5
6
7
8

B:


Control Cell 72hr
1
2
3
5
6
8

C:

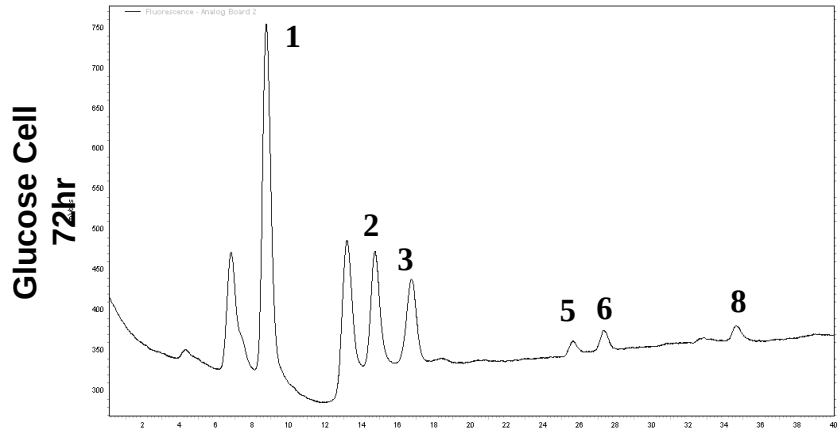

Glucose Cell 72hr
1
2
3
5
6
8

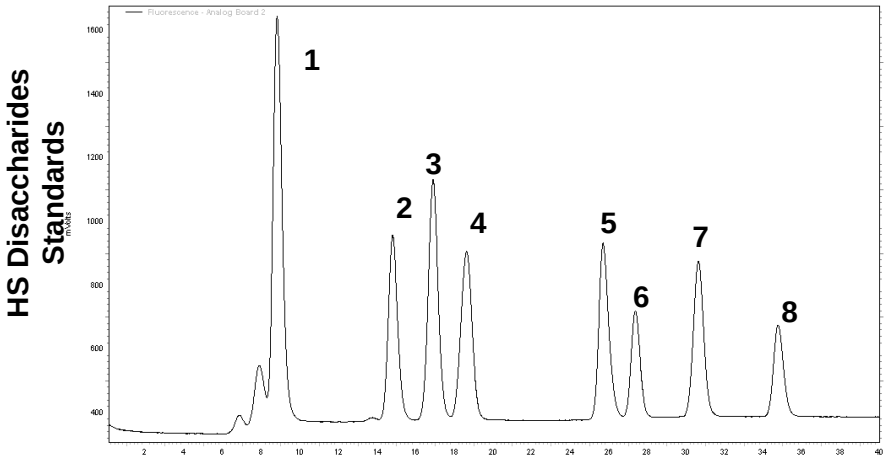
Figure 4.1. HS Disaccharide Peaks Shown in HPLC Analysis for Control and High Glucose-treated Cells at 72 Hours

A: Eight HS disaccharide peaks detected by HPLC in the HS disaccharide standard. **B:** HS disaccharides isolated from control cells for 72 hours. **C:** HS disaccharides isolated from high glucose-treated cells for 72 hours.

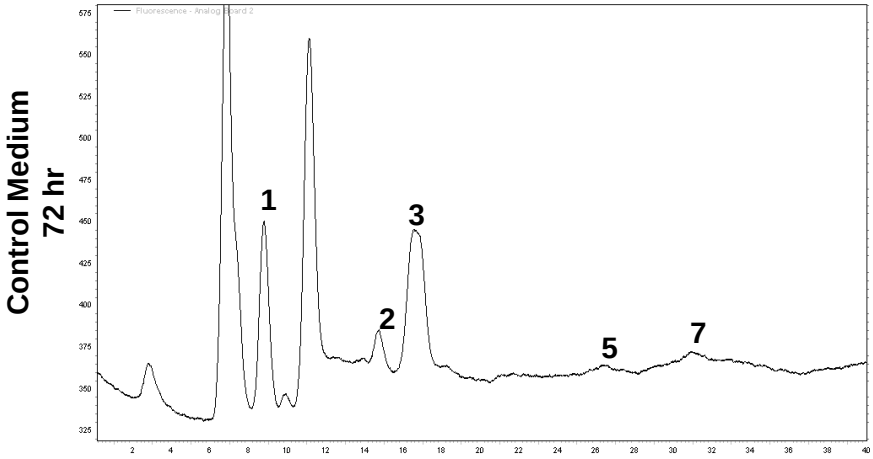
The eight disaccharides are: **1:** ΔUA-GlcNAc, **2:** ΔUA-GlcNS, **3:** ΔUA-GlcNAc6S, **4:** ΔUA2S-GlcNAc*, **5:** ΔUA-GlcNS6S, **6:** ΔUA2S-GlcNS, **7:** ΔUA2S-GlcNAc6S*, **8:** ΔUA2S-GlcNS6S.

* Disaccharides not detected in cells.

A:


HS Disaccharides
Standards
1
2
3
4
5
6
7
8

D:


Control Medium
72 hr
1
2
3
5
7

E:

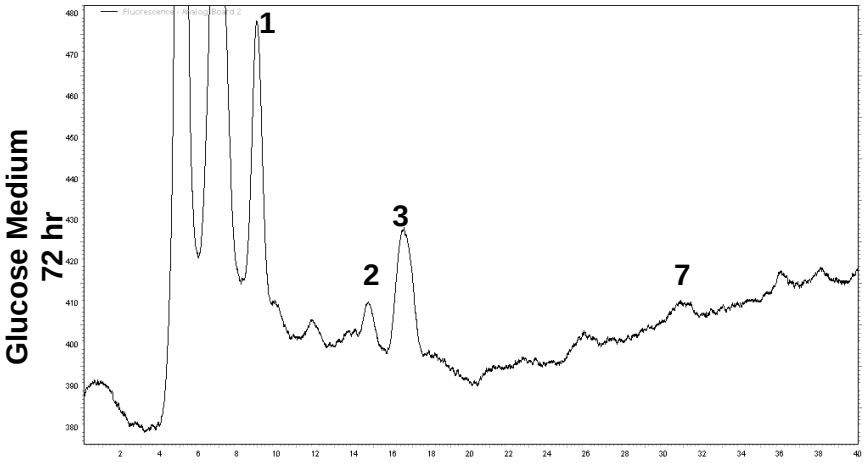

Glucose Medium
72 hr
1
2
3
7

Figure 4.2. HS Disaccharide Peaks Shown in HPLC Analysis for Control and High Glucose-treated Medium at 72 Hours

A: Eight HS disaccharide peaks detected by HPLC in the HS disaccharide standard. **D:** HS disaccharides isolated from control medium for 72 hours. **E:** HS disaccharides isolated from high glucose-treated medium for 72 hours.

The eight disaccharides are: **1:** ΔUA-GlcNAc, **2:** ΔUA-GlcNS, **3:** ΔUA-GlcNAc6S, **4:** ΔUA2S-GlcNAc*, **5:** ΔUA-GlcNS6S**, **6:** ΔUA2S-GlcNS*, **7:** ΔUA2S-GlcNAc6S, **8:** ΔUA2S-GlcNS6S*.

* Disaccharide was not detactable in control medium.

** Additional disaccharide not detected in high glucose-treated medium.

4.4.4. HS Disaccharide in Control Cells and Medium

Cells contained more HS disaccharides than medium as shown in **Figure 4.3** at 24 hours and **Figure 4.4** at 72 hours. At 24 hours, the unsaturated disaccharides UA2S-GlcNAc, UA-GlcNS6S and UA2S-GlcNAc6S were not detected in both cells and medium. As well UA-GlcNS, UA2S-GlcNS and A2S-GlcNS6S were not detected in medium. At 72 hours UA2S-GlcNAc and UA2S-GlcNAc6S were not detected in both cells and medium, while only UA-GlcNS6S was not detected in medium. However, the combination of total individual disacharides was much higher in cells than medium both at 24 and 72 hours.

4.4.5. HS Disaccharides in Cells Treated with/without High Glucose

Control cells had more HS disaccharides than cells treated with high glucose shown in **Figure 4. 5** at 24 hours and **Figure 4.6** at 72 hours. The unsaturated disaccharides UA2S-GlcNAc, UA-GlcNS6S and UA2S-GlcNAc6S were not detected in both control and glucose-treated cells at 24 hours. The unsaturated disaccharides UA2S-GlcNAc and UA2S-GlcNAc6S were not detected in both control and glucose-treated cells at 72 hours. However, the combination of total individual disacharides was much higher in control cells than glucose-treated cells both at 24 and 72 hours.

4.4.6. HS Disaccharidess in Medium Treated with/without High Glucose

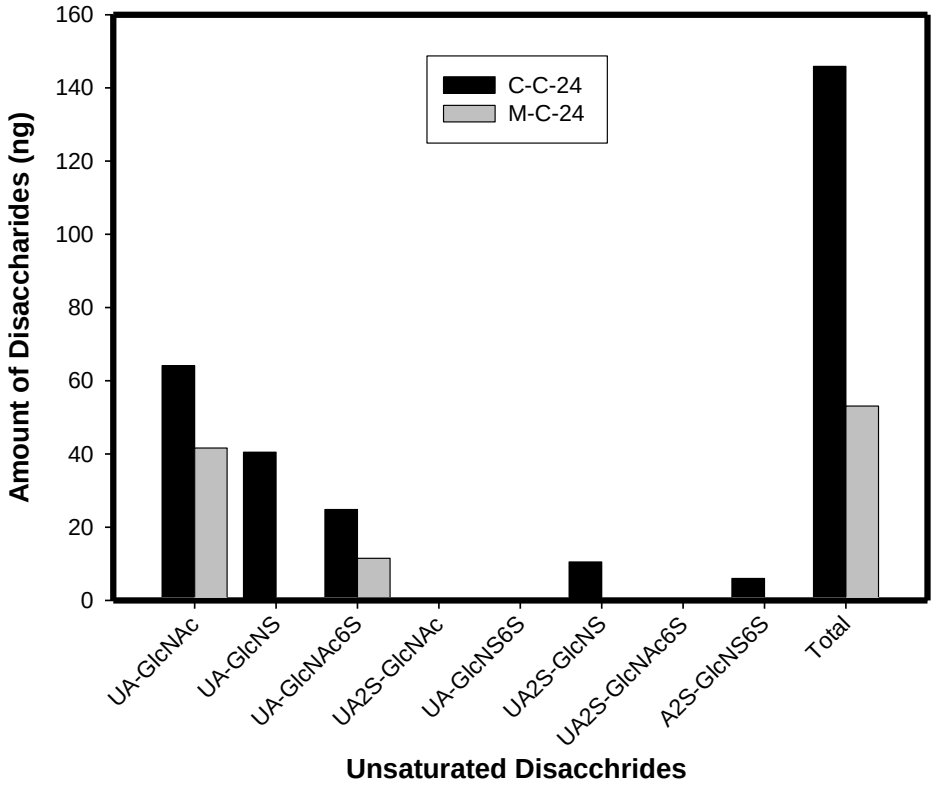


Figure 4.3. HS Disaccharide in Control Cells and Medium at 24 Hours

Total GAGs were isolated from cells and medium and digested by chondroitinases and heparinases. Unsaturated disaccharides were determined by HPLC.

C-C-24: control cells 24 hour treatment; M-C-24: control medium 24 hour treatment

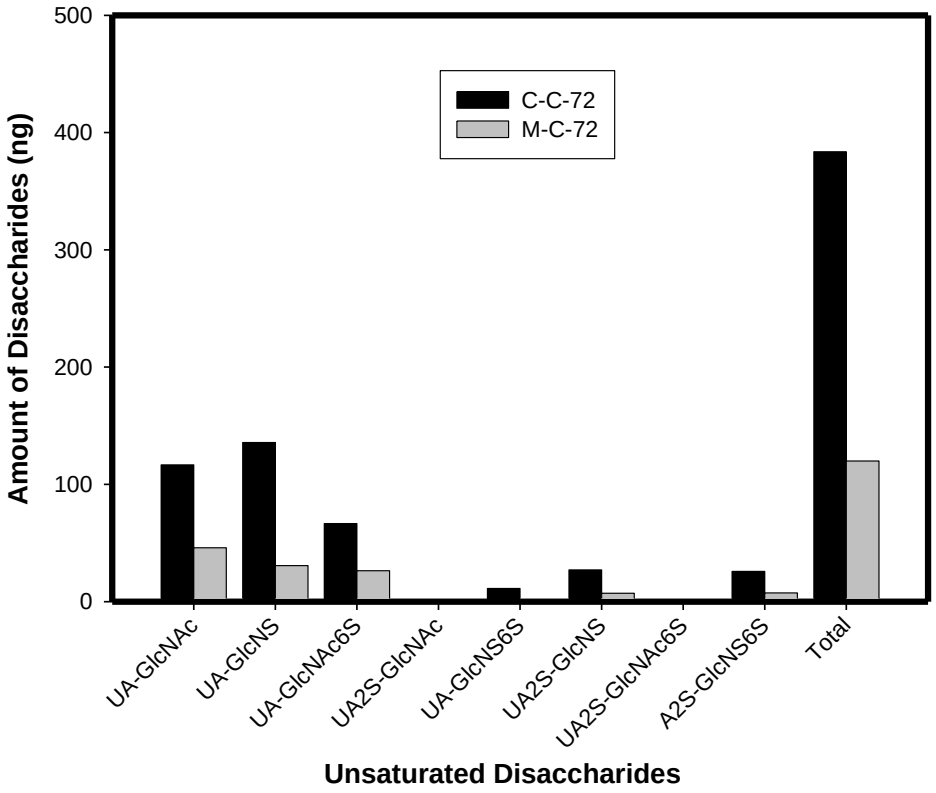


Figure 4.4. HS Disaccharides in Control Cells and Medium at 72 Hours

Total GAGs were isolated from control cells and medium and were digested by chondroitinases and heparinases. Unsaturated disaccharides were determined by HPLC. C-C-24: control cells 72 hour treatment; M-C-72: control medium 72 hour treatment

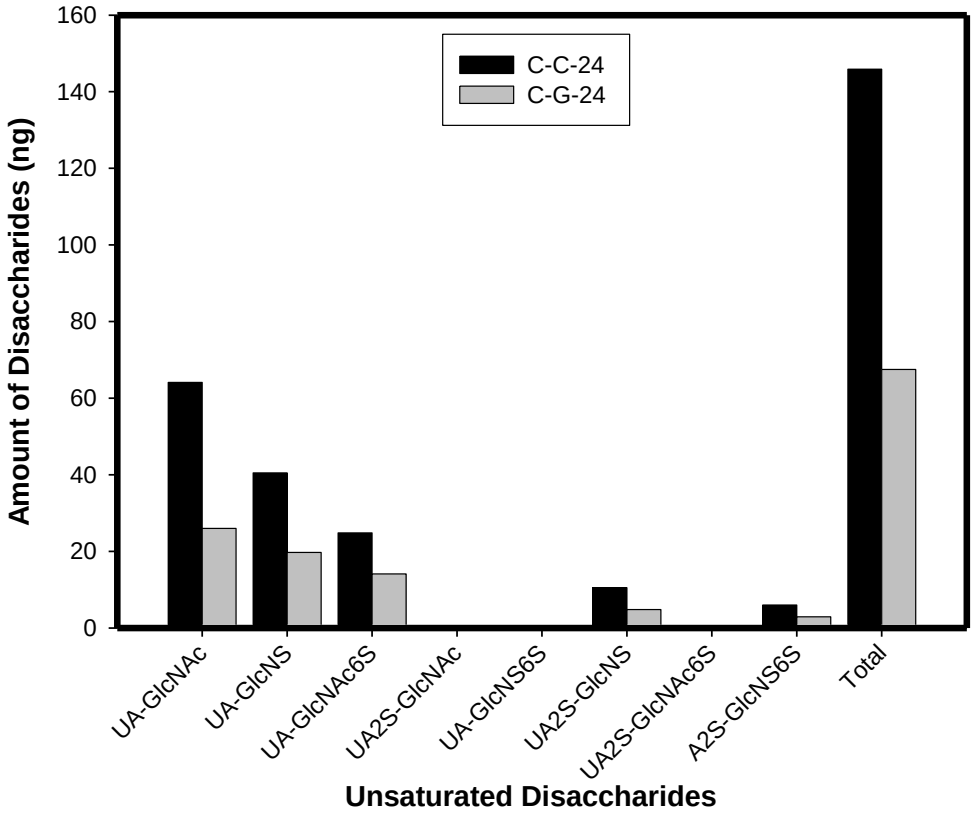


Figure 4.5. HS Disaccharides in Cells Treated with/without High Glucose for 24 Hours

Total GAGs were isolated from control and high glucose-treated cells and medium and were digested by chondroitinases and heparinases. Unsaturated disaccharides were determined by HPLC.

C-C-24: control cells 24 hour treatment; C-G-24: cells with 24 hour high glucose treatment.

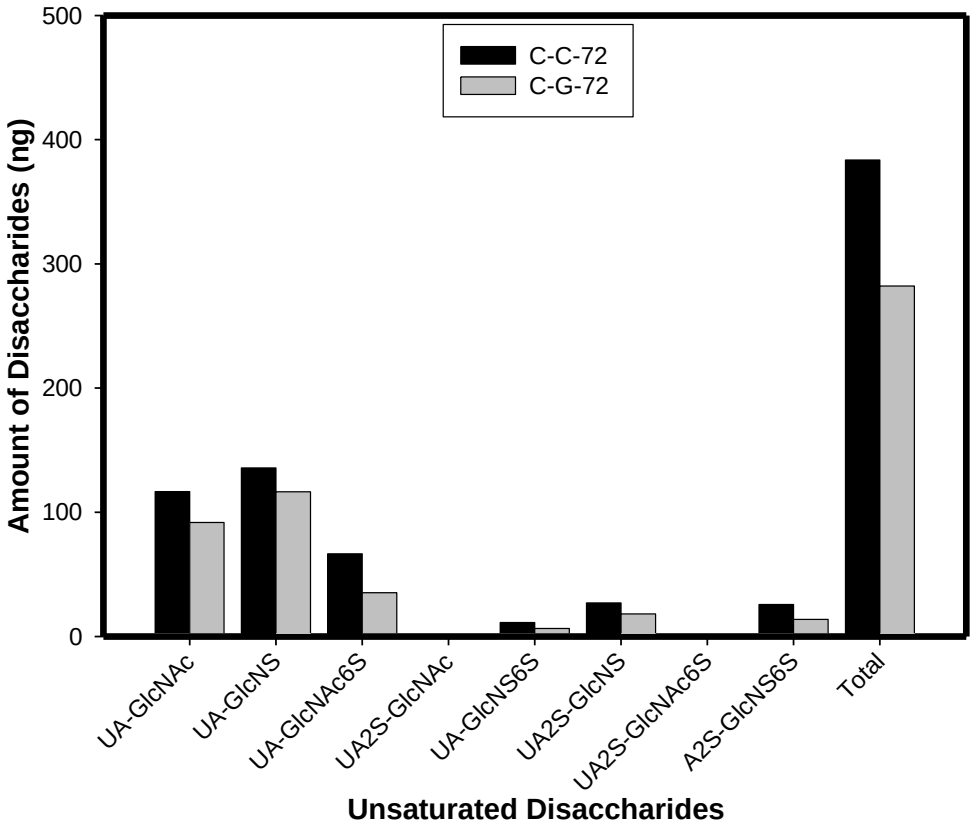


Figure 4.6. HS Disaccharides in Cells Treated with/without High Glucose for 72 Hours

Total GAGs were isolated from control and glucose-treated cells and medium and were digested by chondroitinases and heparinases. Unsaturated disaccharides were determined by HPLC.

C-C-72: control cells 72 hour treatment; C-G-72: cells with 72 hour glucose treatment.

High glucose-treated medium had more total HS disaccharides than control medium at 72 hours as shown in **Figure 4.7**. This was not consistent for all disaccharides. The unsaturated disaccharide UA2S-GlcNAc was not detected in both control and glucose-treated medium, while UA-GlcNS6S, UA2S-GlcNS and A2S-GlcNS6S in glucose-treated medium and UA2S-GlcNAc6S in control medium were not detected.

4.5. Discussion

These preliminary (one experiment) results showed that ECs contained more GAGs than medium in both control and glucose-treated cultures and the amount of GAGs increased with time in both cells and medium indicating that most of GAGs attached to the cell membrane or ECM are not secreted into the medium. These results are consistent with those described in **Chapter 3**.

Analysis of the total amount of HS disaccharides by HPLC in this study showed that more HS disaccharides were found in control than high glucose-treated ECs, while more HS disaccharides were in high glucose-treated medium than control medium. These data further confirmed that high glucose not only decreased total GAGs, but actually decreased the basic structure unit of GAGs, the HS disaccharide. This is an area open to further investigation, particularly the enzymes involved in HS GAG synthesis or degradation under hyperglycemic conditions. The positive correlation

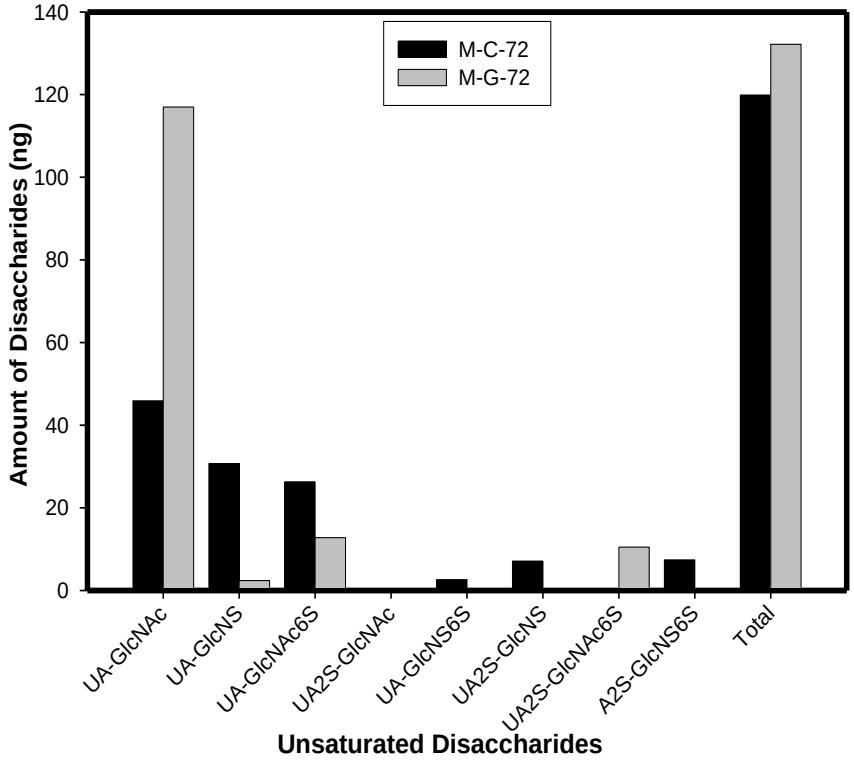


Figure 4.7. HS Disaccharides in Medium for 72 Hours Treated with/without High Glucose

Total GAGs were isolated from control and high glucose-treated cells and medium and were digested by chondroitinases and heparinases. Unsaturated disaccharides were determined by HPLC.

M-C-72: control medium 72 hour treatment; C-G-72: medium 72 hour high glucose treatment.

between total GAGs and total HS disaccharides suggested that changes in GAG content is due to changes in HS disaccharides.

Primarily, HS disaccharides extracted from both control and high glucose-treated ECs and control medium contained six disaccharides and glucose-treated medium contained five disaccharides compared to the HS disaccharide standard which shows eight disaccharides suggesting an effect of high glucose on HS disaccharide composition. These results raise the questions as to whether ECs from different sources or different cells from the same species produce all or some of the eight disaccharides for GAG synthesis.

5. Gene Alteration of Proteoglycans and Heparanase in Endothelial Cells under Hyperglycemic Conditions: The Effect of Insulin and Heparin

5.1. Abstract

HSPGs are macromolecules containing a core protein with GAG chains attached. HSPGs in the vasculature are mainly syndecans on the cell membrane and perlecan in the ECM which are synthesized by ECs. Reduced HSPG is associated with EC dysfunction in hyperglycemia which is a risk factor for diabetic cardiovascular complications. Changes in HSPG core protein and mRNA in diabetes remain controversial. Although heparanase is believed to break down HS, its effect on core protein is unknown. The present study was to determine if mRNA of core proteins of syndecan and perlecan from cultured ECs were altered by high glucose and if this was correlated with heparanase mRNA expression. As well, the effect of heparin and insulin on expression of these three genes was studied. Confluent human aortic endothelial cells (HAECs) were treated with high glucose (30 mM) and/or insulin (0.01 unit/ml) and/or heparin (0.5 μg/ml) for 24 hours. Total RNA was isolated from cells and cDNA was synthesized. Then Real time PCR analysis revealed that high glucose increased heparanase, decreased syndecan and had no effect on perlecan gene

expression. Heparin alone decreased heparanase and perlecan mRNA, and increased syndecan mRNA. Insulin alone decreased mRNA expression of heparanase and perlecan, but had no effect on syndecan. When high glucose was present, heparin decreased mRNA expression of heparanase and perlecan and increased that of syndecan compared to high glucose treatment. Insulin in the presence of high glucose decreased expression of perlecan compared to control, of syndecan compared to control and heparin alone, and of heparanase compared to glucose alone. Heparin and insulin in combination decreased perlecan mRNA compared to control and glucose treatment and increased mRNAs of syndecan and heparanase compared to all other treatments. We conclude that heparin may protect cells from high glucose injury by enhancing syndecan and inhibiting heparanase production. Insulin alone decreased heparanase and perlecan mRNA in the presence and absence of high glucose and syndecan in the presence of high glucose suggesting that insulin may protect cells by inhibiting heparanase mRNA and may be harmful to cells by decreasing perlecan and syndecan mRNA. The combination of insulin and heparin protects cells from high glucose injury by increasing syndecan, but does not decrease heparanase mRNA, suggesting a possible interference of insulin and heparin in gene regulation at the transcriptional levels for heparanase mRNA expression.

5.2. Introduction

Hyperglycemia is an independent risk factor for cardiovascular complications in diabetes mellitus. One of the potential mechanisms of diabetic cardiovascular complications caused by hyperglycemia is the impaired function of ECs. Endothelial cells play a crucial role in the regulation of vascular tone and permeability and in the maintenance of homeostasis. A number of mechanisms have been suggested to mediate endothelial dysfunction in hyperglycemia that may be related to the pathogenesis of diabetic endothelial damage. These include increased tissue oxidative stress, increased production of AGEs, altered coagulation and fibrinolytic processes, and increased production of cytokines (Elliott *et al*., 1993). In addition, degradation of HSPGs could be an important mechanism for endothelial dysfunction due to release of bioactive factors associated with HS chains (Kanwar *et al*., 1980).

HSPGs are macromolecules found in all mammalian tissues and cells. HSPGs normally consist of two to three HS chains positioned in close proximity to each other along protein cores that localize proteoglycans to the cell surface or to ECM (Gallagher *et al*., 1986). HS is a prominent component of blood vessels and the most common GAG found on the EC surface and in the ECM. Typical HSPGs in the vasculature are syndecans and perlecan. Both can be synthesized and secreted by ECs under normal physiological conditions. Syndecans are mainly expressed on the cell surface and

perlecan is presented in the ECM and BM (Kaji *et al*., 2000). It has been shown that the biosynthesis of HSPG and HS chain sulfation is decreased in the hyperglycaemic state (Jensen, 1997; Kjellen *et al*., 1983). HS GAGs were decreased in arteries of diabetic patients (Wasty *et al*., 1993). HS GAGs but not core protein were decreased in cultured ECs and kidney cells treated with high glucose (van Det *et al*., 1996b; Vogl-Willis and Edwards, 2004b). Immunofluorescence studies of renal biopsies also showed a decrease in staining of HS GAGs without changes in core protein staining (Tamsma *et al*., 1994). The concentration of arterial HS was negatively correlated with plasma glucose concentration in diabetic monkeys (Edwards *et al*., 2004). However, it is unknown whether the decrease in GAGs is accompanied by, or independent of, a decrease in the gene expression of core protein under hyperglycemic conditions in vascular ECs.

Heparanase induced by high glucose in ECs could be responsible for the cleavage of HS chains but not core proteins of HSPGs, since heparanase is an endoglucuronidase and breaks down HS chains but not proteins. Heparanase mRNA and protein activity were expressed in ECs treated with high glucose (Han *et al*., 2007). Studies in brain melanoma cells have shown that cell surface syndecan (syndecan-1) and ECM perlecan were targets for heparanase degradation (Reiland *et al*., 2004). However, the correlation between decreased HSPGs such as syndecan and perlecan and increased heparanase in ECs under hyperglycemic conditons has not been studied.

In addition to its antithrombotic activities, heparin is considered a potent vasodilator (Mandal *et al*., 1995) and lowers blood pressure in spontaneously hypertensive rats (Yokokawa *et al*., 1994). Heparin has the ability to inhibit heparanase upregulation induced by high glucose in cultured ECs (Han *et al*., 2007). Insulin can also inhibit heparanase expression in ECs treated with high glucose (Han *et al*., 2007). Although both heparin and insulin affect the expression of heparanase in ECs treated with high glucose, the influence of these two compounds on the core proteins of the HSPGs syndecan and perlecan in ECs under hyperglycaemic conditions is still a mystery. In the present study, cultured HAECs treated with high glucose and heparin and/or insulin were used as an *in vitro* model. Gene expression of syndecan, perlecan and heparanase were determined by real time quantitative PCR to determine if high glucose and insulin or heparin influence expression of these genes. The mechanism of gene regulation at the transcriptional level was also studied.

5.3. Materials and Methods

5.3.1. Human Aortic Endothelial Cell (HAEC) Cultures

HAECs and their basal medium (Medium 200) and cell growth supplement (LSGS kit) were purchased from Cascade Biologics (Portland, OR, USA). HAECs were transferred into 30 ml flasks from a vial of frozen cells and were cultured at 37°C with 5% CO_2/95% air in a humidified environment until confluent. Then confluent HAECs in flasks were washed

twice with magnesium-free Dulbecco's phosphate-buffered saline (CMF-DPBS) and cells were detached by trypsin (0.025% with EDTA in CMF-DPBS) for two or three minutes at room temperature. The cells were resuspended in medium and transferred to 35 mm dishes for further experiments.

5.3.2. Treatment of Cultures

When HAECs were grown to confluence, they were incubated with seven different treatments (3 dishes/group) including control, glucose (30 mM), insulin (0.01 unit/ml), heparin (0.5 μg/ml), glucose plus insulin, glucose plus heparin and glucose plus insulin plus heparin for 24 hours. In a separate study, confluent HAECs cultured in 35 mm dishes were divided into five different groups (3 dishes/group) and were treated with glucose (30 mM), glucose plus actinomycin D (10 μg/ml), glucose plus insulin plus heparin and glucose plus insulin plus heparin plus actinomycin D for 2, 4, 8 and 24 hours. A control group was treated with medium.

5.3.3. Extraction of Total RNA

RNeasy® Mini Kit from QIAGEN (Mississauga, Ontario, CA) was used to isolate total RNAs from HAECs. Briefly, media were removed from dishes, and then cells were washed with cold CMF-DPBS (4°C). Procedural steps from the lysis of cells to obtaining total RNAs were performed according to the manufacturer's instructions. The concentrations of RNAs were determined by a Nanodrop™ 1000 Spectrophotometer (Thermo Scientific). A

ratio of absorbance at 260/280 nm >/= 2.0 indicated RNA pure enough to use for cDNA synthesis.

5.3.4. Synthesis of cDNA

The AffinityScript™ QPCR cDNA Synthesis Kit from Stratagene (La Jolla, CA, USA) was used to create cDNA from RNAs of cells with all treatments. A total of 1 μg RNA from each sample was used for cDNA synthesis in a 20 μl reaction according to the methods described in the manufacturer's instruction. The Oligo (dT) primer (3 μl each reaction) was used. The reaction program was: the primer annealing at 25°C for 5 minutes, cDNA synthesis at 45°C for 15 minutes and the termination of the reaction at 95°C for 5 minutes. The newly synthesized cDNAs were stored at -20°C for later use.

5.3.5. Detection of Gene Expression by Real Time Quantitative PCR

Brilliant® II SYBR® Green QPCR Master Mix Kit (Stratagene) was used for determination of gene expression from samples of synthesized cDNA. The cDNA samples were diluted 20 times prior to the set up of the PCR reactions. The primers were used as following: heparanase sense primer 5'-GGCAAGTATTCTTTGGAGCA-3' and antisense primer 5'-TGGAT TGTCAGTGTTTGTGC-3'; perlecan sense primer 5'-TGCCTGAGGACATAG AGACC-3' and antisense primer 5'-TCGGAATAAACCATCTGGA-3';

syndecan-1 sense primer 5'-TGCAGGTGCTTTGCAAGATA-3' and antisense primer 5'-TTCTGGAGACGTGGGAATA-3'; *β*-actin (internal control) sense primer 5'-GGCATCCTCACCCTGAAGTA-3' and antisense primer 5'-GAAGGTCTCAAACATGATCT-3'. In a 25 *μ*l reagent mixture, assembled according to the manufacture's instructions, final concentrations for each primer was 200 nM alone with 5 *μ*l for each template cDNA. The real time PCR reactions were performed in Mx3005P® QPCR Systems from Stratagene. The PCR cycling programs used were: 1 cycle at 95°C for 10 minutes to activate the DNA polymerase and 40 cycles at 95°C for 30 seconds, 58°C for 1 minute and 72°C for 1 minute to detect and report fluorescence during the annealing and extension step of each cycle.

5.3.6. Statistic Analysis

Relative gene expression data were calculated by using the C_T value of real time quantitative PCR following the $2^{-\Delta\Delta CT}$ method decribed by Livak *et al*., (Livak and Schmittgen, 2001). The formula was $\Delta\Delta C_T = (C_T - C_{TActin})_{Treatment} - (C_T - C_{TActin})_{Control}$. Using the $2^{-\Delta\Delta CT}$ method the data are presented as the fold change in gene expression normalized to the housekeeping reference gene *β*-actin and relative to the untreated control. For the untreated control sample, $\Delta\Delta C_T$ equals zero and 2^0 equals one, so that the fold change in gene expression is relative to the untreated control which equals one. For the treated samples, evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in gene expression relative to the untreated control. For the regulation of gene expression by actinomycin D, relative gene changes at

different times compared to time 0 was also calculated by using the $2^{-\Delta CT}$ method, where $\Delta C_T = C_{T.time\ 0} - C_{T.time\ n}$.

All data is expressed as mean +/- standard deviation (SD) from three culture dishes per group. A one-way ANOVA was used to determine significant differences between groups with $p<0.05$ considered significant. A two-tailed t-test was used to test difference between two groups with $p<0.05$ considered significant. The regression analysis shows relative mRNA inhibited by actinomycin D with time.

5.4. Results

5.4.1. Gene Expression

To determine the correlation of expression of the genes perlecan, syndecan and heparanase under hyperglycemic conditions and the effect of insulin and/or heparin, total RNAs were isolated from seven groups of cultured cells with different treatments including control, high glucose, heparin, insulin, high glucose plus insulin, high glucose plus heparin, and high glucose plus insulin plus heparin. Then, cDNAs were synthesized from RNAs and gene expression was detected by quantitative real time PCR. The right products of real time PCR for heparanase, syndecana and perlecan were confirmed by gel electrophoresis and dissociation curve as shown in **Figure 5.1**.

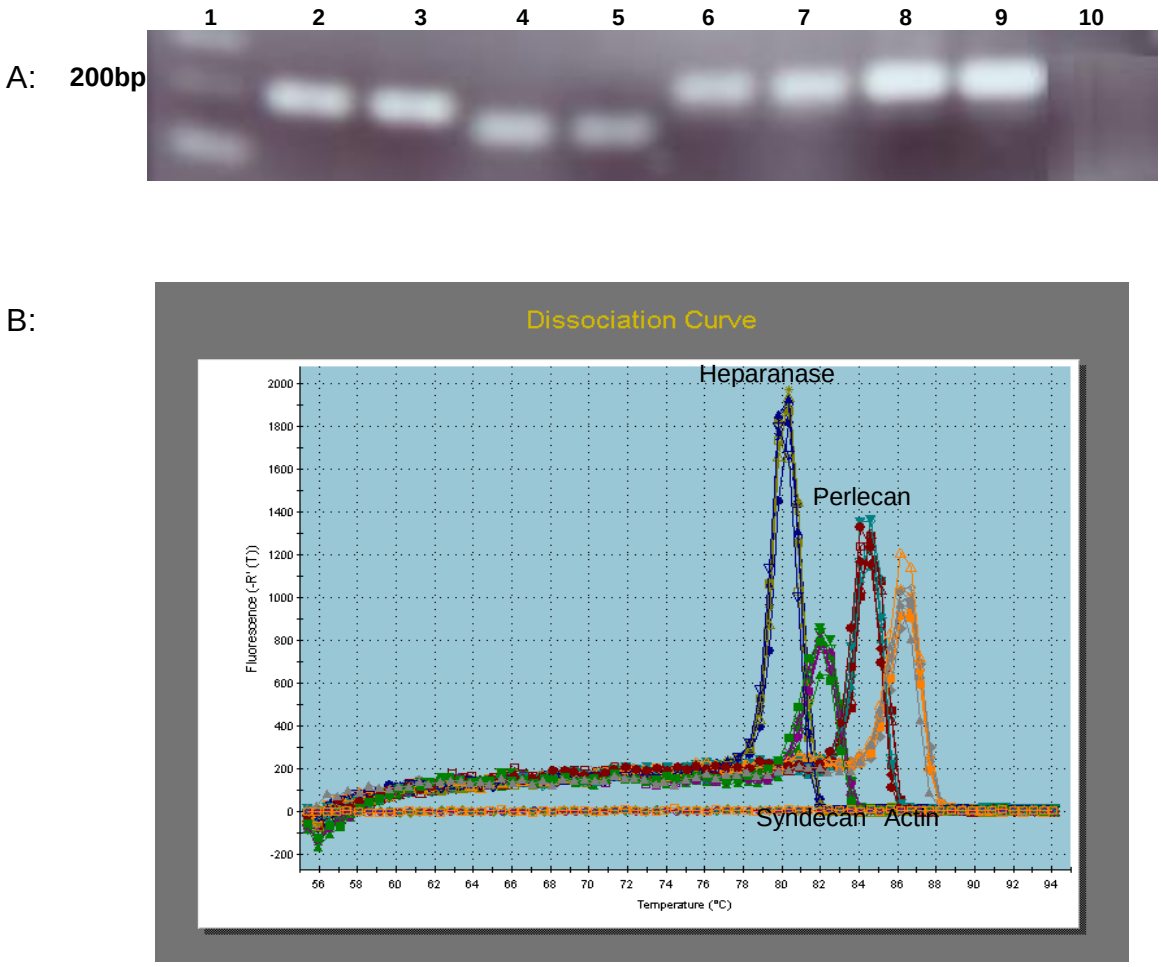


Figure 5.1. Confirmation of Real Time PCR Products

Control endothelial cells and cells treated with high glucose for 24 hours. Total RNAs were isolated from cells. cDNAs were synthesized. Real time quantitative PCR was performed with cDNA to amplify heparanase, syndecan and perlecan mRNAs. A: Gel electrophoresis shown: Lane 1: 100bp DNA ladder; Lane 2 (control) and Lane 3 (glucose) for Heparanase mRNA; Lane 4 (control) and Lane 5 (glucose) for Perlecan; Lane 6 (control) and Lane 7 (glucose) for Syndecan; Lane 8 (control) and Lane 9 (glucose) for β-actin; Lane 10: no template control. B: Dissociation curve for heparanase, perlecan, syndecan and β-actin.

Heparanase mRNA Expression: As shown in **Figure 5.2.**, heparanase mRNA was expressed in control cultured ECs. High glucose significantly increased heparanase mRNA expression compared to control. Heparin or insulin alone had no effect on heparanase mRNA expression. In the presence of high glucose, insulin or heparin alone decreased heparanase mRNA expression compared to high glucose alone, while the combination of insulin and heparin enhanced heparanase mRNA expression even further than high glucose alone.

Syndecan mRNA Expression: As shown in **Figure 5.3.**, syndecan mRNA was normally expressed in control cultured ECs. High glucose alone inhibited syndecan mRNA expression. Insulin or heparin alone did not have any effect on syndecan mRNA expression. When high glucose was present, heparin, but not insulin, restored syndecan mRNA to control levels. Insulin plus heparin increased syndecan mRNA expression significantly compared to all other groups.

Perlecan mRNA Expression: As shown in **Figure 5.4.**, perlecan mRNA was normally expressed in control cultured ECs. Glucose alone did not significantly affect perlecan mRNA expression. Insulin or heparin alone significantly decreased perlecan mRNA expression compared to control. When high glucose was present, insulin, heparin and insulin plus heparin significantly decreased perlecan mRNA expression. Also when high glucose was present, heparin and insulin plus heparin significantly decreased perlecan mRNA expression compared to glucose alone.

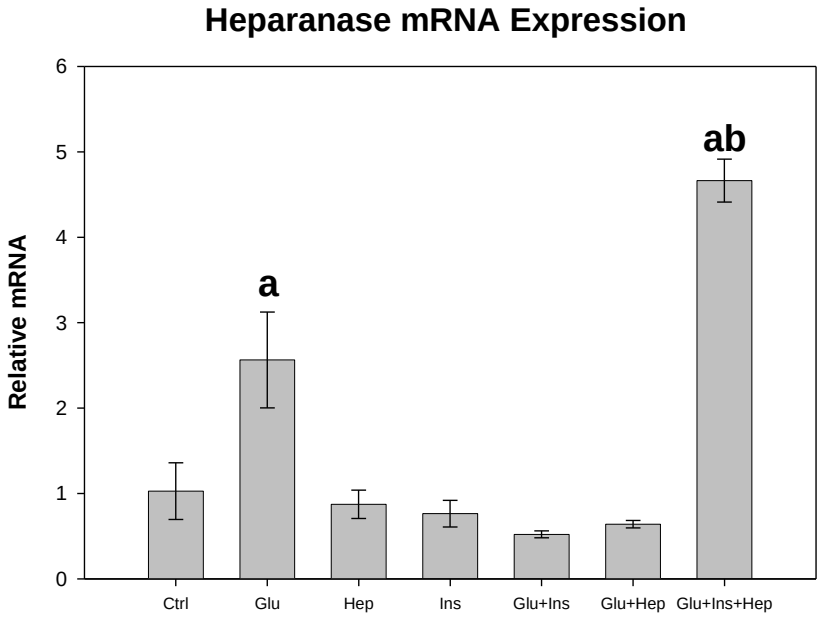


Figure 5.2. Heparanase mRNA Expression in Endothelial Cells Treated with High Glucose and/or Insulin and/or Heparin

Endothelial cells were treated with high glucose (Glu) and/or insulin (Ins) and/or heparin (Hep) for 24 hours. Total RNAs were isolated from cells. cDNAs were synthesized. Real time quantitative PCR was performed with cDNA to amplify heparanase mRNA.

a, significantly different from all other groups; b, significantly different from glucose treatment, $p<0.05$, one-way ANOVA.

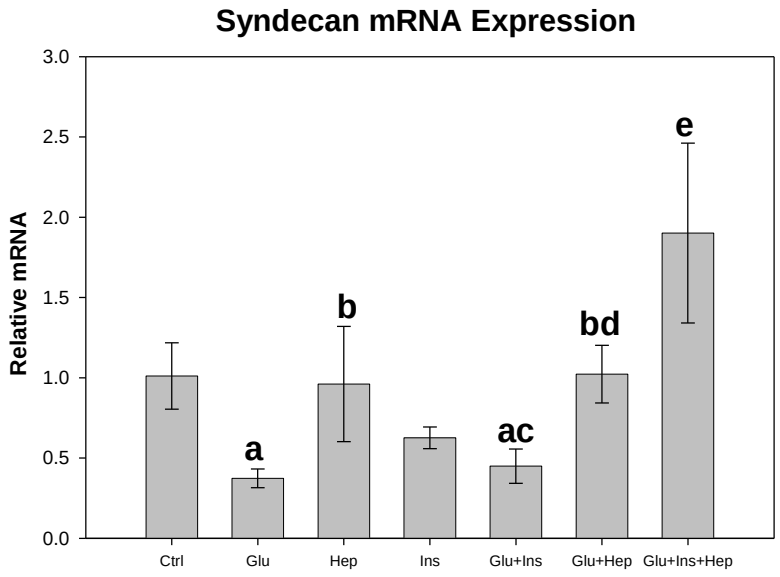


Figure 5.3. Syndecan mRNA Expression in Endothelial Cells Treated with High Glucose and/or Insulin and/or Heparin

Endothelial cells were treated with high glucose (Glu) and/or insulin (Ins) and/or heparin (Hep) for 24 hours. Total RNAs were isolated from cells. cDNAs were synthesized. Real time quantitative PCR were performed with cDNAs to amplify the syndecan mRNA.

a, significantly different from control (Ctrl); b, significantly different from glucose (Glu) treatment; c, significantly different from heparin (Hep); e, significantly different from all other groups, $p<0.05$, one-way ANOVA.

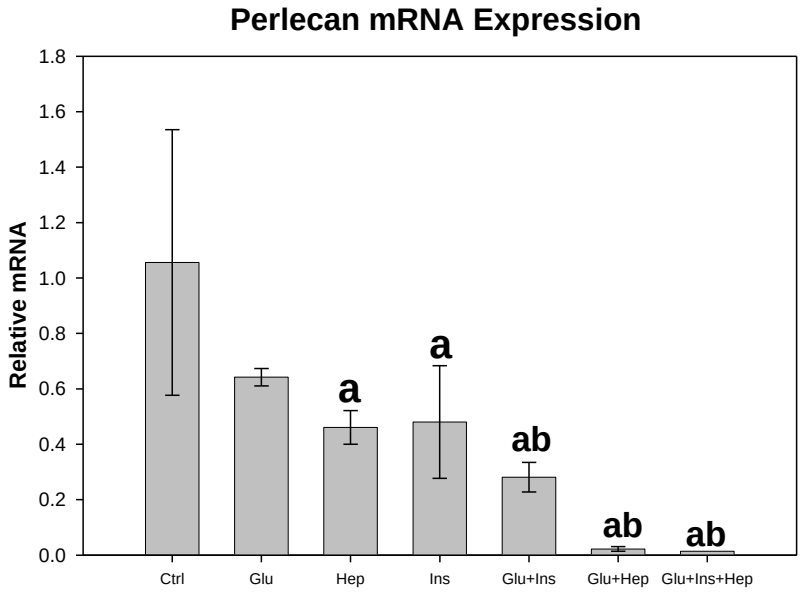


Figure 5.4. Perlecan mRNA Expression in Endothelial Cells Treated with High Glucose and/or Insulin and/or Heparin

Endothelial cells were treated with high glucose (Glu) and/or insulin (Ins) and/or heparin (Hep) for 24 hours. Total RNAs were isolated from cells. cDNAs were synthesized. Real time quantitative PCR were performed with cDNAs to amplify the perlecan mRNA.

a, Significantly different from control (Ctrl); b, significantly different from glucose (Glu) treatment, $p<0.05$, one-way ANOVA.

5.4.2. Mechanism of Gene Regulation

To determine the mechanism of gene expression, the samples with highest levels of mRNAs from real time PCR detection, the heparanase mRNA treated with high glucose or high glucose plus insulin plus heparin and syndecan mRNA treated with high glucose plus insulin plus heparin, were treated with (+) or without (-) addition of actinomycin D.

The results from the effect of actinomycin D on heparanase mRNA expression induced by high glucose are shown in **Figure 5.5.A**. Heparanase mRNA expression relative to the housekeeping gene *β*-actin was increased with time in high glucose-treated cells with or without actinomycin D and was significantly increased at 24 hours compared to 0 hours. The expression of heparanase mRNA was increased and *β*-actin mRNA did not change in cells not treated with actinomycin D **(Figure 5.5.B)**, while the expression of *β*-actin steadily decreased with time, and heparanase increased and then decreased to slightly below control levels at 24 hours in cells treated with actinomycin D **(Figure 5.5.C)**.

In cells treated with high glucose plus insulin plus heparin, heparanase mRNA expression was significantly increased in cells without actinomycin D at 24 hours compared to 0 hours but not in cells with actinomycin D as shown in **Figure 5.6.A**. At 24 hours, heparanase mRNA in cells without actinomycin D was significantly different from cells with actinomycin D, when compared 2, 4, 8, and 24 hour to their own expression

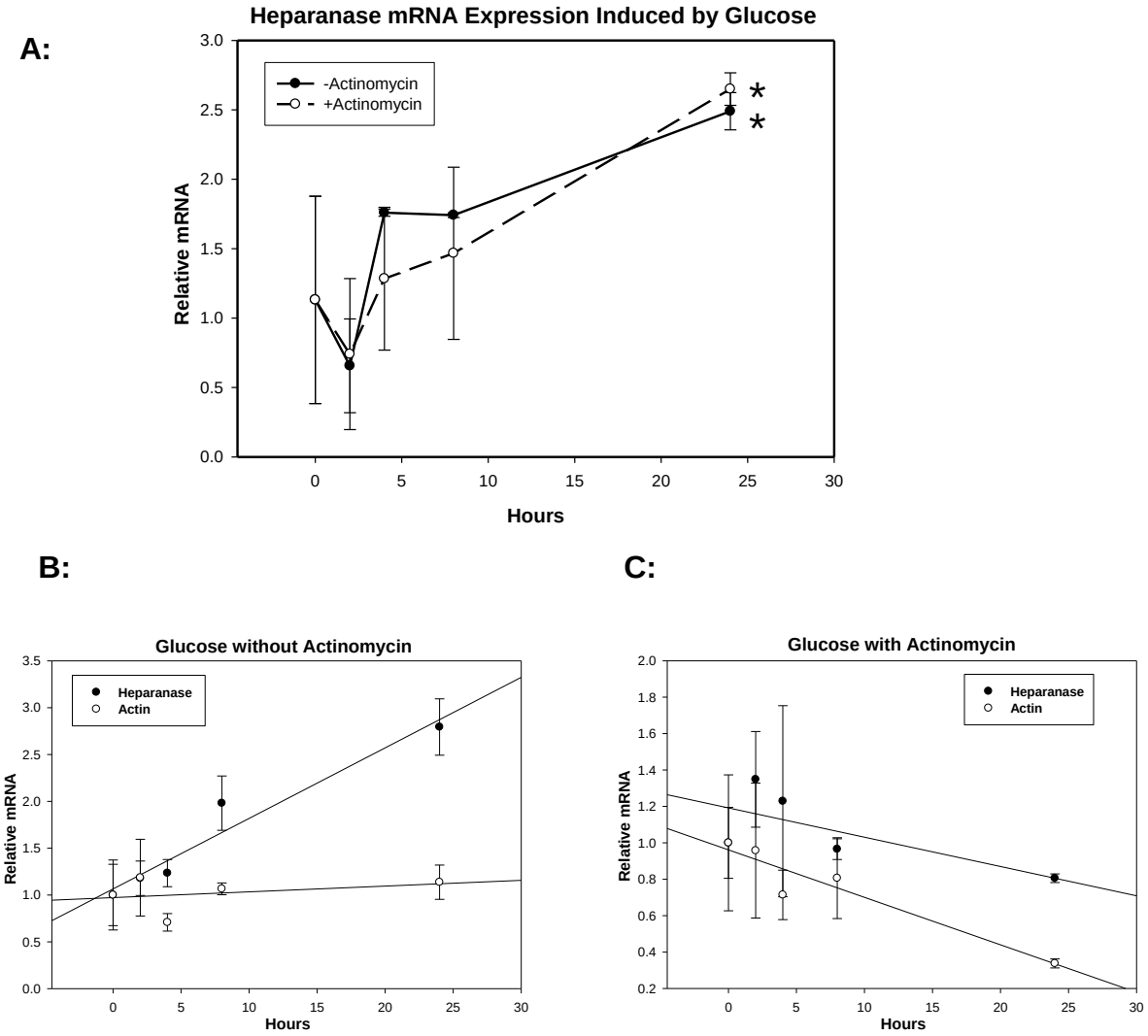


Figure 5.5. Effect of Actinomycin D on Heparanase mRNA Expression Induced by High Glucose

Two groups of ECs were treated with high glucose and high glucose+actinomycin D for 0, 2, 4, 8 and 24 hours. Total RNAs were isolated and cDNAs were synthesized. Real time quantitative PCR was used to amplify heparanase gene. **A:** Relative heparanase mRNA expression to β-actin; **B:** Heparanase and β-actin mRNA at 2, 4, 8, and 24 hours relative to time 0 -actinomycin; **C:** Heparanase and β-actin mRNA at 2, 4, 8, and 24 hours relative to time 0 +actinomycin. *****Significantly different from 0 hours, $p<0.05$, two-tailed t-test.

at time 0. The expression of heparanase mRNA was increased and β-actin mRNA did not change in cells without actinomycin D treatment **(Figure 5.6.B)**, while the expression of both heparanase and β-actin was decreased (β-actin declined faster than heparanase) with the time in cells treated with actinomycin D **(Figure 5.6.C)**.

The results from the effect of actinomycin D on syndecan mRNA expression induced by high glucose plus insulin plus heparin are shown in **Figure 5.7.A.** Syndecan mRNA expression was increased significantly in cells without actinomycin D, but not in cells with actinomycin D when 24 hours is compared to 0 hours. At 24 hours syndecan mRNA expression was significantly greater in cells without actinomycin D than in cells with actinomycin D. When comparing expression at 2, 4, 8, and 24 hours relative to time 0, the expression of syndecan mRNA increased and β-actin mRNA did not change in cells without actinomycin D **(Figure 5.7.B)**, while the expression of syndecan and β-actin both decreased with the time in cells with actinomycin D **(Figure 5.7.C)**.

5.5. Discussion

HSPGs derived from ECs are important components in the vasculature. Alteration of HSPGs is a cause of endothelial dysfunction or injury which is involved in vascular complications. HSPG metabolism in general can be affected at different levels including core protein synthesis and GAG chain addition, elongation, sulfation and degradation. Core protein

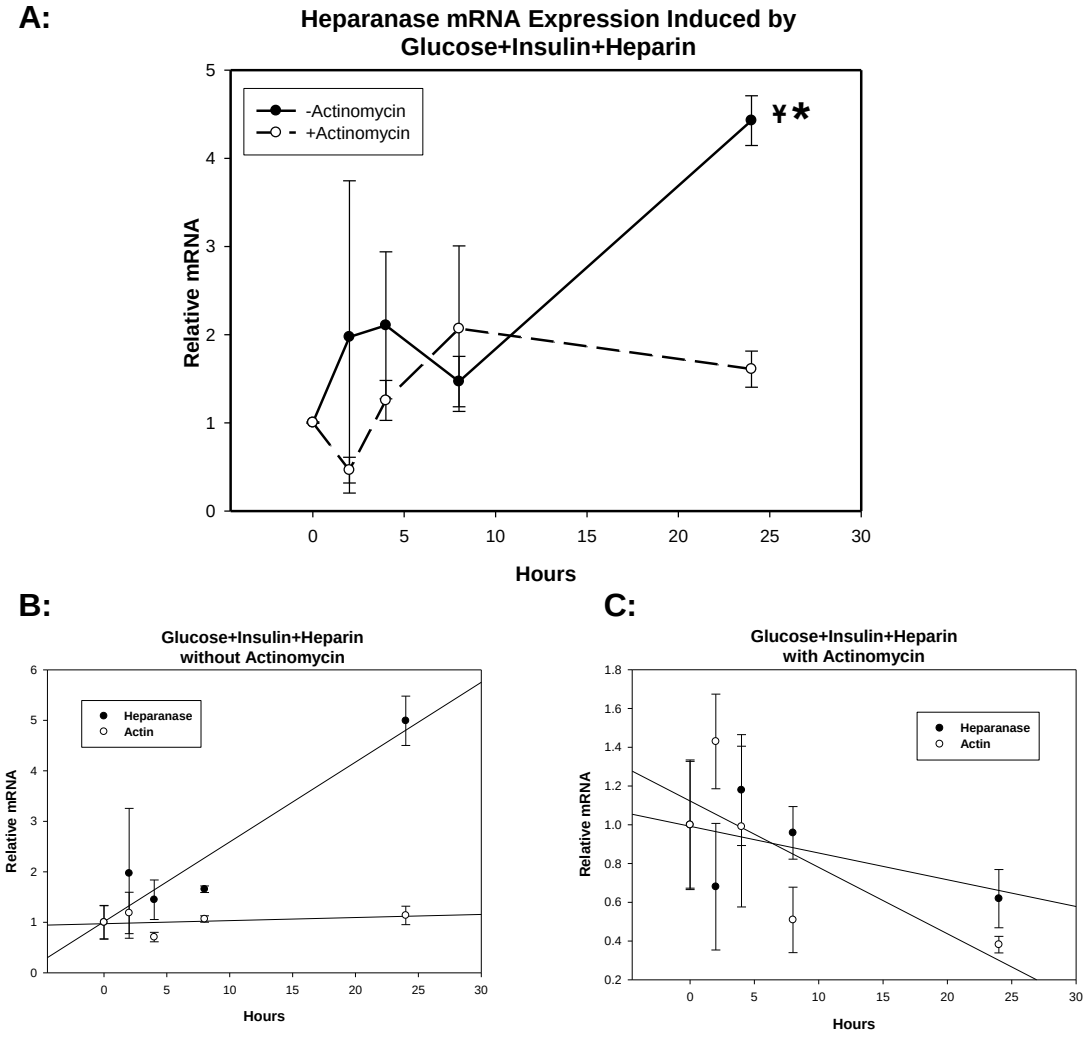


Figure 5.6. Effect of Actinomycin D on Heparanase mRNA Expression Induced by Glucose plus Insulin plus Heparin

Two groups of ECs were treated with high glucose and high glucose+actinomycin D for 2, 4, 8 and 24 hours. Total RNAs were isolated and cDNAs were synthesized. Real time quantitative PCR was used to amplify the heparanase gene. **A:** Relative heparanase mRNA expression to β-actin; **B:** Heparanase and β-actin mRNA at 2, 4, 8, and 24 hours relative to time 0 -actinomycin; **C:** Heparanase and β-actin mRNA at 2, 4, 8, and 24 hours relative to time 0 of +actinomycin. *****Significantly different from 0 hour, **¥** significantly different between actinomycin D addition and non-addition, $p<0.05$, two-tailed t-test.

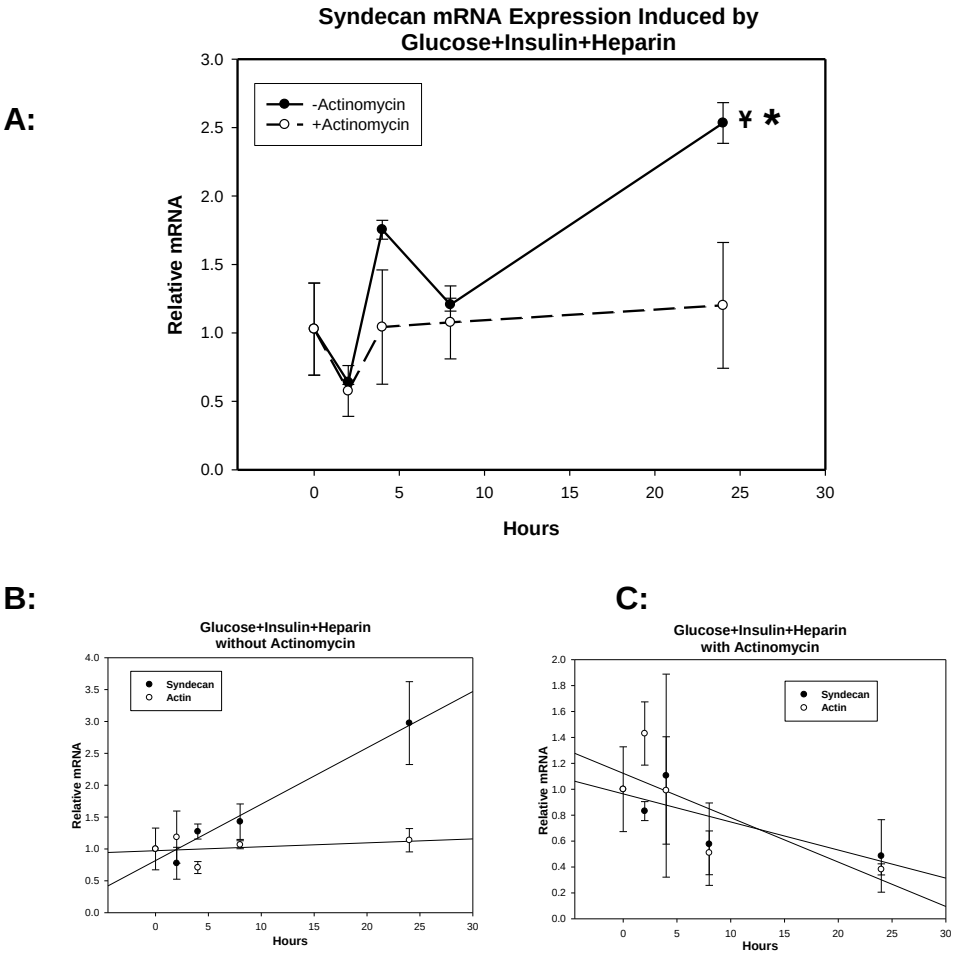


Figure 5.7. Effect of Actinomycin D on Syndecan mRNA Expression Induced by Glucose plus Insulin plus Heparin

Two groups of ECs were treated with glucose+insulin+heparin +/-actinomycin D for 2, 4, 8 and 24 hours. Total RNAs were isolated and cDNAs were synthesized. Real time quantitative PCR was used to amplify syndecan gene. **A:** Relative syndecan mRNA expression to *β*-actin; **B:** Syndecan and *β*-actin mRNA at 2, 4, 8, and 24 hours relative to time 0 -actinomycin; **C:** Syndecan and *β*-actin mRNA at 2, 4, 8, and 24 hours relative to time 0+actinomycin. *Significantly different from 0 hours,¥ significantly different between actinomycin D addition and non-addition. $p<0.05$, two-tailed t-test.

synthesis is controlled by transcription of DNA to mRNA and translation of mRNA to protein. Changes in HSPG synthesis and turnover will affect the endothelium to a large extent. There is accumulating evidence in diabetic patients, animal models, and in cultured cells from different sources under hyperglycemic conditions to suggest that decreased HSPG is an important factor contributing to diabetic cardiovascular complications. HS chains are believed to be degraded by heparanase, an endo-*β*-D-glucuronidase capable of cleaving glycosidic bonds of HS side chains at a limited number of sites with a hydrolase mechanism (Vlodavsky and Friedmann, 2001). Induction of heparanase by high glucose is at least partly responsible for HS degradation in diabetes, however, it is still unknown if core proteins of syndecan on the cell surface or perlecan in the ECM are also affected by high glucose or correlated with heparanase induction in ECs under hyperglycemic conditions.

In the present study, the effect of high glucose on gene expression and regulation of heparanase, syndecan and perlecan in HAECs was determined by real time quantitative PCR. We found that high glucose increased heparanase, decreased syndecan and had no effect on perlecan mRNA expression compared to control.

Upregulation of heparanase under hyperglycaemic conditions has been shown in diabetic animal models and human patients as well as in cultured cells from various sources. Previous studies localized heparanase protein both in glomeruli capillaries and tubular epithelium and detected heparanase activity in the urine of diabetic patients (Katz *et al*., 2002; Shafat

et al., 2006). The reduction of HS in the glomeruli of patients with overt diabetic nephropathy in type 2 diabetes was correlated with an upregulation of heparanase (van den Hoven *et al*., 2006). Heparanase protein expression was inversely correlated with HS expression in mouse renal cortex with STZ-induced diabetes (van den Hoven *et al*., 2006). Upregulated heparanase mRNA by high glucose in HAEC is consistent with our previous results where heparanase mRNA was upregulated by high glucose in PAECs (Han *et al*., 2007) suggesting that there is no species specificity in terms of heparanase upregulation by high glucose in ECs. This result also confirmed that ECs are an important cell type that expresses and releases heparanase in patients with overt diabetic nephropathy (Levidiotis *et al*., 2001; 2004). HS chains on syndecan or perlecan in melanoma cells were the targets of heparanase degradation, and syndecan core protein was not changed by heparanase (Reiland *et al*., 2004). Heparanase inhibitors, PI-88 and heparin, reversed the degradation of cell surface HSPG by heparanase upregulation under hyperglycaemic conditions in rat and human glomerular epithelial cells as well as human tubular epithelial cells (Maxhimer *et al*., 2005). Therefore, HS reduction is at least partly due to accelerated degradation by increased heparanase expression invoked by the hyperglycaemic environment. This also confirmed our previous study that total GAGs were decreased in cells and increased in medium by high glucose (**Chapter 3** of this thesis).

Expression of both heparanase gene and protein was detected in human tumor cell lines, head and neck tumors and metastic pancreatic

cancer, suggesting the possibility of transcriptional and translational regulation of heparanase (Koliopanos *et al*., 2001; Simizu *et al*., 2003; Vlodavsky *et al*., 2002). Previous studies have demonstrated that high glucose is able to activate several transcription factors that are involved in regulation of the heparanase promoter, including Sp1, early growth response-1 (Egr-1) and the ETS family transcription factors (Jiang *et al*., 2002). A study in renal epithelial cells showed that concerted activation of these transcription factors by high glucose leads to the activation of heparanase promoter and induction of heparanase gene expression (Maxhimer *et al*., 2005). Transcription factors of the Sp1 and ETS families play a role in controlling the basal activity of human heparanase gene expression (Jiang *et al*., 2002; Lu *et al*., 2003). Egr-1 is a key activator of heparanase gene expression and binds to a specific element in the heparanase promoter regulating inducible transcription. Inducible expression of heparanase mRNA and the activity of heparanase promoter element are dependent on the ERK1/2 (MEK1/2) pathway. This pathway is critical for induction of Egr-1 expression at both the mRNA and protein level in T cells (Jiang *et al*., 2002; Lu *et al*., 2003). Our study showed that actinomycin D inhibited both *β*-actin and heparanase mRNA expression under high glucose conditions **(Figure 5.4)** suggesting transcriptional regulation of high glucose on heparanase gene expression occurs in ECs. Additionally, all the aforementioned transcriptional factors may be involved in the process of

heparanase gene regulation, but the action of each factor on the regulation of heparanase gene in ECs needs further investigation.

Syndecan is lost from the cell surface under a number of physiological conditions, and degradation of HS can result in changes in syndecan distribution (Sanderson, 2001; Yang *et al*., 2003). High glucose decreased the expression of syndecan mRNA, shown in this study **(Figure 5.2)** suggesting that some factors induced by high glucose interfered with syndecan gene transcription. Syndecan expression can be regulated at both transcriptional and translational levels (Hayashida *et al*., 2006). The syndecan promoter has multiple proximal Sp1 binding sites (Larrain *et al*., 1997) and Sp1-like transcription factors have a critical role in regulation of the transcriptional activity of the syndecan gene (Vihinen *et al*., 1996). Sp1 is ubiquitously expressed, and its activation and recruitment of distinct transduction pathways and signaling molecules may serve as mechanisms to promote transactivation of selected responsive genes. Regulation of Sp1-dependent transcription may be conveyed by changes in DNA binding activity, by association with other transcription factors, and by changes in Sp1 abundance or in transactivation due to biochemical modification, such as phosphorylation (Black *et al*., 2001; Philipsen and Suske, 1999). Sp1 is phosphorylated by a number of cellular kinases, including DNA-dependent protein kinase, protein kinase A (PKA) and different members of the PKC family (Black *et al*., 2001). In a study on the terminal differentiation of liver, an increase in Sp1 phosphorylation decreased its DNA binding activity (Leggett

et al., 1995). Decrease in Sp1 activity may cause down-regulation of syndecan expression in ECs under hyperglycaemic conditions, since glucose can activate and upregulate PKC through formation of DAG and AGEs (Scivittaro *et al*., 2000). PKC is a ubiquitous family of serine-threonine phosphorylating enzymes (Koya and King, 1998). The distribution of over 10 isoforms varies among cell types with PKCα and *β* most prevalent in the vasculature (Trial Research Group, 1993) where hyperglycemia predominately activates PKC*β* (Inoguchi *et al*., 1992). The effects of PKC activation are variable including alterations in cell signalling, production of vasoconstrictor substances, and conversion of VSMCs and ECs to a proliferative phenotype in the retinal microcirculation and conduit vessels. Sp1 phosphorylation by PKC on the syndecan gene may decrease its transcription under hyperglycaemic conditions. In addition, an increase in serum syndecan was shown in early nephropathy in a type 1 diabetic patient (Svennevig *et al*., 2006). Heparanase was shown responsible for syndecan shedding in myeloma and breast cancer cells (Yang *et al*., 2007) which may lead to increased serum syndecan in the diabetic patient. Therefore, heparanase induction may play a role in decreased syndecan and HS in vessels. There is no evidence to show the effect of heparanase on gene expression of syndecan. However, the present study showed that high glucose increased heparanase and decreased syndecan gene expression which may suggest the effect of heparanase on syndecan gene expression.

The expression of perlecan mRNA was not changed by high glucose even though there was a trend to a decrease compared to control **(Figure 5.3)**. Changes in perlecan mRNA and protein under hyperglycemia is controversial in different diabetic models. A study of HS in mesangial and epithelial cells cultured in high glucose medium showed no changes in HSPG made up of mainly perlecan core protein (van Det *et al*., 1996b). A decrease in the synthesis and mRNA levels of perlecan was demonstrated in retinas from STZ-diabetic rats (Bollineni *et al*., 1997). A significant decrease in perlecan core protein expression and somewhat moderate reductions in its mRNA expression under high glucose conditions was also reported (Ha *et al*., 2004). A study of bovine myocardial ECs exposed to high glucose showed increased sulfate incorporation into the proteoglycan with no changes in core protein synthesis (Klein *et al*., 1995). The perlecan mRNA was reduced in glomerular epithelial cells exposed to medium containing 30 mM glucose which resulted in reduction in synthesis of BM HSPG at 24 hour and 7 days (Kasinath *et al*., 1996). There were no absolute changes in kidney cortex levels of perlecan mRNA observed in a mouse model of type 2 diabetes (Raats *et al*., 2000). The differences in perlecan mRNA expression between studies may also be related to the cell type or source, exposure time to glucose, and culture conditions in general. Regulation of perlecan gene expression under hyperglycaemic conditions is beginning to be understood. The promoter of human perlecan has been partially sequenced, revealing potential binding sites for several transcription factors, including Sp1, AP-2

and ETF (Cohen *et al*., 1993). cAMP, a regulator of a wide variety of genes, caused a less reduction in perlecan mRNA than perlecan core protein in glomerular epithelial cells, indicating that both transcriptional and posttranscriptional mechanisms may be involved and that cAMP regulates posttranscriptional events in proteoglycan synthesis (Ko *et al*., 1996). Since regulation of perlecan gene expression by high glucose may be at transcriptional levels, the core protein or HS chains may be altered despite our observations of no significant change in perlecan mRNA.

Exogenous heparin is capable of ameliorating increased vascular permeability caused by various polycationic substances (Fairman *et al*., 1987; Peterson *et al*., 1987). Heparin also has antiviral activity, binds to a variety of growth factors, inhibits complement activation and regulates angiogenesis (Casu, 1985; Lane and Lindahl, 1989). Low molecular weight heparin (LMWH) may prolong the survival in patients with advanced cancer (Kakkar *et al*., 2004). Therefore, heparin can protect or prevent diseases of the cardiovascular system and cancer processes by variety of mechanisms, but the exact mechanism of heparin's therapeutic function is still not clear, especially in hyperglycemia induced cardiovascular complications. Therefore, the effect of heparin on the gene expression of syndecan, perlecan and heparanase may reveal a possible mechanism of therapeutic action. Heparin alone decreased heparanase and perlecan, and increased syndecan mRNA expression in this study, indicating that heparin is capable of inhibiting heparanase expression and enhancing the synthesis of syndecan, but not

perlecan. Previous studies have shown that heparin increased HS synthesis and prevented heparanase upregulation in ECs, and inhibited HSPG degradation by heparanase induction (Bar-Ner *et al*., 1987; Han *et al*., 2007; Nader *et al*., 1991). These results indicated that, at least in part, heparin may protect endothelium and the vasculature by increasing syndecan synthesis and preventing heparanase induction. However, there is no direct information about the regulatory mechanisms of heparin on the gene expression of heparanase, syndecan or perlecan in ECs. It has been shown that heparin or HS suppressed proliferation of mesangial and vascular smooth muscle cells partly by interfering with PKC signalling (Castellot, Jr. *et al*., 1982; Pukac *et al*., 1992). Heparin inhibits apoptosis of mesangial cells via intervention in the AP-1 pathway (Ishikawa *et al*., 1997) and the activity of AP-1 is regulated by both production of AP-1 protein and its activation via c-Jun N-terminal kinase (JNK). The inhibitory effect of heparin on the AP-1 pathway may be mainly through reduction in the mRNA and protein levels of AP-1 components (Ishikawa and Kitamura, 1999). Since mesangial cells are the main cell type in kidney to produce HSPG and show pathogenic changes in diabetic nephropathy, heparin may also influence the factors or pathways involved in regulation of HSPG or heparanase induction. LMWH prevented the binding of nuclear proteins to the regulatory AP-1 site but not to a Sp1 site in a study of heparin inhibiting expression of prosclerotic TGF-β1 which has been implicated in the pathogenesis of diabetic nephropathy (Weigert *et al*., 2001). The regulation of gene expression by heparin through AP-1 is different from

that of high glucose, through activating Sp1 transcription factor, suggesting that the possible mechanism by which heparin protects cells from high glucose injury is to activate AP-1 and inhibit Sp1.

Insulin regulates the blood glucose concentration and the metabolism of fats and proteins in cells. Binding of insulin to its receptors results in diverse signalling processes that mediate many of insulin's actions in vascular cells including modulating transcription, altering cell content of numerous mRNAs and stimulating cell growth, DNA synthesis, and replication (Bornfeldt *et al*., 1992; Sowers, 1997). In this study, insulin decreased heparanase and perlecan mRNA, but had no effect on syndecan. Insulin upregulated PAI-1 by activiting Sp1 transcription factor binding to the PAI-1 promoter (Banfi *et al*., 2001). Rat apolipoprotein (Apo) AI gene expression is induced by insulin activating a promoter with a Sp1 binding site (Murao *et al*., 1998). The genes of heparanase, perlecan and syndecan express Sp1 binding sites on their promoter regions, therefore decreased mRNA expression of heparanase and perlecan may be due to Sp1 regulation by insulin. Activation of Sp1 may play a negative transcriptional role at promoter regions of heparanase and perlecan, since over-expression of Sp1 in rat hepatocytes leads to a decrease in fat acid synthase (FAS)-promoter activity (Fukada *et al*., 1997). Syndecan mRNA expression wasn't affected by insulin in the present study. However, previous evidence has shown that insulin promoted shedding of syndecan ectodomains from 3T3-L1 adipocytes (Reizes *et al*., 2006) and may reduce the amount and bioactivity of syndecan

acting on EC surface. The similar sequences between human insulin receptor and mouse syndecan (Ebina *et al*., 1985) may lead insulin to affect the pathways involved in syndecan biosynthesis or turn over. Therefore, insulin may play a role in posttranscriptional regulation of syndecan expression and reduce syndecan on the cell surface although syndecan mRNA wasn't effected by insulin in this study.

When high glucose was present, heparin decreased mRNA expression of heparanase and perlecan and increased that of syndecan compared to high glucose treatment while insulin decreased expression of perlecan compared to control, syndecan compared to control and heparin alone, and heparanase compared to glucose alone and heparin plus insulin with high glucose treatment. Heparin and insulin in combination decreased perlecan mRNA compared to control and glucose treatment and increased mRNAs of syndecan and heparanase compared to all other treatments. These results suggest that the influence of heparin and/or insulin on expression of these three genes is complicated in the presence of high glucose. As mentioned previously in the discussion, heparin or insulin regulate gene expression by interacting with different transcriptional factors although these transcriptional binding sites are present on all these genes. The action of the same transcriptional factor on different genes may show positive or negative regulation. Additionally, the glucose response element (GRE) has been identified in the promoters of a variety of glucose regulated genes and involved in upregulation of the gene expression (Shih and Towle,

1992; Thompson and Towle, 1991; Vaulont and Kahn, 1994). It is possible there are alternative sites regulated by heparin, insulin or glucose in the promoter regions of heparanase, syndecan, and perlecan genes. As shown in actinomycin D treated cells under the treatment of high glucose plus insulin and heparin **(Figure 5.5 and 5.6)**, both syndecan and heparanase genes are regulated at the transcriptional level. However, transcriptional regulation of heparanase in cells treated with high glucose alone was not shown when relative heparanase mRNA was compared to the housekeeping gene *β*-actin. However, transcriptional regulation was confirmed by comparing relative heparanase mRNA to its own expression at time 0. These results indicate that the expression of heparanase gene was alternatively regulated by high glucose or by high glucose plus insulin plus heparin. Further investigation is needed to identify intermediary steps and effectors involved in genetic regulation of HSPG core proteins by high glucose.

We observed in this study that heparin decreased heparanase and increased syndecan mRNA expression in high glucose-treated cells indicating the protective effect on ECs. Perlecan mRNA in cells treated with high glucose was decreased by heparin and/or insulin and suggests that regulation of ECM perlecan is different from that of cell surface syndecan. Since perlecan upregulation may be part of the thickening of BM in the vasculature which contributes to endothelial dysfunction, insulin or heparin may have the ability to protect endothelium. Interestingly, we found the

combination of heparin and insulin not only increased mRNA of syndecan, but also heparanase in the presence of high glucose. It is understandable that heparin and insulin may synergistically increase syndecan production to protect ECs, since heparin and insulin in combination protected cells from high glucose and heparinase injury (Han *et al*., 2005). However, the mechanism of heparin and insulin increased heparanase mRNA can not be elucidated in the present study. It is possible that high glucose, insulin or heparin regulate heparanase gene expression through synergistically activating different transcriptional factors on the heparanase promoter region. In addition, the protective effect of heparin and/or insulin on ECs injured by high glucose is shown by reduced EC intercellular gaps and inhibition of heparanase upregulation (Mandal *et al*., 2000; Han *et al*., 2007). As mentioned above, heparin and insulin interact with and function by different mechanisms on HSPGs, and may collectively and synergistically activate or inactivate gene expression of syndecan, perlecan and heparanase under hyperglycaemic conditions. Increased mRNA levels may not reflect increased active protein in these experiments, or increases may occur that are insufficient to affect proteoglycan synthesis. This is an area that requires further experimentation through investigation of gene regulation at the transcriptional level.

In summary, the investigation of gene expression of heparanase, syndecan and perlecan in ECs under hyperglycemic condition in the present study revealed high glucose induced heparanase, decreased syndecan and

had no effect on perlecan gene expression. The effect of insulin and heparin alone or in combination on gene expression varied depending on the presence or absence of high glucose, indicating that the regulatory complexity of these genes by high glucose and/or insulin and/or heparin. Further studies in transcriptional factors involved in the mechanism of gene regulation will lay the foundation for understanding genetic alteration in diabetic vascular complications and the therapeutic functions of insulin and heparin at the level of gene regulation.

6. Prediction of Promoter Regions and Possible Transcription Factors on the Genes of Heparanase, Perlecan and Syndecan

To better understand the mechanisms of gene expression of heparanase, perlecan and syndecan, the promoter regions and possible transcription factor binding sites for these three genes were analyzed by Proscan from BIMAS (BioInformatics and Molecular Analysis Section)

Heparanase: The promoter region was found on the forward strand up to 3000 bp before the gene transcriptional region. Possible transcriptional factor binding sites found at this region are JCV_repeated_sequence, two Sp1 and EARLY-SEQ1. It has been shown that the heparanase promoter does not contain TATA or CAAT box but contains a GC-rich sequence in the proximal promoter region. The basal promoter activity of the heparanase gene is largely located in a 0.3-kb region upstream of the translation start site (Maxhimer *et al*., 2005). Three Sp1 sites and four ETS (transcription factor family) relevant elements were identified in this region (Jiang *et al*., 2002). The ability of glucose to transcriptionally activate heparanase promoter rests largely within the 0.3-kb region upstream of the translational start site (Jiang *et al*., 2002). Previous studies have demonstrated that high glucose is able to activate several transcription factors that are involved in regulation of the

heparanase promoter, including Sp1, Egr-1 (Early growth response factor 1) and the ETS family transcription factors (Jiang *et al*., 2002). A study of induction of heparanase expression in renal epithelial cells by high glucose showed that concerted activation of these transcription factors by high glucose leads to the activation of heparanase promoter and induction of heparanase gene expression (Maxhimer *et al*., 2005).

Perlecan: Promoter region was predicted on the perlecan forward strand up to 2000 bp before the gene transcriptional region. The possible transcriptional factors that bind to this region are Sp1, JCV_repeated_sequence, GCF, AP-2, EARLY-SEQ1 and NGFI-C. A study on perlecan promoter by Cohen *et al* showed that a characteristic of the human perlecan promoter is the presence of potential sites for binding of several transcription factors, including Sp1, Ap-2 and ETF. The promoter is highly enriched in GC content, contains four GC boxes and three GGGCGG hexanucleotide sequences, which are known to bind the transcription factor Sp1 (Cohen *et al*., 1993). However, direct examination of regulation of the perlecan gene by these transcriptional factors remains to be performed.

Syndecan: The prediction of the human syndecan promoter region by Proscan from BIMAS resulted in no promoter regions on the syndecan forward strand up to 2000-4000 bp before the gene transcriptional region. This result indicates that the studies on promoter regions of human syndecans are limited. Studies on mouse cell surface HSPG genes have

shown that potential binding sites for several transcription factors have been identified in the upstream sequence of these genes including NF-kB, Myo-D, Antennapedia-binding site and multiple proximal Sp-1 binding sites on syndecan-1. Sp1-like transcription factors have an essential role in the regulation of the transcriptional activity of the syndecan-1 gene (Hinkes *et al*., 1993; Larrain *et al*., 1997; Tsuzuki *et al*., 1997; Vihinen *et al*., 1996). The expression of syndecan-1 during myoblast terminal differentiation was down-regulated due to the variations in Sp1 activity (Vihinen *et al*., 1996). Similarly, the expression of perlecan during myogenesis is also down-regulated (Larrain *et al*., 1997). The promoter region of perlecan resembles that of mouse syndecan-1, suggesting that similar regulatory mechanisms are involved in the regulation of these two macromolecules (Cohen *et al*., 1993; Vihinen *et al*., 1996).

The prediction and studies of promoter and transcription factors for genes of heparanase, perlecan and syndecan imply that the transcription factor Sp1 is a common factor for the regulation of the three genes. Sp1 is a human transcription factor involved in gene expression in the early development of an organism. The protein is 785 amino acids long, with a molecular weight of 81 KDa. It contains a zinc finger protein motif, by which it binds directly to DNA and enhances gene transcription. Its zinc finger binds the consensus sequence 5’-(G/T)GGGCGG(G/A)(C/T)-3’(GC box element). Sp1 has been shown to be involved in regulation of heparanase promoter by high glucose activation. Sp1 may play a role in regulation of promoters of

perlecan and syndecan by high glucose activation. Elucidation of transcriptional functions of Sp1 on the promoters of heparanase, perlecan and syndecan may provide the fundamental mechanisms of alterations of HSPG in hyperglycemia providing further understanding of diabetic vascular complications due to endothelial injury.

7. GENERAL DISCUSSION

HSPGs are macromolecules containing a core protein with GAG chains attached that are mostly HS. ECs synthesize two main types of HSPG in the vasculature, syndecans on the cell surface and perlecan in ECM. HSPG metabolism and degradation can be pathophysiologically affected at different levels including synthesis of core protein and GAG chains (Esko and Lindahl, 2001). Core protein synthesis is controlled by transcription of DNA to mRNA and translation of mRNA to protein. GAG chain synthesis is directed by chain addition, elongation, sulfation and degradation. Changes in HSPG synthesis and turnover will affect the endothelium to a large extent. HSPGs are believed to be the endogenous receptors for circulating growth factors and chemokines that regulate cell proliferation, differentiation and migration and possesses functions of membrane filtering and cell signaling. Therefore, degradation of HSPG may cause increased vascular permeability and damage to the vascular wall. One of the pathological mechanisms of diabetic cardiovascular complications is endothelial dysfunction or injury, caused by HSPG degradation induced by hyperglycemia.

In the present study, our results show for the first time in ECs, that high glucose decreased GAG content in PAECs and decreased syndecan gene and increased heparanase gene expression in PAECs and that insulin decreased GAGs in PAECs and medium suggesting insulin may inhibit GAG

synthesis and play a role in GAG degradation. As well our results showed that insulin or heparin protected GAGs from high glucose degradation in PAECs; and insulin and/or heparin upregulated syndecan and heparanase, and downregulated perlecan gene expression in the presence of high glucose in HAECs. In addition, HPLC analysis of HS disaccharides in PAECs showed different missing disaccharides in cells versus medium and control medium versus glucose treated medium.

Decreased total GAGs in high glucose-treated ECs is consistent with previous studies that the content of HS and HSPG was reduced in glomerular and skin BM and in the aortic intima of diabetic patients. It is possible that reduction of total GAGs by high glucose was partly caused by decreased syndecan mRNA and increased heparanase mRNA shown in this study **(Figure 5.1** and **5.2)**, since heparanase degrades HS chains on syndecans or perlecan (Reiland *et al*., 2004). Heparanase protein had been localized in both glomeruli capillaries and tubular epithelium and heparanase activity was detected in the urine of diabetic patients and high glucose-treated PAECs (Han *et al*., 2007; Katz *et al*., 2002; Shafat *et al*., 2006). Since heparanase does not change syndecan core protein (Reiland *et al*., 2004), HS reduction due to decreased syndecan mRNA by high glucose may not correlate with heparanase induction. In addition, the degradation of cell surface HSPG was reversed by heparanase inhibitors, PI-88 and heparin, in epithelial cells under hyperglycaemic conditions (Maxhimer *et al*., 2005) further confirming that heparanase induction is the cause of HS chain degradation. However,

an increase in serum syndecan was shown in early nephropathy of diabetic patients (Svennevig *et al*., 2006) and heparanase was responsible for syndecan shedding in myeloma and breast cancer cells (Yang *et al*., 2007). These observations suggest that increased serum syndecan in the diabetic patient may be due to heparanase induction which may play a role in decreased syndecan core protein and HS, but not expression of syndecan mRNA in ECs.

Vascular endothelium is a physiological target of insulin and a potential link between insulin resistance and atherosclerosis (Hsueh and Law, 1999; Mather *et al*., 2001a). Insulin acts on vascular cells by modulating transcription, altering cell content of numerous mRNAs and stimulating cell growth, DNA synthesis and replication (Bornfeldt *et al*., 1992; Sowers, 1997). Our studies demonstrated that insulin also plays a role in GAG synthesis or degradation. There was a trend that insulin alone increased GAGs in culture medium as culturing time increased (**Figure 3.4.B**) which may indicate HSPG degradation, turnover or shedding of HS or syndecan into medium. GAGs were significantly reduced in 72 hour insulin alone treated cultures compared to control, suggesting that GAG synthesis may also be inhibited (**Figure 3.4.C**). Insulin alone decreased heparanase and perlecan mRNA in the presence and absence of high glucose and syndecan in the presence of high glucose, suggesting that insulin may protect cells by inhibiting heparanase mRNA and may be harmful to cells by decreasing perlecan and syndecan mRNA. These data suggest the dual actions of insulin on ECs. On

the one hand, insulin inhibited heparanase mRNA expression in high glucose-treated cells and protected cell injury by HS degradation caused by heparanase upregulation. As well, insulin alone inhibited perlecan and syndecan mRNA expression and may shed syndecan ectodomains from the cell surface which are harmful to ECs, since insulin promoted shedding of syndecan ectodomains from 3T3-L1 adipocytes (Reizes *et al*., 2006). In a study of regulation of HSPG metabolism and hepatocyte growth by insulin, insulin markedly stimulated the rate of internalization of matrix HSPG and phospholipase C and therefore may control cell surface or matrix HSPG turnover (Ishihara *et al*., 1987).

Previous studies have shown that heparin increased HS synthesis, prevented heparanase upregulation in ECs and HSPG degradation from heparanase induction (Bar-Ner *et al*., 1987; Han *et al*., 2007; Nader *et al*., 1991). In the present study, heparin increased GAGs in high glucose-treated cultures **(Figure 3.6)** and protected syndecan mRNA from high glucose degradation which are consistent with the previous observations. Heparin alone or in the presence of high glucose decreased heparanase and perlecan, increased syndecan mRNA expression and further confirmed that heparin is capable of inhibiting heparanase expression under high glucose conditions and enhancing the synthetic formation of syndecan, but not perlecan. These results suggest that heparin plays a role in the protection of endothelium and vasculature by increasing syndecan synthesis and preventing of heparanase induction.

Addition of actinomycin D inhibited both *β*-actin and heparanase mRNA expression under high glucose conditions **(Figure 5.4),** suggesting transcriptional regulation of high glucose on heparanase gene expression occurs in ECs. Previous studies have demonstrated that high glucose is able to activate several transcriptional factors that are involved in regulation of the heparanase promoter, including Sp1, Egr-1 and the ETS family transcription factors (Jiang *et al*., 2002). These transcription factors play a role in controlling the basal activity of human heparanase gene expression (Jiang *et al*., 2002; Lu *et al*., 2003). Increased mRNAs of syndecan and heparanase and decreased perlecan mRNA in high glucose plus insulin plus heparin treated cultures indicated that the influence of heparin and insulin on expression of these three genes was complicated in the presence of high glucose. Addition of actinomycin D in high glucose plus insulin plus heparin treated cultures inhibited genes of heparanase and syndecan as well as *β*-actin **(Figure 5.5 and 5.6),** suggesting transcriptional regulation of these two genes by a combination of insulin and heparin under hyperglycemic conditions. The syndecan gene is regulated by Sp1-like transcription factors (Vihinen *et al*., 1996). Phosphorylation of Sp1 by the PKC family may change its abundance or transactivation activity (Black *et al*., 2001). Phosphorylation of Sp1 by PKC decreased its activity (Leggett *et al*., 1995). A decrease in Sp1 activity may cause down-regulation of syndecan gene expression in ECs under hyperglycaemic conditions, since glucose can activate and upregulate PKC (Scivittaro *et al*., 2000). Although perlecan mRNA was not changed by

high glucose in the present study, expression may be posttranscriptionally regulated, since perlecan expression was down-regulated by cAMP alone in glomerular epithelial cells through posttranscriptional events (Ko *et al*., 1996). Heparin prevented binding of AP-1, but not Sp1 to their binding site on TGF-*β* and circumvented the prosclerotic pathogenesis in diabetic nephropathy (Weigert *et al*., 2001). Insulin upregulated PAI-1 by activating Sp1 transcription factor (Banfi *et al*., 2001). In contrast, over-expression of Sp1 in rat hepatocytes leads to a decrease in FAS promoter activity (Fukada *et al*., 1997). Heparin or insulin may regulate gene expression by interacting with different transcriptional factors or acting differently on common transcriptional binding sites such as Sp1 through diverse pathways with positive or negative regulations. It is possible that there are alternative sites regulated by heparin, insulin or glucose on the promoters of heparanase, syndecan, and perlecan genes. The decreased mRNA expression of heparanase and perlecan by insulin alone may be due to Sp1 activated by insulin, which may play a negative transcriptional role at promoter regions of heparanase and perlecan. Syndecan mRNA expression decreased by high glucose and not changed by insulin in this study may suggest opposite actions of glucose and insulin on Sp1 regulation. However, insulin promoted shedding of syndecan, and the similar sequences between the human insulin receptor and mouse syndecan (Ebina *et al*., 1985) may mean that insulin affects the pathways involved in syndecan biosynthesis or turnover. Therefore, insulin may play a role in posttranscriptional regulation of syndecan expression and reduce syndecan

on cell surface although syndecan mRNA wasn't effected by insulin in the present study.

In conclusion, alteration of HSPGs, either the core protein or GAG chains by high glucose, influences EC functions and activities. Decreased total GAG content and syndecan mRNA, and increased heparanase mRNA by high glucose suggests that high glucose is detrimental to ECs. Dysfunction or injury of ECs induced by HSPG degradation is responsible for diabetic vascular complications. Interestingly, we also found that insulin, an effective glucose lowering therapy, also reduced total GAGs in cells and medium suggesting a potential mechanism of insulin resistance or hyperinsulinemia. Heparin showed a protective effect on ECs by increasing GAG content and syndecan mRNA as well as decreasing heparanase mRNA. Combination of insulin and heparin synergistically enhanced syndecan and heparanase mRNA expression, suggesting complex the gene regulation mechanisms by several transcriptional factors are involved in the regulation of these genes. Furthermore, enhanced heparanase mRNA expression by high glucose plus insulin plus heparin may raise precautions when using insulin and heparin for therapeutic purposes in diabetic treatment.

The present study has several limitations. The size of cell culture dishes is important for extracting enough GAGs from cells for analysis. Enough GAGs could not be obtained from a single 60 mm dish, but three 60 mm dishes combined provide enough GAGs for HPLC analysis of HS

disaccharides. More primary PAECs were needed for passing cells to bigger (100 mm) dishes. When using these large dishes, it takes a longer time for cells to grow to confluence and become evenly distributed which may affect cell health and viability as well as GAG synthesis. It is even harder to grow ample uniform human ECs for GAG studies due to the unstable quality or diverse sources of commercially available cultured HAECs.

Heparin interferes with the GAG content in ECs. The carbazole assay determines all GAGs including heparin and can not be performed in heparin treated cells, therefore the effect of heparin and/or insulin addition on total GAG content could not be measured and evaluated by the carbazole assay in this study. HAECs but not PAECs were used to determine gene expression in this study due to gene sequences for heparanase, syndecan and perlecan being available for human but not porcine species. There is a limitation to cooperatively integrate and interpret the two studies mentioned above because we used cells from two different species. These are *in vitro* studies where results cannot be completely applied to elucidate the *in vivo* situation, since ECs in the vasculature reside in enviroments different from culture medium. Further investigations in *in vivo* studies will provide more accurate consequences of GAG changes and gene expression in pathological mechanisms of hyperglycemia and the therapeutic outcome of heparin and/or insulin on diabetes.

Future studies: A dose response determination for heparin and insulin needs to be performed in order to select the right dose for obvious

effects of these two compounds on GAG changes and gene expression. Analysis of enzymes involved in HS synthesis such as 3OSTs, NDSTs, and 6OSTs in high glucose-treated ECs will provide information on the fundamental mechanisms of HSPG degadation under hyperglycemic conditions. Determining changes in different HS disaccharides by HPLC in high glucose-treated ECs will confirm the enzyme analysis. Elucidation of transcriptional factors on the promoters of heparanase, perlecan and syndecan, especially some common expressed factors such as Sp1 and the effect of insulin and heparin will help to further understand gene regulatory mechanisms of HSPG core proteins in hyperglycemia as well as the therapeutic funtions of insulin and heparin in diabetes.

8. REFERENCES

Adler S: Heparin Alters Epidermal Growth Factor Metabolism in Cultured Rat Glomerular Epithelial Cells. ***American Journal of Pathology*** 139(1): 169-175, 1991

Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, and Park JE. Vascular Endothelial Growth Factor in Ocular Fluid of Patients With Diabetic Retinopathy and Other Retinal Disorders. ***The New England Journal of Medicine*** 331(22): 1480-1487, 1994

Aikawa J and Esko JD: Molecular Cloning and Expression of a Third Member of the Heparan Sulfate/Heparin GlcNAc N-Deacetylase/ N-Sulfotransferase Family. ***Journal of Biological Chemistry*** 274(5): 2690-2695, 1999

Aikawa J, Grobe K, Tsujimoto M and Esko JD: Multiple Isozymes of Heparan Sulfate/Heparin GlcNAc N-Deacetylase/GlcN N-Sulfotransferase. Structure and Activity of the Fourth Member, NDST4. ***Journal of Biological Chemistry*** 276(8): 5876-5882, 2001

Aird WC: Vascular Bed-Specific Hemostasis: Role of Endothelium in Sepsis Pathogenesis. ***Critical Care Medicine*** 29(7 Suppl): S28-S34, 2001

Aird WC: Endothelium As an Organ System. ***Critical Care Medicine*** 32(5 Suppl): S271-S279, 2004

Alpert E, Gruzman A, Riahi Y, Blejter R, Aharoni P, Weisinger G, Eckel J, Kaiser N and Sasson S: Delayed Autoregulation of Glucose Transport in Vascular Endothelial Cells. ***Diabetologia*** 48(4): 752-755, 2005

Arap W, Kolonin MG, Trepel M, Lahdenranta J, Cardo-Vila M, Giordano RJ, Mintz PJ, Ardelt PU, Yao VJ, Vidal CI, Chen L, Flamm A, Valtanen H, Weavind LM, Hicks ME, Pollock RE, Botz GH, Bucana CD, Koivunen E, Cahill D, Troncoso P, Baggerly KA, Pentz RD, Do KA, Logothetis CJ and Pasqualini R: Steps Toward Mapping the Human Vasculature by Phage Display. ***Nature Medicine*** 8(2): 121-127, 2002

Arikawa-Hirasawa E, Watanabe H, Takami H, Hassell JR and Yamada Y: Perlecan Is Essential for Cartilage and Cephalic Development. ***Nature Genetics*** 23(3): 354-358, 1999

Bai X and Esko JD: An Animal Cell Mutant Defective in Heparan Sulfate Hexuronic Acid 2-O-Sulfation. ***Journal of Biological Chemistry*** 271(30): 17711-17717, 1996

Bandtlow CE and Zimmermann DR: Proteoglycans in the Developing Brain: New Conceptual Insights for Old Proteins. ***Physiological Reviews*** 80(4): 1267-1290, 2000

Banfi C, Eriksson P, Giandomenico G, Mussoni L, Sironi L, Hamsten A and Tremoli E: Transcriptional Regulation of Plasminogen Activator Inhibitor Type 1 Gene by Insulin: Insights into the Signaling Pathway. ***Diabetes*** 50(7): 1522-1530, 2001

Bank N, Mower P, Aynedjian HS, Wilkes BM and Silverman S: Sorbinil Prevents Glomerular Hyperperfusion in Diabetic Rats. ***American Journal of Physiology*** 256(6 Pt 2): F1000-F1006, 1989

Bar-Ner M, Eldor A, Wasserman L, Matzner Y, Cohen IR, Fuks Z and Vlodavsky I: Inhibition of Heparanase-Mediated Degradation of Extracellular Matrix Heparan Sulfate by Non-Anticoagulant Heparin Species. ***Blood*** 70(2): 551-557, 1987

Bartlett MR, Underwood PA and Parish CR: Comparative Analysis of the Ability of Leucocytes, Endothelial Cells and Platelets to Degrade the Subendothelial Basement Membrane: Evidence for Cytokine Dependence and Detection of a Novel Sulfatase. ***Immunology & Cell Biology*** 73(2): 113-124, 1995

Barzu T, van Rijn JL, Petitou M, Tobelem G and Caen JP: Heparin Degradation in the Endothelial Cells. ***Thrombosis Research*** 47(5): 601-609, 1987

Basta G, Lazzerini G, Massaro M, Simoncini T, Tanganelli P, Fu C, Kislinger T, Stern DM, Schmidt AM and De Caterina R: Advanced Glycation End Products Activate Endothelium Through Signal-Transduction Receptor RAGE: a Mechanism for Amplification of Inflammatory Responses. ***Circulation*** 105(7): 816-822, 2002

Bazzoni G, Beltran NA, Mascellani G, Bianchini P, Dejana E and Del Maschio A: Effect of Heparin, Dermatan Sulfate, and Related Oligo-Derivatives on Human Polymorphonuclear Leukocyte Functions. ***The Journal of Laboratory and Clinical Medicine*** 121(2): 268-275, 1993

Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J and Zako M: Functions of Cell Surface Heparan Sulfate Proteoglycans. ***Annual Review of Biochemistry*** 68: 729-777, 1999

Bernfield M, Kokenyesi R, Kato M, Hinkes MT, Spring J, Gallo RL and Lose EJ: Biology of the Syndecans: a Family of Transmembrane Heparan Sulfate Proteoglycans. ***Annual Review of Cell and Developmental Biology*** 8: 365-393, 1992

Bernfield M and Sanderson RD: Syndecan, a Developmentally Regulated Cell Surface Proteoglycan That Binds Extracellular Matrix and Growth Factors. ***Philosophical Transactions of the Royal Society B: Biological Sciences*** 327(1239): 171-186, 1990

Bertelsen M, Anggard EE and Carrier MJ: Oxidative Stress Impairs Insulin Internalization in Endothelial Cells *in vitro*. ***Diabetologia*** 44(5): 605-613, 2001

Berthoud HR, Bereiter DA, Trimble ER, Siegel EG and Jeanrenaud B: Cephalic Phase, Reflex Insulin Secretion. Neuroanatomical and Physiological Characterization. ***Diabetologia*** 20 Suppl 393-401, 1981

Beyer-Mears A, Cruz E, Edelist T and Varagiannis E: Diminished Proteinuria in Diabetes Mellitus by Sorbinil, an Aldose Reductase Inhibitor. ***Pharmacology*** 32(1): 52-60, 1986

Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, Stern DM and Nawroth PP: Understanding RAGE, the Receptor for Advanced Glycation End Products. ***Journal of Molecular Medicine*** 83(11): 876-886, 2005

Black AR, Black JD and Azizkhan-Clifford J: Sp1 and Kruppel-Like Factor Family of Transcription Factors in Cell Growth Regulation and Cancer. ***Journal of Cellular Physiology*** 188(2): 143-160, 2001

Bollineni JS, Alluru I and Reddi AS: Heparan Sulfate Proteoglycan Synthesis and Its Expression Are Decreased in the Retina of Diabetic Rats. ***Current Eye Research*** 16(2): 127-130, 1997

Bombeli T, Schwartz BR and Harlan JM: Adhesion of Activated Platelets to Endothelial Cells: Evidence for a GPIIbIIIa-Dependent Bridging Mechanism and Novel Roles for Endothelial Intercellular Adhesion Molecule 1 (ICAM-1), Alphavbeta3 Integrin, and GPIbalpha. ***The Journal of Experimental Medicine*** 187(3): 329-339, 1998

Bornfeldt KE, Skottner A and Arnqvist HJ: In-Vivo Regulation of Messenger RNA Encoding Insulin-Like Growth Factor-I (IGF-I) and Its Receptor by Diabetes, Insulin and IGF-I in Rat Muscle. ***Journal of Endocrinology*** 135(2): 203-211, 1992

Borsum T: Biochemical Properties of Vascular Endothelial Cells. ***Virchows Archive B: Cell Pathology Including Molecular Pathology*** 60(5): 279-286, 1991

Bouchard JF, Moore SW, Tritsch NX, Roux PP, Shekarabi M, Barker PA and Kennedy TE: Protein Kinase A Activation Promotes Plasma Membrane Insertion of DCC From an Intracellular Pool: A Novel Mechanism Regulating Commissural Axon Extension. ***The Journal of Neuroscience*** 24(12): 3040-3050, 2004

Boulanger C and Luscher TF: Release of Endothelin From the Porcine Aorta. Inhibition by Endothelium-Derived Nitric Oxide. ***Journal of Clinical Investigation*** 85(2): 587-590, 1990

Bourin MC and Lindahl U: Glycosaminoglycans and the Regulation of Blood Coagulation. ***Biochemical Journal*** 289 (Pt 2): 313-330, 1993

Bradbury NA and Bridges RJ: Endocytosis Is Regulated by Protein Kinase A, but Not Protein Kinase C in a Secretory Epithelial Cell Line. ***Biochemical and Biophysical Research Communications*** 184(3): 1173-1180, 1992

Brennan MJ, Oldberg A, Hayman EG and Ruoslahti E: Effect of a Proteoglycan Produced by Rat Tumor Cells on Their Adhesion to Fibronectin-Collagen Substrata. ***Cancer Research*** 43(9): 4302-4307, 1983

Bresnick GH, Davis MD, Myers FL and de Venecia G: Clinicopathologic Correlations in Diabetic Retinopathy. II. Clinical and Histologic Appearances of Retinal Capillary Microaneurysms. ***Archives of Ophthalmology*** 95(7): 1215-1220, 1977

Brown DM, Klein DJ, Michael AF and Oegema TR: 35S-Glycosaminoglycan and 35S-Glycopeptide Metabolism by Diabetic Glomeruli and Aorta. ***Diabetes*** 31(5 Pt 1): 418-425, 1982

Brown JC, Sasaki T, Gohring W, Yamada Y and Timpl R: The C-Terminal Domain V of Perlecan Promotes Beta1 Integrin-Mediated Cell Adhesion, Binds Heparin, Nidogen and Fibulin-2 and Can Be Modified by Glycosaminoglycans. ***European Journal of Biochemistry*** 250(1): 39-46, 1997

Brownlee M: Biochemistry and Molecular Cell Biology of Diabetic Complications. ***Nature*** 414(6865): 813-820, 2001

Brownlee M and Spiro RG: Glomerular Basement Membrane Metabolism in the Diabetic Rat. *In vivo* Studies. ***Diabetes*** 28(2): 121-125, 1979

Bucala R, Tracey KJ and Cerami A: Advanced Glycosylation Products Quench Nitric Oxide and Mediate Defective Endothelium-Dependent Vasodilatation in Experimental Diabetes. ***Journal of Clinical Investigation*** 87(2): 432-438, 1991

Cai H, Wang XL and Wilcken DE: Genetic Polymorphism of Heparan Sulfate Proteoglycan (Perlecan, HSPG2), Lipid Profiles and Coronary Artery Disease in the Australian Population. ***Atherosclerosis*** 148(1): 125-129, 2000

Carey DJ, Evans DM, Stahl RC, Asundi VK, Conner KJ, Garbes P and Cizmeci-Smith G: Molecular Cloning and Characterization of N-Syndecan, a Novel Transmembrane Heparan Sulfate Proteoglycan. ***Journal of Cell Biology*** 117(1): 191-201, 1992

Castellot JJ, Jr., Favreau LV, Karnovsky MJ and Rosenberg RD: Inhibition of Vascular Smooth Muscle Cell Growth by Endothelial Cell-Derived Heparin. Possible Role of a Platelet Endoglycosidase. ***Journal of Biological Chemistry*** 257(19): 11256-11260, 1982

Castellot JJ, Jr., Hoover RL, Harper PA and Karnovsky MJ: Heparin and Glomerular Epithelial Cell-Secreted Heparin-Like Species Inhibit Mesangial-Cell Proliferation. ***American Journal of Pathology*** 120(3): 427-435, 1985

Castellot JJ, Jr., Hoover RL and Karnovsky MJ: Glomerular Endothelial Cells Secrete a Heparinlike Inhibitor and a Peptide Stimulator of Mesangial Cell Proliferation. ***American Journal of Pathology*** 125(3): 493-500, 1986

Casu B: Structure and Biological Activity of Heparin. ***Advances in Carbohydrate Chemistry & Biochemistry*** 43: 51-134, 1985

Chakravarti S, Hassell JR and Phillips SL: Perlecan Gene Expression Precedes Laminin Gene Expression During Differentiation of F9 Embryonal Carcinoma Cells. ***Developmental Dynamics*** 197(2): 107-114, 1993

Charonis AS, Reger LA, Dege JE, Kouzi-Koliakos K, Furcht LT, Wohlhueter RM and Tsilibary EC: Laminin Alterations After *in vitro* Nonenzymatic Glycosylation. ***Diabetes*** 39(7): 807-814, 1990

Chen S, Hong SW, Iglesias-de la Cruz MC, Isono M, Casaretto A and Ziyadeh FN: The Key Role of the Transforming Growth Factor-Beta System in the Pathogenesis of Diabetic Nephropathy. ***Renal Failure*** 23(3-4): 471-481, 2001

Chon JH, Wang HS and Chaikof EL: Role of Fibronectin and Sulfated Proteoglycans in Endothelial Cell Migration on a Cultured Smooth Muscle Layer. ***Journal of Surgical Research*** 72(1): 53-59, 1997

Cizmeci-Smith G, Asundi V, Stahl RC, Teichman LJ, Chernousov M, Cowan K and Carey DJ: Regulated Expression of Syndecan in Vascular Smooth Muscle Cells and Cloning of Rat Syndecan Core Protein CDNA. ***Journal of Biological Chemistry*** 267(22): 15729-15736, 1992

Cizmeci-Smith G, Stahl RC, Showalter LJ and Carey DJ: Differential Expression of Transmembrane Proteoglycans in Vascular Smooth Muscle Cells. ***Journal of Biological Chemistry*** 268(25): 18740-18747, 1993

Clark CM, Jr. and Lee DA: Prevention and Treatment of the Complications of Diabetes Mellitus. ***The New England Journal of Medicine*** 332(18): 1210-1217, 1995

Cohen IR, Grassel S, Murdoch AD and Iozzo RV: Structural Characterization of the Complete Human Perlecan Gene and Its Promoter. ***Proceedings of the National Academy of Sciences of the United States of America*** 90(21): 10404-10408, 1993

Cohen MP: Aldose Reductase, Glomerular Metabolism, and Diabetic Nephropathy. ***Metabolism*** 35(4 Suppl 1): 55-59, 1986

Colwell JA and Lopes-Virella MF: A Review of the Development of Large-Vessel Disease in Diabetes Mellitus. ***The American Journal of Medicine*** 85(5A): 113-118, 1988b

Colwell JA and Lopes-Virella MF: A Review of the Development of Large-Vessel Disease in Diabetes Mellitus. ***The American Journal of Medicine*** 85(5A): 113-118, 1988a

Conrad, H. E. In: Heparin-Binding proteins. Academic Press, San Diego, CL, Pp. 1998

Craven PA and DeRubertis FR: Protein Kinase C Is Activated in Glomeruli From Streptozotocin Diabetic Rats. Possible Mediation by Glucose. ***Journal of Clinical Investigation*** 83(5): 1667-1675, 1989a

Craven PA and DeRubertis FR: Sorbinil Suppresses Glomerular Prostaglandin Production in the Streptozotocin Diabetic Rat. ***Metabolism*** 38(7): 649-654, 1989b

Crawford BE, Olson SK, Esko JD and Pinhal MA: Cloning, Golgi Localization, and Enzyme Activity of the Full-Length Heparin/Heparan Sulfate-Glucuronic Acid C5-Epimerase. ***Journal of Biological Chemistry*** 276(24): 21538-21543, 2001

Creager MA, Luscher TF, Cosentino F and Beckman JA: Diabetes and Vascular Disease: Pathophysiology, Clinical Consequences, and Medical Therapy: Part I. ***Circulation*** 108(12): 1527-1532, 2003

Danielson KG, Martinez-Hernandez A, Hassell JR and Iozzo RV: Establishment of a Cell Line From the EHS Tumor: Biosynthesis of Basement Membrane Constituents and Characterization of a Hybrid Proteoglycan Containing Heparan and Chondroitin Sulfate Chains. ***Matrix*** 12(1): 22-35, 1992

David G, Lories V, Decock B, Marynen P, Cassiman JJ and Van den BH: Molecular Cloning of a Phosphatidylinositol-Anchored Membrane Heparan Sulfate Proteoglycan From Human Lung Fibroblasts. ***Journal of Cell Biology*** 111(6 Pt 2): 3165-3176, 1990

David G, Van der SB, Marynen P, Cassiman JJ and Van den BH: Molecular Cloning of Amphiglycan, a Novel Integral Membrane Heparan Sulfate Proteoglycan Expressed by Epithelial and Fibroblastic Cells. ***Journal of Cell Biology*** 118(4): 961-969, 1992

Deckert T, Feldt-Rasmussen B, Borch-Johnsen K, Jensen T and Kofoed-Enevoldsen A: Albuminuria Reflects Widespread Vascular Damage. The Steno Hypothesis. ***Diabetologia*** 32(4): 219-226, 1989

Deeney JT, Prentki M and Corkey BE: Metabolic Control of Beta-Cell Function. ***Seminars in Cell and Developmental Biology*** 11(4): 267-275, 2000

Dempsey LA, Brunn GJ and Platt JL: Heparanase, a Potential Regulator of Cell-Matrix Interactions. ***Trends in Biochemical Sciences*** 25(8): 349-351, 2000

Despres JP, Lamarche B, Mauriege P, Cantin B, Dagenais GR, Moorjani S and Lupien PJ: Hyperinsulinemia As an Independent Risk Factor for Ischemic Heart Disease. ***The New England Journal of Medicine*** 334(15): 952-957, 1996

Dodge GR, Boesler EW and Jimenez SA: Expression of the Basement Membrane Heparan Sulfate Proteoglycan (Perlecan) in Human Synovium and in Cultured Human Synovial Cells. ***Laboratory Investigation*** 73(5): 649-657, 1995

Dodge GR, Kovalszky I, Hassell JR and Iozzo RV: Transforming Growth Factor Beta Alters the Expression of Heparan Sulfate Proteoglycan in Human Colon Carcinoma Cells. ***Journal of Biological Chemistry*** 265(29): 18023-18029, 1990

Dodson G and Steiner D: The Role of Assembly in Insulin's Biosynthesis. ***Current Opinion in Structural Biology*** 8(2): 189-194, 1998

Dolan M, Horchar T, Rigatti B and Hassell JR: Identification of Sites in Domain I of Perlecan That Regulate Heparan Sulfate Synthesis. ***Journal of Biological Chemistry*** 272(7): 4316-4322, 1997

Doolittle, R. F. In: New perspectives on evolution provided by protein sequences. (Warren, L. eds.) New Perspectives on Evolution. Wiley-Liss, New York, Pp. 165-173, 1991

Duncan G, McCormick C and Tufaro F: The Link Between Heparan Sulfate and Hereditary Bone Disease: Finding a Function for the EXT Family of Putative Tumor Suppressor Proteins. ***Journal of Clinical Investigation*** 108(4): 511-516, 2001

Ebina Y, Ellis L, Jarnagin K, Edery M, Graf L, Clauser E, Ou JH, Masiarz F, Kan YW and Goldfine ID: The Human Insulin Receptor CDNA: the Structural Basis for Hormone-Activated Transmembrane Signalling. ***Cell*** 40(4): 747-758, 1985

Edge AS and Spiro RG: Characterization of Novel Sequences Containing 3-O-Sulfated Glucosamine in Glomerular Basement Membrane Heparan Sulfate and Localization of Sulfated Disaccharides to a Peripheral Domain. ***Journal of Biological Chemistry*** 265(26): 15874-15881, 1990

Edwards IJ, Wagner JD, Vogl-Willis CA, Litwak KN and Cefalu WT: Arterial Heparan Sulfate Is Negatively Associated With Hyperglycemia and Atherosclerosis in Diabetic Monkeys. ***Cardiovascular Diabetology*** 3: 6 doi: 10.1186/1475-2840-3-6, 2004

Elenius K, Maatta A, Salmivirta M and Jalkanen M: Growth Factors Induce 3T3 Cells to Express BFGF-Binding Syndecan. ***Journal of Biological Chemistry*** 267(9): 6435-6441, 1992

Elkin M, Ilan N, Ishai-Michaeli R, Friedmann Y, Papo O, Pecker I and Vlodavsky I: Heparanase As Mediator of Angiogenesis: Mode of Action. ***FASEB Journal*** 15(9): 1661-1663, 2001

Elliott TG, Cockcroft JR, Groop PH, Viberti GC and Ritter JM: Inhibition of Nitric Oxide Synthesis in Forearm Vasculature of Insulin-Dependent Diabetic Patients: Blunted Vasoconstriction in Patients With Microalbuminuria. ***Clinical Science (London)*** 85(6): 687-693, 1993

Endo K, Takino T, Miyamori H, Kinsen H, Yoshizaki T, Furukawa M and Sato H: Cleavage of Syndecan-1 by Membrane Type Matrix Metalloproteinase-1 Stimulates Cell Migration. ***Journal of Biological Chemistry*** 278(42): 40764-40770, 2003

Engerman RL and Kern TS: Experimental Galactosemia Produces Diabetic-Like Retinopathy. ***Diabetes*** 33(1): 97-100, 1984

Engerman RL and Kern TS: Hyperglycemia and Development of Glomerular Pathology: Diabetes Compared With Galactosemia. ***Kidney International*** 36(1): 41-45, 1989

Ernst S, Rhomberg AJ, Biemann K and Sasisekharan R: Direct Evidence for a Predominantly Exolytic Processive Mechanism for Depolymerization of Heparin-Like Glycosaminoglycans by Heparinase I. ***Proceedings of the National Academy of Sciences of the United States of America*** 95(8): 4182-4187, 1998

Esko JD and Lindahl U: Molecular Diversity of Heparan Sulfate. ***Journal of Clinical Investigation*** 108(2): 169-173, 2001

Esko JD and Zhang L: Influence of Core Protein Sequence on Glycosaminoglycan Assembly. ***Current Opinion in Structural Biology*** 6(5): 663-670, 1996

Evans-Molina C, Garmey JC, Ketchum R, Brayman KL, Deng S and Mirmira RG: Glucose Regulation of Insulin Gene Transcription and Pre-MRNA Processing in Human Islets. ***Diabetes*** 56(3): 827-835, 2007

Fairbanks MB, Mildner AM, Leone JW, Cavey GS, Mathews WR, Drong RF, Slightom JL, Bienkowski MJ, Smith CW, Bannow CA and Heinrikson RL: Processing of the Human Heparanase Precursor and Evidence That the Active Enzyme Is a Heterodimer. ***Journal of Biological Chemistry*** 274(42): 29587-29590, 1999

Fairman RP, Sessler CN, Bierman M and Glauser FL: Protamine Sulfate Causes Pulmonary Hypertension and Edema in Isolated Rat Lungs. ***Journal of Applied Physiology*** 62(4): 1363-1367, 1987

Fanning AS and Anderson JM: Protein-Protein Interactions: PDZ Domain Networks. ***Current Biology*** 6(11): 1385-1388, 1996

Feener EP and King GL: Vascular Dysfunction in Diabetes Mellitus. ***Lancet*** 350: Suppl 1 SI9-13, 1997

Ferri C, Carlomagno A, Coassin S, Baldoncini R, Cassone Faldetta MR, Laurenti O, Properzi G, Santucci A and De Mattia G: Circulating Endothelin-1 Levels Increase During Euglycemic Hyperinsulinemic Clamp in Lean NIDDM Men. ***Diabetes Care*** 18(2): 226-233, 1995a

Ferri C, Pittoni V, Piccoli A, Laurenti O, Cassone MR, Bellini C, Properzi G, Valesini G, De Mattia G and Santucci A: Insulin Stimulates Endothelin-1 Secretion From Human Endothelial Cells and Modulates Its Circulating Levels *in vivo*. ***The Journal of Clinical Endocrinology & Metabolism*** 80(3): 829-835, 1995b

Fischer VW, Barner HB and Leskiw ML: Capillary Basal Laminar Thichness in Diabetic Human Myocardium. ***Diabetes*** 28(8): 713-719, 1979

Fisslthaler B, Benzing T, Busse R and Fleming I: Insulin Enhances the Expression of the Endothelial Nitric Oxide Synthase in Native Endothelial Cells: a Dual Role for Akt and AP-1. ***Nitric.Oxide.*** 8(4): 253-261, 2003

Fitzgerald ML, Wang Z, Park PW, Murphy G and Bernfield M: Shedding of Syndecan-1 and -4 Ectodomains Is Regulated by Multiple Signaling Pathways and Mediated by a TIMP-3-Sensitive Metalloproteinase. ***Journal of Cell Biology*** 148(4): 811-824, 2000

Freeman C, Browne AM and Parish CR: Evidence That Platelet and Tumour Heparanases Are Similar Enzymes. ***Biochemical Journal*** 342 (Pt 2) 361-368, 1999

Freeman C and Parish CR: Human Platelet Heparanase: Purification, Characterization and Catalytic Activity. ***Biochemical Journal*** 330 (Pt 3) 1341-1350, 1998

Fukada H, Iritani N and Noguchi T: Transcriptional Regulatory Regions for Expression of the Rat Fatty Acid Synthase. ***FEBS Letters*** 406 243-248, 1997

Gallagher JT, Lyon M and Steward WP: Structure and Function of Heparan Sulphate Proteoglycans. ***Biochemical Journal*** 236(2): 313-325, 1986

Gallo RL, Ono M, Povsic T, Page C, Eriksson E, Klagsbrun M and Bernfield M: Syndecans, Cell Surface Heparan Sulfate Proteoglycans, Are Induced by a Proline-Rich Antimicrobial Peptide From Wounds. ***Proceedings of the National Academy of Sciences of the United States of America*** 91(23): 11035-11039, 1994

Garg HG, Thompson BT and Hales CA: Structure Determinants of Antiproliferativeity of Heparin on Pulmonary Artery Smooth Muscle Cells. ***American Journal of Phydiology-Lung Cellular and Molecular Physiology*** 279(5): L779-L789,2000

Genuth, S. M. In: Hormones of the pancreatic islets. (3) (Berne R.M. and Levy M.N. eds.) Physiology. Mosby-Year Book, Inc, St. Louis, MO, Pp. 851-876, 1993

Gharagozlian S, Borrebaek J, Henriksen T, Omsland TK, Shegarfi H and Kolset SO: Effect of Hyperglycemic Condition on Proteoglycan Secretion in Cultured Human Endothelial Cells. ***European Journal of Nutrition*** 45(7): 369-375, 2006

Godder K, Vlodavsky I, Eldor A, Weksler BB, Haimovitz-Freidman A and Fuks Z: Heparanase Activity in Cultured Endothelial Cells. ***Journal of Cellular Physiology*** 148(2): 274-280, 1991

Goldin A, Beckman JA, Schmidt AM and Creager MA: Advanced Glycation End Products: Sparking the Development of Diabetic Vascular Injury. ***Circulation*** 114(6): 597-605, 2006

Gosmanov AR, Stentz FB and Kitabchi AE: De Novo Emergence of Insulin-Stimulated Glucose Uptake in Human Aortic Endothelial Cells Incubated With High Glucose. ***The American Journal of Physiology - Endocrinology and Metabolism*** 290(3): E516-E522, 2006

Gotlieb AI and Spector W: Migration into an *in vitro* Experimental Wound: a Comparison of Porcine Aortic Endothelial and Smooth Muscle Cells and the Effect of Culture Irradiation. ***American Journal of Pathology*** 103(2): 271-282, 1981

Gould SE, Upholt WB and Kosher RA: Syndecan 3: a Member of the Syndecan Family of Membrane-Intercalated Proteoglycans That Is Expressed in High Amounts at the Onset of Chicken Limb Cartilage Differentiation. ***Proceedings of the National Academy of Sciences of the United States of America*** 89(8): 3271-3275, 1992

Greene DA, Lattimer SA and Sima AA: Sorbitol, Phosphoinositides, and Sodium-Potassium-ATPase in the Pathogenesis of Diabetic Complications. ***The New England Journal of Medicine*** 316(10): 599-606, 1987

Guzik TJ, Mussa S, Gastaldi D, Sadowski J, Ratnatunga C, Pillai R and Channon KM: Mechanisms of Increased Vascular Superoxide Production in Human Diabetes Mellitus: Role of NAD(P)H Oxidase and Endothelial Nitric Oxide Synthase. ***Circulation*** 105(14): 1656-1662, 2002

Ha TS, Song CJ and Lee JH: Effects of Advanced Glycosylation Endproducts on Perlecan Core Protein of Glomerular Epithelium. ***Pediatric Nephrology*** 19(11): 1219-1224, 2004

Habuchi H, Tanaka M, Habuchi O, Yoshida K, Suzuki H, Ban K and Kimata K: The Occurrence of Three Isoforms of Heparan Sulfate 6-O-Sulfotransferase Having Different Specificities for Hexuronic Acid Adjacent to the Targeted N-Sulfoglucosamine. ***Journal of Biological Chemistry*** 275(4): 2859-2868, 2000

Haffner SM, Stern MP, Hazuda HP, Mitchell BD and Patterson JK: Cardiovascular Risk Factors in Confirmed Prediabetic Individuals. Does the Clock for Coronary Heart Disease Start Ticking Before the Onset of Clinical Diabetes? ***The Journal Of the American Medical Association*** 263(21): 2893-2898, 1990

Hammes HP, Du X, Edelstein D, Taguchi T, Matsumura T, Ju Q, Lin J, Bierhaus A, Nawroth P, Hannak D, Neumaier M, Bergfeld R, Giardino I and Brownlee M: Benfotiamine Blocks Three Major Pathways of Hyperglycemic Damage and Prevents Experimental Diabetic Retinopathy. ***Nature Medicine*** 9(3): 294-299, 2003

Han J, Mandal AK and Hiebert LM: Endothelial Cell Injury by High Glucose and Heparanase Is Prevented by Insulin, Heparin and Basic Fibroblast Growth Factor. ***Cardiovascular Diabetology*** 4(1): 12 doi:10.1186/1475-2840-4-12. 2005

Han J, Woytowich AE, Mandal AK and Hiebert LM: Heparanase Upregulation in High Glucose-Treated Endothelial Cells Is Prevented by Insulin and Heparin. ***Experimental Biology and Medicine*** 232(7): 927-934, 2007

Hanefeld M, Fischer S, Julius U, Schulze J, Schwanebeck U, Schmechel H, Ziegelasch HJ and Lindner J: Risk Factors for Myocardial Infarction and Death in Newly Detected NIDDM: the Diabetes Intervention Study, 11-Year Follow-Up. ***Diabetologia*** 39(12): 1577-1583, 1996

Hansen PM, Chowdhury T, Deckert T, Hellgren A, Bain SC and Pociot F: Genetic Variation of the Heparan Sulfate Proteoglycan Gene (Perlecan Gene). Association With Urinary Albumin Excretion in IDDM Patients. ***Diabetes*** 46(10): 1658-1659, 1997

Hardebo JE and Kahrstrom J: Endothelial Negative Surface Charge Areas and Blood-Brain Barrier Function. ***Acta Physiologica Scandinavica*** 125(3): 495-499, 1985

Harpel PC, Zhang X and Borth W: Homocysteine and Hemostasis: Pathogenic Mechanisms Predisposing to Thrombosis. ***The Journal of Nutrition*** 126(4 Suppl): 1285S-1289S, 1996

Hautanen A, Gailit J, Mann DM and Ruoslahti E: Effects of Modifications of the RGD Sequence and Its Context on Recognition by the Fibronectin Receptor. ***Journal of Biological Chemistry*** 264(3): 1437-1442, 1989

Hayashi K, Madri JA and Yurchenco PD: Endothelial Cells Interact With the Core Protein of Basement Membrane Perlecan Through Beta 1 and Beta 3 Integrins: an Adhesion Modulated by Glycosaminoglycan. ***Journal of Cell Biology*** 119(4): 945-959, 1992

Hayashida K, Johnston DR, Goldberger O and Park PW: Syndecan-1 Expression in Epithelial Cells Is Induced by Transforming Growth Factor Beta Through a PKA-Dependent Pathway. ***Journal of Biological Chemistry*** 281(34): 24365-24374, 2006

Hayat SA, Patel B, Khattar RS and Malik RA: Diabetic Cardiomyopathy: Mechanisms, Diagnosis and Treatment. ***Clinical Science (London)*** 107(6): 539-557, 2004

Heickendorff L, Ledet T and Rasmussen LM: Glycosaminoglycans in the Human Aorta in Diabetes Mellitus: a Study of Tunica Media From Areas With and Without Atherosclerotic Plaque. ***Diabetologia*** 37(3): 286-292, 1994

Henquin JC: Glucose-Induced Electrical Activity in Beta-Cells. Feedback Control of ATP-Sensitive K+ Channels by Ca2+? [Corrected]. ***Diabetes*** 39(11): 1457-1460, 1990

Hiebert LM and Liu JM: Heparin Protects Cultured Arterial Endothelial Cells From Damage by Toxic Oxygen Metabolites. ***Atherosclerosis*** 83(1): 47-51, 1990

Hiebert LM, Wice SM, McDuffie NM and Jaques LB: The Heparin Target Organ--the Endothelium. Studies in a Rat Model. ***QJM: An International Journal of Medicine*** 86(5): 341-348, 1993

Hileman RE, Fromm JR, Weiler JM and Linhardt RJ: Glycosaminoglycan-Protein Interactions: Definition of Consensus Sites in Glycosaminoglycan Binding Proteins. ***Bioessays*** 20(2): 156-167, 1998

Hinkes MT, Goldberger OA, Neumann PE, Kokenyesi R and Bernfield M: Organization and Promoter Activity of the Mouse Syndecan-1 Gene. ***Journal of Biological Chemistry*** 268(15): 11440-11448, 1993

Hirschberg CB, Robbins PW and Abeijon C: Transporters of Nucleotide Sugars, ATP, and Nucleotide Sulfate in the Endoplasmic Reticulum and Golgi Apparatus. ***Annual Review of Biochemistry*** 67 49-69, 1998

Hofer G, Grimmer C, Sukhatme VP, Sterzel RB and Rupprecht HD: Transcription Factor Egr-1 Regulates Glomerular Mesangial Cell Proliferation. ***Journal of Biological Chemistry*** 271(45): 28306-28310, 1996

Hook M, Kjellen L and Johansson S: Cell-Surface Glycosaminoglycans. ***Annual Review of Biochemistry*** 53 847-869, 1984

Hopf M, Gohring W, Kohfeldt E, Yamada Y and Timpl R: Recombinant Domain IV of Perlecan Binds to Nidogens, Laminin-Nidogen Complex, Fibronectin, Fibulin-2 and Heparin. ***European Journal of Biochemistry*** 259(3): 917-925, 1999

Horowitz A and Simons M: Phosphorylation of the Cytoplasmic Tail of Syndecan-4 Regulates Activation of Protein Kinase Calpha. ***Journal of Biological Chemistry*** 273(40): 25548-25551, 1998

Howard G, O'Leary DH, Zaccaro D, Haffner S, Rewers M, Hamman R, Selby JV, Saad MF, Savage P and Bergman R: Insulin Sensitivity and Atherosclerosis. The Insulin Resistance Atherosclerosis Study (IRAS) Investigators. ***Circulation*** 93(10): 1809-1817, 1996

Hsueh WA and Law RE: Insulin Signaling in the Arterial Wall. ***American Journal of Cardiology*** 84(1A): 21J-24J, 1999

Hubbard SR, Wei L, Ellis L and Hendrickson WA: Crystal Structure of the Tyrosine Kinase Domain of the Human Insulin Receptor. ***Nature*** 372(6508): 746-754, 1994

Hulett MD, Freeman C, Hamdorf BJ, Baker RT, Harris MJ and Parish CR: Cloning of Mammalian Heparanase, an Important Enzyme in Tumor Invasion and Metastasis. ***Nature Medicine*** 5(7): 803-809, 1999

Ihrcke NS, Parker W, Reissner KJ and Platt JL: Regulation of Platelet Heparanase During Inflammation: Role of PH and Proteinases. ***Journal of Cellular Physiology*** 175(3): 255-267, 1998

Imano E, Kadowaki H, Kadowaki T, Iwama N, Watarai T, Kawamori R, Kamada T and Taylor SI: Two Patients With Insulin Resistance Due to Decreased Levels of Insulin-Receptor MRNA. ***Diabetes*** 40(5): 548-557, 1991

Inoguchi T, Battan R, Handler E, Sportsman JR, Heath W and King GL: Preferential Elevation of Protein Kinase C Isoform Beta II and Diacylglycerol Levels in the Aorta and Heart of Diabetic Rats: Differential Reversibility to Glycemic Control by Islet Cell Transplantation. ***Proceedings of the National Academy of Sciences of the United States of America*** 89(22): 11059-11063, 1992

Inoue S, Grant D and Leblond CP: Heparan Sulfate Proteoglycan Is Present in Basement Membrane As a Double-Tracked Structure. ***Journal of Histochemistry and Cytochemistry*** 37(5): 597-602, 1989

Iozzo RV: Matrix Proteoglycans: From Molecular Design to Cellular Function. ***Annual Review of Biochemistry*** 67 609-652, 1998

Iozzo RV and Cohen I: Altered Proteoglycan Gene Expression and the Tumor Stroma. ***Experientia*** 49(5): 447-455, 1993

Iozzo RV, Cohen IR, Grassel S and Murdoch AD: The Biology of Perlecan: the Multifaceted Heparan Sulphate Proteoglycan of Basement Membranes and Pericellular Matrices. ***Biochemical Journal*** 302 (Pt 3) 625-639, 1994

Iozzo RV, Pillarisetti J, Sharma B, Murdoch AD, Danielson KG, Uitto J and Mauviel A: Structural and Functional Characterization of the Human Perlecan Gene Promoter. Transcriptional Activation by Transforming Growth Factor-Beta Via a Nuclear Factor 1-Binding Element. ***Journal of Biological Chemistry*** 272(8): 5219-5228, 1997

Ishihara M, Fedarko NS and Conrad HE: Involvement of Phosphatidylinositol and Insulin in the Coordinate Regulation of Proteoheparan Sulfate Metabolism and Hepatocyte Growth. ***Journal of Biological Chemistry*** 262(10): 4708-4716, 1987

Ishikawa Y and Kitamura M: Inhibition of Glomerular Cell Apoptosis by Heparin. ***Kidney International*** 56(3): 954-963, 1999

Ishikawa Y, Yokoo T and Kitamura M: C-Jun/AP-1, but Not NF-Kappa B, Is a Mediator for Oxidant-Initiated Apoptosis in Glomerular Mesangial Cells. ***Biochemical and Biophysical Research Communications*** 240(2): 496-501, 1997

Jackson DG: Human Leucocyte Heparan Sulphate Proteoglycans and Their Roles in Inflammation. ***Biochemical Society Transactions*** 25(1): 220-224, 1997

Jalkanen M, Rapraeger A, Saunders S and Bernfield M: Cell Surface Proteoglycan of Mouse Mammary Epithelial Cells Is Shed by Cleavage of Its Matrix-Binding Ectodomain From Its Membrane-Associated Domain. ***Journal of Cell Biology*** 105(6 Pt 2): 3087-3096, 1987

Jaques LB, Wice SM and Hiebert LM: Determination of Absolute Amounts of Heparin and of Dextran Sulfate in Plasma in Microgram Quantities. ***The Journal of Laboratory and Clinical Medicine*** 115(4): 422-432, 1990

Jarrett RJ, McCartney P and Keen H: The Bedford Survey: Ten Year Mortality Rates in Newly Diagnosed Diabetics, Borderline Diabetics and Normoglycaemic Controls and Risk Indices for Coronary Heart Disease in Borderline Diabetics. ***Diabetologia*** 22(2): 79-84, 1982

Jensen T: Pathogenesis of Diabetic Vascular Disease: Evidence for the Role of Reduced Heparan Sulfate Proteoglycan. ***Diabetes*** 46: Suppl 2 S98-100, 1997

Jiang P, Kumar A, Parrillo JE, Dempsey LA, Platt JL, Prinz RA and Xu X: Cloning and Characterization of the Human Heparanase-1 (HPR1) Gene Promoter: Role of GA-Binding Protein and Sp1 in Regulating HPR1 Basal Promoter Activity. ***Journal of Biological Chemistry*** 277(11): 8989-8998, 2002

Johansson S and Hook M: Substrate Adhesion of Rat Hepatocytes: on the Mechanism of Attachment to Fibronectin. ***Journal of Cell Biology*** 98(3): 810-817, 1984

Jokel R and Colwell J.A.: Arterial Thrombosis and Atherosclerosis in Diabetes. ***Diabetes Reviews*** 5: 316-330, 1997

Joris I, Zand T, Nunnari JJ, Krolikowski FJ and Majno G: Studies on the Pathogenesis of Atherosclerosis. I. Adhesion and Emigration of Mononuclear

Cells in the Aorta of Hypercholesterolemic Rats. ***American Journal of Pathology*** 113(3): 341-358, 1983

Juhasz P and Biemann K: Mass Spectrometric Molecular-Weight Determination of Highly Acidic Compounds of Biological Significance Via Their Complexes With Basic Polypeptides. ***Proceedings of the National Academy of Sciences of the United States of America*** 91(10): 4333-4337, 1994

Kaiser N, Sasson S, Feener EP, Boukobza-Vardi N, Higashi S, Moller DE, Davidheiser S, Przybylski RJ and King GL: Differential Regulation of Glucose Transport and Transporters by Glucose in Vascular Endothelial and Smooth Muscle Cells. ***Diabetes*** 42(1): 80-89, 1993

Kaji T, Yamada A, Miyajima S, Yamamoto C, Fujiwara Y, Wight TN and Kinsella MG: Cell Density-Dependent Regulation of Proteoglycan Synthesis by Transforming Growth Factor-Beta(1) in Cultured Bovine Aortic Endothelial Cells. ***Journal of Biological Chemistry*** 275(2): 1463-1470, 2000

Kakkar AK, Levine MN, Kadziola Z, Lemoine NR, Low V, Patel HK, Rustin G, Thomas M, Quigley M and Williamson RC: Low Molecular Weight Heparin, Therapy With Dalteparin, and Survival in Advanced Cancer: the Fragmin Advanced Malignancy Outcome Study (FAMOUS). ***Journal of Clinical Oncology*** 22(10): 1944-1948, 2004

Kakuta Y, Sueyoshi T, Negishi M and Pedersen LC: Crystal Structure of the Sulfotransferase Domain of Human Heparan Sulfate N-Deacetylase/ N-Sulfotransferase 1. ***Journal of Biological Chemistry*** 274(16): 10673-10676, 1999

Kamata K, Miyata N, Abiru T and Kasuya Y: Functional Changes in Vascular Smooth Muscle and Endothelium of Arteries During Diabetes Mellitus. ***Life Sciences*** 50(19): 1379-1387, 1992

Kanwar YS, Linker A and Farquhar MG: Increased Permeability of the Glomerular Basement Membrane to Ferritin After Removal of Glycosaminoglycans (Heparan Sulfate) by Enzyme Digestion. ***Journal of Cell Biology*** 86(2): 688-693, 1980

Karlsson K and Marklund SL: Heparin-Induced Release of Extracellular Superoxide Dismutase to Human Blood Plasma. ***Biochemical Journal*** 242(1): 55-59, 1987

Kasinath BS: Effect of Insulin on High-Glucose Medium-Induced Changes in Rat Glomerular Epithelial Cell Metabolism of Glycoconjugates. ***Archives of Biochemistry and Biophysics*** 318(2): 286-294, 1995

Kasinath BS, Grellier P, Choudhury GG and Abboud SL: Regulation of Basement Membrane Heparan Sulfate Proteoglycan, Perlecan, Gene Expression in Glomerular Epithelial Cells by High Glucose Medium. ***Journal of Cellular Physiology*** 167(1): 131-136, 1996

Kassab E, McFarlane SI and Sower JR: Vascular Complications in Diabetes and Their Prevention. ***Vascular Medicine*** 6(4): 249-255, 2001

Kato M, Wang H, Kainulainen V, Fitzgerald ML, Ledbetter S, Ornitz DM and Bernfield M: Physiological Degradation Converts the Soluble Syndecan-1 Ectodomain From an Inhibitor to a Potent Activator of FGF-2. ***Nature Medicine*** 4(6): 691-697, 1998

Katsura T, Gustafson CE, Ausiello DA and Brown D: Protein Kinase A Phosphorylation Is Involved in Regulated Exocytosis of Aquaporin-2 in Transfected LLC-PK1 Cells. ***American Journal of Physiology*** 272(6 Pt 2): F817-F822, 1997

Katz A, Van Dijk DJ, Aingorn H, Erman A, Davies M, Darmon D, Hurvitz H and Vlodavsky I: Involvement of Human Heparanase in the Pathogenesis of Diabetic Nephropathy. ***Israeli Medical Association*** 4(11): 996-1002, 2002

Kefalides NA: Basement Membrane Research in Diabetes Mellitus. ***Collagen Related Research*** 1(3): 295-299, 1981

Kido Y, Nakae J and Accili D: Clinical Review 125: The Insulin Receptor and Its Cellular Targets. ***The Journal of Clinical Endocrinology & Metabolism*** 86(3): 972-979, 2001

Kim CW, Goldberger OA, Gallo RL and Bernfield M: Members of the Syndecan Family of Heparan Sulfate Proteoglycans Are Expressed in Distinct Cell-, Tissue-, and Development-Specific Patterns. ***Molcualr Biology of the Cell*** 5(7): 797-805, 1994

Kim JA, Montagnani M, Koh KK and Quon MJ: Reciprocal Relationships Between Insulin Resistance and Endothelial Dysfunction: Molecular and Pathophysiological Mechanisms. ***Circulation*** 113(15): 1888-1904, 2006

Kinoshita A and Sugahara K: Microanalysis of Glycosaminoglycan-Derived Oligosaccharides Labeled With a Fluorophore 2-Aminobenzamide by High-Performance Liquid Chromatography: Application to Disaccharide Composition Analysis and Exosequencing of Oligosaccharides. ***Analytical Biochemistry*** 269(2): 367-378, 1999

Kitamura M, Maruyama N, Mitarai T, Nagasawa R, Yokoo T and Sakai O: Heparin Selectively Inhibits Gene Expression of Matrix Metalloproteinase Transin in Cultured Mesangial Cells. ***Biochemical and Biophysical Research Communications*** 203(2): 1333-1338, 1994

Kjellen L, Bielefeld D and Hook M: Reduced Sulfation of Liver Heparan Sulfate in Experimentally Diabetic Rats. ***Diabetes*** 32(4): 337-342, 1983

Kjellen L and Lindahl U: Proteoglycans: Structures and Interactions. ***Annual Review of Biochemistry*** 60: 443-475, 1991

Klein DJ, Brown DM, Oegema TR, Brenchley PE, Anderson JC, Dickinson MA, Horigan EA and Hassell JR: Glomerular Basement Membrane Proteoglycans Are Derived From a Large Precursor. ***Journal of Cell Biology*** 106(3): 963-970, 1988

Klein DJ, Cohen RM and Rymaszewski Z: Proteoglycan Synthesis by Bovine Myocardial Endothelial Cells Is Increased by Long-Term Exposure to High Concentrations of Glucose. ***Journal of Cellular Physiology*** 165(3): 493-502, 1995

Ko CW, Bhandari B, Yee J, Terhune WC, Maldonado R and Kasinath BS: Cyclic AMP Regulates Basement Membrane Heparan Sulfate Proteoglycan, Perlecan, Metabolism in Rat Glomerular Epithelial Cells. ***Molecular and Cellular Biochemistry*** 162(1): 65-73, 1996

Koda-kimble, M. A. and Carlisle, B. A. In: Diabetes mellitus. (6) (Young L.Y., Koda-kimble M.A., Kradjan W.A., and Guglielmo B.J. eds.) Applied therapeutics: the clinical use of drugs. Mack publishing Co., Vancouver, WA, Pp. 1-58, 1995

Kohno M, Yasunari K, Murakawa K, Yokokawa K, Horio T, Fukui T and Takeda T: Plasma Immunoreactive Endothelin in Essential Hypertension. ***The American Journal of Medicine*** 88(6): 614-618, 1990

Kojima T, Shworak NW and Rosenberg RD: Molecular Cloning and Expression of Two Distinct CDNA-Encoding Heparan Sulfate Proteoglycan Core Proteins

From a Rat Endothelial Cell Line. ***Journal of Biological Chemistry*** 267(7): 4870-4877, 1992

Kokenyesi R and Bernfield M: Core Protein Structure and Sequence Determine the Site and Presence of Heparan Sulfate and Chondroitin Sulfate on Syndecan-1. ***Journal of Biological Chemistry*** 269(16): 12304-12309, 1994

Koliopanos A, Friess H, Kleeff J, Shi X, Liao Q, Pecker I, Vlodavsky I, Zimmermann A and Buchler MW: Heparanase Expression in Primary and Metastatic Pancreatic Cancer. ***Cancer Research*** 61(12): 4655-4659, 2001

Koya D and King GL: Protein Kinase C Activation and the Development of Diabetic Complications. ***Diabetes*** 47(6): 859-866, 1998

Kreuger J, Salmivirta M, Sturiale L, Gimenez-Gallego G and Lindahl U: Sequence Analysis of Heparan Sulfate Epitopes With Graded Affinities for Fibroblast Growth Factors 1 and 2. ***Journal of Biological Chemistry*** 276(33): 30744-30752, 2001

Kusche-Gullberg M, Eriksson I, Pikas DS and Kjellen L: Identification and Expression in Mouse of Two Heparan Sulfate Glucosaminyl N-Deacetylase/N-Sulfotransferase Genes. ***Journal of Biological Chemistry*** 273(19): 11902-11907, 1998

Laghi PF, Pasqui AL, Ceccatelli L, Capecchi PL, Orrico A and Di Perri T: Heparin Inhibition of Polymorphonuclear Leukocyte Activation *in vitro*. A Possible Pharmacological Approach to Granulocyte-Mediated Vascular Damage. ***Thrombosis Research*** 35(5): 527-537, 1984

Lander AD and Selleck SB: The Elusive Functions of Proteoglycans: *in vivo* Veritas. ***Journal of Cell Biology*** 148(2): 227-232, 2000

Lane AD and Lindahl U: Heparin, Chemical and Biological Properties, Clinical Applications. CRC Press Inc., Boca Raton, FL, 1989

Larrain J, Cizmeci-Smith G, Troncoso V, Stahl RC, Carey DJ and Brandan E: Syndecan-1 Expression Is Down-Regulated During Myoblast Terminal Differentiation. Modulation by Growth Factors and Retinoic Acid. ***Journal of Biological Chemistry*** 272(29): 18418-18424, 1997

Lebakken CS and Rapraeger AC: Syndecan-1 Mediates Cell Spreading in Transfected Human Lymphoblastoid (Raji) Cells. ***Journal of Cell Biology*** 132(6): 1209-1221, 1996

Ledbetter SR, Tyree B, Hassell JR and Horigan EA: Identification of the Precursor Protein to Basement Membrane Heparan Sulfate Proteoglycans. ***Journal of Biological Chemistry*** 260(13): 8106-8113, 1985

Lee TS, MacGregor LC, Fluharty SJ and King GL: Differential Regulation of Protein Kinase C and (Na,K)-Adenosine Triphosphatase Activities by Elevated Glucose Levels in Retinal Capillary Endothelial Cells. ***Journal of Clinical Investigation*** 83(1): 90-94, 1989a

Lee TS, Saltsman KA, Ohashi H and King GL: Activation of Protein Kinase C by Elevation of Glucose Concentration: Proposal for a Mechanism in the Development of Diabetic Vascular Complications. ***Proceedings of the National Academy of Sciences of the United States of America*** 86(13): 5141-5145, 1989b

Leggett RW, Armstrong SA, Barry D and Mueller CR: Sp1 Is Phosphorylated and Its DNA Binding Activity Down-Regulated Upon Terminal Differentiation of the Liver. ***Journal of Biological Chemistry*** 270(43): 25879-25884, 1995

Levidiotis V, Freeman C, Tikellis C, Cooper ME and Power DA: Heparanase Is Involved in the Pathogenesis of Proteinuria As a Result of Glomerulonephritis. ***Journal of the American Society of Nephrology*** 15(1): 68-78, 2004

Levidiotis V, Kanellis J, Ierino FL and Power DA: Increased Expression of Heparanase in Puromycin Aminonucleoside Nephrosis. ***Kidney International*** 60(4): 1287-1296, 2001

Levy P, Picard J and Bruel A: Evidence for Diabetes-Induced Alterations in the Sulfation of Heparin Sulfate Intestinal Epithelial Cells. ***Life Sciences*** 35(26): 2613-2620, 1984

Ley K, Cerrito M and Arfors KE: Sulfated Polysaccharides Inhibit Leukocyte Rolling in Rabbit Mesentery Venules. ***American Journal of Physiology*** 260(5 Pt 2): H1667-H1673, 1991

Li JP, Gong F, El Darwish K, Jalkanen M and Lindahl U: Characterization of the D-Glucuronyl C5-Epimerase Involved in the Biosynthesis of Heparin and Heparan Sulfate. ***Journal of Biological Chemistry*** 276(23): 20069-20077, 2001

Li Q, Park PW, Wilson CL and Parks WC: Matrilysin Shedding of Syndecan-1 Regulates Chemokine Mobilization and Transepithelial Efflux of Neutrophils in Acute Lung Injury. ***Cell*** 111(5): 635-646, 2002

Lindahl, U. In: Biosynthesis of heparin and related polysaccharides. (Lane, D. A. and Lindahl, U eds.) Heparin, chemical and biological properties, clinical applications. CRC Press, Inc., Boca Raton, FL, Pp. 159-189, 1989

Lindahl U, Thunberg L, Backstrom G, Riesenfeld J, Nordling K and Bjork I: Extension and Structural Variability of the Antithrombin-Binding Sequence in Heparin. ***Journal of Biological Chemistry*** 259(20): 12368-12376, 1984

Linhardt, R. J. and Toida, T. In: Carbohydrates as Drugs. (Witczak, Z. B. and Nieforth, K. A. eds.) Heparin analogs-development and applications. Marcel Dekker, New York, NY, Pp. 277-341, 1997

Linhardt, R. J. and Toida, T. In: Characterization of glycosaminoglycans by capillary electrophoresis. (Honda S. and Thibaull, P. eds.) Capillary electrophoresis of carbohydrates. Humana Press, New York, NY, Pp. 131-147, 1999

Liu J, Shriver Z, Blaiklock P, Yoshida K, Sasisekharan R and Rosenberg RD: Heparan Sulfate D-Glucosaminyl 3-O-Sulfotransferase-3A Sulfates N-Unsubstituted Glucosamine Residues. ***Journal of Biological Chemistry*** 274(53): 38155-38162, 1999a

Liu J, Shworak NW, Sinay P, Schwartz JJ, Zhang L, Fritze LM and Rosenberg RD: Expression of Heparan Sulfate D-Glucosaminyl 3-O-Sulfotransferase Isoforms Reveals Novel Substrate Specificities. ***Journal of Biological Chemistry*** 274(8): 5185-5192, 1999b

Livak KJ and Schmittgen TD: Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T)) Method. ***Methods*** 25(4): 402-408, 2001

Lu WC, Liu YN, Kang BB and Chen JH: Trans-Activation of Heparanase Promoter by ETS Transcription Factors. ***Oncogene*** 22(6): 919-923, 2003

Lupu F, Bergonzelli GE, Heim DA, Cousin E, Genton CY, Bachmann F and Kruithof EK: Localization and Production of Plasminogen Activator Inhibitor-1 in Human Healthy and Atherosclerotic Arteries. ***Arteriosclerosis, Thrombosis, and Vascular Biology.*** 13(7): 1090-1100, 1993

Malmberg K, Yusuf S, Gerstein HC, Brown J, Zhao F, Hunt D, Piegas L, Calvin J, Keltai M and Budaj A: Impact of Diabetes on Long-Term Prognosis in Patients With Unstable Angina and Non-Q-Wave Myocardial Infarction: Results of the OASIS (Organization to Assess Strategies for Ischemic Syndromes) Registry. ***Circulation*** 102(9): 1014-1019, 2000

Mamula PW, McDonald AR, Brunetti A, Okabayashi Y, Wong KY, Maddux BA, Logsdon C and Goldfine ID: Regulating Insulin-Receptor-Gene Expression by Differentiation and Hormones. ***Diabetes Care*** 13(3): 288-301, 1990

Mandal AK, Lyden TW, Fazel A, Saklayen MG, Mehrotra B, Mehling B, Taylor CA, Yokokawa K and Colvin RV: Heparin-Induced Endothelial Cell Cytoskeletal Reorganization: a Potential Mechanism for Vascular Relaxation. ***Kidney International*** 48(5): 1508-1516, 1995

Mandal AK, Puchalski JT, Lemley-Gillespie S, Taylor CA and Kohno M: Effect of Insulin and Heparin on Glucose-Induced Vascular Damage in Cell Culture. ***Kidney International*** 57(6): 2492-2501, 2000

Marcum JA, Atha DH, Fritze LM, Nawroth P, Stern D and Rosenberg RD: Cloned Bovine Aortic Endothelial Cells Synthesize Anticoagulantly Active Heparan Sulfate Proteoglycan. ***Journal of Biological Chemistry*** 261(16): 7507-7517, 1986

Marynen P, Zhang J, Cassiman JJ, Van den BH and David G: Partial Primary Structure of the 48- and 90-Kilodalton Core Proteins of Cell Surface-Associated Heparan Sulfate Proteoglycans of Lung Fibroblasts. Prediction of an Integral Membrane Domain and Evidence for Multiple Distinct Core Proteins at the Cell Surface of Human Lung Fibroblasts. ***Journal of Biological Chemistry*** 264(12): 7017-7024, 1989

Mather K, Anderson TJ and Verma S: Insulin Action in the Vasculature: Physiology and Pathophysiology. ***Journal of Vascular Research*** 38(5): 415-422, 2001a

Mather KJ, Verma S and Anderson TJ: Improved Endothelial Function With Metformin in Type 2 Diabetes Mellitus. ***Journal of the American College of Cardiology*** 37(5): 1344-1350, 2001b

Maxhimer JB, Somenek M, Rao G, Pesce CE, Baldwin D, Jr., Gattuso P, Schwartz MM, Lewis EJ, Prinz RA and Xu X: Heparanase-1 Gene Expression and Regulation by High Glucose in Renal Epithelial Cells: a Potential Role in the Pathogenesis of Proteinuria in Diabetic Patients. ***Diabetes*** 54(7): 2172-2178, 2005

McNeil PL, Muthukrishnan L, Warder E and D'Amore PA: Growth Factors Are Released by Mechanically Wounded Endothelial Cells. ***Journal of Cell Biology*** 109(2): 811-822, 1989

Meglasson MD and Matschinsky FM: Pancreatic Islet Glucose Metabolism and Regulation of Insulin Secretion. ***Diabetes/Metabolism Research and Reviews*** 2(3-4): 163-214, 1986

Merry CL, Lyon M, Deakin JA, Hopwood JJ and Gallagher JT: Highly Sensitive Sequencing of the Sulfated Domains of Heparan Sulfate. ***Journal of Biological Chemistry*** 274(26): 18455-18462, 1999

Miller WL, Redfield MM and Burnett JC, Jr.: Integrated Cardiac, Renal, and Endocrine Actions of Endothelin. ***Journal of Clinical Investigation*** 83(1): 317-320, 1989

Milstien S and Katusic Z: Oxidation of Tetrahydrobiopterin by Peroxynitrite: Implications for Vascular Endothelial Function. ***Biochemical and Biophysical Research Communications*** 263(3): 681-684, 1999

Miralem T, Wang A, Whiteside CI and Templeton DM: Heparin Inhibits Mitogen-Activated Protein Kinase-Dependent and -Independent C-Fos Induction in Mesangial Cells. ***Journal of Biological Chemistry*** 271(29): 17100-17106, 1996

Mizushige K, Yao L, Noma T, Kiyomoto H, Yu Y, Hosomi N, Ohmori K and Matsuo H: Alteration in Left Ventricular Diastolic Filling and Accumulation of Myocardial Collagen at Insulin-Resistant Prediabetic Stage of a Type II Diabetic Rat Model. ***Circulation*** 101(8): 899-907, 2000

Mogensen CE, Schmitz A and Christensen CK: Comparative Renal Pathophysiology Relevant to IDDM and NIDDM Patients. ***Diabetes/Metabolism Research and Reviews*** 4(5): 453-483, 1988

Mollace V, Salvemini D, Anggard E and Vane J: Nitric Oxide From Vascular Smooth Muscle Cells: Regulation of Platelet Reactivity and Smooth Muscle Cell Guanylate Cyclase. ***British Journal of Pharmacology*** 104(3): 633-638, 1991

Moncada S, Palmer RM and Higgs EA: The Discovery of Nitric Oxide As the Endogenous Nitrovasodilator. ***Hypertension*** 12(4): 365-372, 1988

Mongiat M, Taylor K, Otto J, Aho S, Uitto J, Whitelock JM and Iozzo RV: The Protein Core of the Proteoglycan Perlecan Binds Specifically to Fibroblast Growth Factor-7. ***Journal of Biological Chemistry*** 275(10): 7095-7100, 2000

Monnier, V. M. and Cerami, A. In: The malliard reaction in foods and nutrition. (Waller G.R. and Feathers M.S. eds.) The American chemical society symposium series. 431-439, 1983

Moran A, Brown DM, Kim Y and Klein DJ: Effects of IGF-I and Glucose on Protein and Proteoglycan Synthesis by Human Fetal Mesangial Cells in Culture. ***Diabetes*** 40(10): 1346-1354, 1991

Murao K, Wada Y, Nakamura T, Taylor AH, Mooradian AD and Wong NC: Effects of Glucose and Insulin on Rat Apolipoprotein A-I Gene Expression. ***Journal of Biological Chemistry*** 273(30): 18959-18965, 1998

Murdoch AD, Dodge GR, Cohen I, Tuan RS and Iozzo RV: Primary Structure of the Human Heparan Sulfate Proteoglycan From Basement Membrane (HSPG2/Perlecan). A Chimeric Molecule With Multiple Domains Homologous to the Low Density Lipoprotein Receptor, Laminin, Neural Cell Adhesion Molecules, and Epidermal Growth Factor. ***Journal of Biological Chemistry*** 267(12): 8544-8557, 1992

Mykkanen L, Laakso M, Uusitupa M and Pyorala K: Prevalence of Diabetes and Impaired Glucose Tolerance in Elderly Subjects and Their Association With Obesity and Family History of Diabetes. ***Diabetes Care*** 13(11): 1099-1105, 1990

Nader HB, Buonassisi V, Colburn P and Dietrich CP: Heparin Stimulates the Synthesis and Modifies the Sulfation Pattern of Heparan Sulfate Proteoglycan From Endothelial Cells. ***Journal of Cellular Physiology*** 140(2): 305-310, 1989

Nader HB, Toma L, Pinhal MA, Buonassisi V, Colburn P and Dietrich CP: Effect of Heparin and Dextran Sulfate on the Synthesis and Structure of Heparan Sulfate From Cultured Endothelial Cells. ***Seminars in Thrombosis and Hemostasis*** 17 Suppl 1 47-56, 1991

Nakajima M, Irimura T and Nicolson GL: A Solid-Phase Substrate of Heparanase: Its Application to Assay of Human Melanoma for Heparan Sulfate Degradative Activity. ***Analytical Biochemistry*** 157(1): 162-171, 1986

Nakajima M, Irimura T and Nicolson GL: Heparanases and Tumor Metastasis. ***Journal of Cellular Biochemistry*** 36(2): 157-167, 1988

Nieuwdorp M, Mooij HL, Kroon J, Atasever B, Spaan JA, Ince C, Holleman F, Diamant M, Heine RJ, Hoekstra JB, Kastelein JJ, Stroes ES and Vink H: Endothelial Glycocalyx Damage Coincides With Microalbuminuria in Type 1 Diabetes. ***Diabetes*** 55(4): 1127-1132, 2006a

Nieuwdorp M, van Haeften TW, Gouverneur MC, Mooij HL, van Lieshout MH, Levi M, Meijers JC, Holleman F, Hoekstra JB, Vink H, Kastelein JJ and Stroes ES: Loss of Endothelial Glycocalyx During Acute Hyperglycemia Coincides With Endothelial Dysfunction and Coagulation Activation *in vivo*. ***Diabetes*** 55(2): 480-486, 2006b

Nishio Y, Warren CE, Buczek-Thomas JA, Rulfs J, Koya D, Aiello LP, Feener EP, Miller TB, Jr., Dennis JW and King GL: Identification and Characterization of a Gene Regulating Enzymatic Glycosylation Which Is Induced by Diabetes and Hyperglycemia Specifically in Rat Cardiac Tissue. ***Journal of Clinical Investigation*** 96(4): 1759-1767, 1995

Noonan DM, Fulle A, Valente P, Cai S, Horigan E, Sasaki M, Yamada Y and Hassell JR: The Complete Sequence of Perlecan, a Basement Membrane Heparan Sulfate Proteoglycan, Reveals Extensive Similarity With Laminin A Chain, Low Density Lipoprotein-Receptor, and the Neural Cell Adhesion Molecule. ***Journal of Biological Chemistry*** 266(34): 22939-22947, 1991

Nugent MA and Iozzo RV: Fibroblast Growth Factor-2. ***The International Journal of Biochemistry & Cell Biology*** 32(2): 115-120, 2000

Oh P, Li Y, Yu J, Durr E, Krasinska KM, Carver LA, Testa JE and Schnitzer JE: Subtractive Proteomic Mapping of the Endothelial Surface in Lung and Solid Tumours for Tissue-Specific Therapy. ***Nature*** 429(6992): 629-635, 2004

Okuda S, Languino LR, Ruoslahti E and Border WA: Elevated Expression of Transforming Growth Factor-Beta and Proteoglycan Production in Experimental Glomerulonephritis. Possible Role in Expansion of the Mesangial Extracellular Matrix. ***Journal of Clinical Investigation*** 86(2): 453-462, 1990

Olgemoller B, Schwaabe S, Gerbitz KD and Schleicher ED: Elevated Glucose Decreases the Content of a Basement Membrane Associated Heparan Sulphate Proteoglycan in Proliferating Cultured Porcine Mesangial Cells. ***Diabetologia*** 35(2): 183-186, 1992

Olsen BR: Life Without Perlecan Has Its Problems. ***Journal of Cell Biology*** 147(5): 909-912, 1999

Osterby R, Parving HH, Hommel E, Jorgensen HE and Lokkegaard H: Glomerular Structure and Function in Diabetic Nephropathy. Early to Advanced Stages. ***Diabetes*** 39(9): 1057-1063, 1990

Ott VL and Rapraeger AC: Tyrosine Phosphorylation of Syndecan-1 and -4 Cytoplasmic Domains in Adherent B82 Fibroblasts. ***Journal of Biological Chemistry*** 273(52): 35291-35298, 1998

Paka L, Kako Y, Obunike JC and Pillarisetti S: Apolipoprotein E Containing High Density Lipoprotein Stimulates Endothelial Production of Heparan Sulfate Rich in Biologically Active Heparin-Like Domains. A Potential Mechanism for the Anti-Atherogenic Actions of Vascular Apolipoprotein e. ***Journal of Biological Chemistry*** 274(8): 4816-4823, 1999

Parish CR, Hindmarsh EJ, Bartlett MR, Staykova MA, Cowden WB and Willenborg DO: Treatment of Central Nervous System Inflammation With Inhibitors of Basement Membrane Degradation. ***Immunology & Cell Biology*** 76(1): 104-113, 1998

Parthasarathy N and Spiro RG: Effect of Diabetes on the Glycosaminoglycan Component of the Human Glomerular Basement Membrane. ***Diabetes*** 31(8 Pt 1): 738-741, 1982

Pearson JD: Endothelial Cell Biology. ***Radiology*** 179(1): 9-14, 1991

Pekala P, Marlow M, Heuvelman D and Connolly D: Regulation of Hexose Transport in Aortic Endothelial Cells by Vascular Permeability Factor and Tumor Necrosis Factor-Alpha, but Not by Insulin. ***Journal of Biological Chemistry*** 265(30): 18051-18054, 1990

Perrimon N and Bernfield M: Specificities of Heparan Sulphate Proteoglycans in Developmental Processes. ***Nature*** 404(6779): 725-728, 2000

Peterson MW, Stone P and Shasby DM: Cationic Neutrophil Proteins Increase Transendothelial Albumin Movement. ***Journal of Applied Physiology*** 62(4): 1521-1530, 1987

Petitou M, Duchaussoy P, Lederman I, Choay J, Sinay P, Jacquinet JC and Torri G: Synthesis of Heparin Fragments. A Chemical Synthesis of the Pentasaccharide O-(2-Deoxy-2-Sulfamido-6-O-Sulfo-Alpha-D-Glucopyranosyl)-(1-4)-O-(Beta-D-Glucopyranosyluronic Acid)-(1-4)-O-(2-Deoxy-2-Sulfamido-3,6-Di-O-Sulfo-Alpha-D-Glu Copyranosyl)-(1-4)-O-(2-O-Sulfo-Alpha-L-Idopyranosyluronic Acid)-(1-4)-2-Deoxy-2-Sulfamido-6-O-Sulfo-D-Glucopyranose Decasodium Salt, a Heparin Fragment Having High Affinity for Antithrombin III. ***Carbohydrate Research*** 147(2): 221-236, 1986

Philipsen S and Suske G: A Tale of Three Fingers: the Family of Mammalian Sp/XKLF Transcription Factors. ***Nucleic Acids Research*** 27(15): 2991-3000, 1999

Piedagnel R, Prie D, Cassingena R, Ronco PM and Lelongt B: SV40 Large-T Oncogene Inhibits Transcription of Perlecan-Related Proteoglycans but Stimulates Hyaluronan Synthesis in a Temperature-Sensitive Renal-Tubule Principal Cell Line. ***Journal of Biological Chemistry*** 269(26): 17469-17476, 1994

Pirart J: [Diabetes Mellitus and Its Degenerative Complications: a Prospective Study of 4,400 Patients Observed Between 1947 and 1973 (2nd Part) (Author's Transl)]. ***Diabetes & Metabolism*** 3(2): 97-107; 3(3): 173-182; 3(4): 245-256, 1977

Pope RM, Raska CS, Thorp SC and Liu J: Analysis of Heparan Sulfate Oligosaccharides by Nano-Electrospray Ionization Mass Spectrometry. ***Glycobiology*** 11(6): 505-513, 2001

Pugliese G, Tilton RG, Speedy A, Chang K, Province MA, Kilo C and Williamson JR: Vascular Filtration Function in Galactose-Fed Versus Diabetic Rats: the Role of Polyol Pathway Activity. ***Metabolism*** 39(7): 690-697, 1990

Pukac LA, Ottlinger ME and Karnovsky MJ: Heparin Suppresses Specific Second Messenger Pathways for Protooncogene Expression in Rat Vascular Smooth Muscle Cells. ***Journal of Biological Chemistry*** 267(6): 3707-3711, 1992

Raats CJ, van den BJ and Berden JH: Glomerular Heparan Sulfate Alterations: Mechanisms and Relevance for Proteinuria. ***Kidney International*** 57(2): 385-400, 2000

Radomski MW, Palmer RM and Moncada S: The Anti-Aggregating Properties of Vascular Endothelium: Interactions Between Prostacyclin and Nitric Oxide. ***British Journal of Pharmacology*** 92(3): 639-646, 1987

Rapraeger A, Jalkanen M, Endo E, Koda J and Bernfield M: The Cell Surface Proteoglycan From Mouse Mammary Epithelial Cells Bears Chondroitin Sulfate and Heparan Sulfate Glycosaminoglycans. ***Journal of Biological Chemistry*** 260(20): 11046-11052, 1985

Rapraeger AC and Ott VL: Molecular Interactions of the Syndecan Core Proteins. ***Current Opinion in Cell Biology*** 10(5): 620-628, 1998

Rasch R: Studies on the Prevention of Glomerulopathy in Diabetic Rats. ***Acta Endocrinologica.Supplement (Copenhagen)*** 242 43-44, 1981

Ratner L, Thielan B and Collins T: Sequences of the 5' Portion of the Human C-Sis Gene: Characterization of the Transcriptional Promoter and Regulation of Expression of the Protein Product by 5' Untranslated MRNA Sequences. ***Nucleic Acids Research*** 15(15): 6017-6036, 1987

Reiland J, Sanderson RD, Waguespack M, Barker SA, Long R, Carson DD and Marchetti D: Heparanase Degrades Syndecan-1 and Perlecan Heparan Sulfate: Functional Implications for Tumor Cell Invasion. ***Journal of Biological Chemistry*** 279(9): 8047-8055, 2004

Reizes O, Goldberger O, Smith AC, Xu Z, Bernfield M and Bickel PE: Insulin Promotes Shedding of Syndecan Ectodomains From 3T3-L1 Adipocytes: a Proposed Mechanism for Stabilization of Extracellular Lipoprotein Lipase. ***Biochemistry*** 45(18): 5703-5711, 2006

Rhomberg AJ, Ernst S, Sasisekharan R and Biemann K: Mass Spectrometric and Capillary Electrophoretic Investigation of the Enzymatic Degradation of Heparin-Like Glycosaminoglycans. ***Proceedings of the National Academy of Sciences of the United States of America*** 95(8): 4176-4181, 1998a

Rhomberg AJ, Shriver Z, Biemann K and Sasisekharan R: Mass Spectrometric Evidence for the Enzymatic Mechanism of the Depolymerization of Heparin-Like Glycosaminoglycans by Heparinase II. ***Proceedings of the National Academy of Sciences of the United States of America*** 95(21): 12232-12237, 1998b

Richardson JV, Wright CB and Hiratzka LF: The Role of Endothelium in the Patency of Small Venous Substitutes. ***Journal of Surgical Research*** 28(6): 556-562, 1980

Robinson HC, Horner AA, Hook M, Ogren S and Lindahl U: A Proteoglycan Form of Heparin and Its Degradation to Single-Chain Molecules. ***Journal of Biological Chemistry*** 253(19): 6687-6693, 1978

Robison WG, Jr., Nagata M, Laver N, Hohman TC and Kinoshita JH: Diabetic-Like Retinopathy in Rats Prevented With an Aldose Reductase Inhibitor. ***Investigative Ophthalmology & Visual Science*** 30(11): 2285-2292, 1989

Rohrbach DH, Wagner CW, Star VL, Martin GR, Brown KS and Yoon JW: Reduced Synthesis of Basement Membrane Heparan Sulfate Proteoglycan in Streptozotocin-Induced Diabetic Mice. ***Journal of Biological Chemistry*** 258(19): 11672-11677, 1983

Rong J, Habuchi H, Kimata K, Lindahl U and Kusche-Gullberg M: Substrate Specificity of the Heparan Sulfate Hexuronic Acid 2-O-Sulfotransferase. ***Biochemistry*** 40(18): 5548-5555, 2001

Rops AL, van d, V, Lensen JF, Wijnhoven TJ, van den Heuvel LP, van Kuppevelt TH and Berden JH: Heparan Sulfate Proteoglycans in Glomerular Inflammation. ***Kidney International*** 65(3): 768-785, 2004

Rosenberg RD, Shworak NW, Liu J, Schwartz JJ and Zhang L: Heparan Sulfate Proteoglycans of the Cardiovascular System. Specific Structures Emerge but How Is Synthesis Regulated? ***Journal of Clinical Investigation*** 100(11 Suppl): S67-S75, 1997

Rosenzweig LJ and Kanwar YS: Removal of Sulfated (Heparan Sulfate) or Nonsulfated (Hyaluronic Acid) Glycosaminoglycans Results in Increased Permeability of the Glomerular Basement Membrane to 125I-Bovine Serum Albumin. ***Laboratory Investigation*** 47(2): 177-184, 1982

Rostand KS and Esko JD: Microbial Adherence to and Invasion Through Proteoglycans. ***Infection and Immunity*** 65(1): 1-8, 1997

Ruderman NB, Williamson JR and Brownlee M: Glucose and Diabetic Vascular Disease. ***FASEB Journal*** 6(11): 2905-2914, 1992

Saito Y, Kida H, Takeda S, Yoshimura M, Yokoyama H, Koshino Y and Hattori N: Mesangiolysis in Diabetic Glomeruli: Its Role in the Formation of Nodular Lesions. ***Kidney International*** 34(3): 389-396, 1988

Saksela O, Moscatelli D, Sommer A and Rifkin DB: Endothelial Cell-Derived Heparan Sulfate Binds Basic Fibroblast Growth Factor and Protects It From Proteolytic Degradation. ***Journal of Cell Biology*** 107(2): 743-751, 1988

Saku T and Furthmayr H: Characterization of the Major Heparan Sulfate Proteoglycan Secreted by Bovine Aortic Endothelial Cells in Culture. Homology to the Large Molecular Weight Molecule of Basement Membranes. ***Journal of Biological Chemistry*** 264(6): 3514-3523, 1989

Salazar G and Gonzalez A: Novel Mechanism for Regulation of Epidermal Growth Factor Receptor Endocytosis Revealed by Protein Kinase A Inhibition. ***Molcualr Biology of the Cell*** 13(5): 1677-1693, 2002

Salmivirta M and Jalkanen M: Syndecan Family of Cell Surface Proteoglycans: Developmentally Regulated Receptors for Extracellular Effector Molecules. ***Experientia*** 51(9-10): 863-872, 1995

Sanderson RD: Heparan Sulfate Proteoglycans in Invasion and Metastasis. ***Seminars in Cell and Developmental Biology*** 12(2): 89-98, 2001

Sanderson RD, Hinkes MT and Bernfield M: Syndecan-1, a Cell-Surface Proteoglycan, Changes in Size and Abundance When Keratinocytes Stratify. ***Journal of Investigative Dermatology*** 99(4): 390-396, 1992

Sasson S, Gorowits N, Joost HG, King GL, Cerasi E and Kaiser N: Regulation by Metformin of the Hexose Transport System in Vascular Endothelial and Smooth Muscle Cells. ***British Journal of Pharmacology*** 117(6): 1318-1324, 1996

Saunders S, Jalkanen M, O'Farrell S and Bernfield M: Molecular Cloning of Syndecan, an Integral Membrane Proteoglycan. ***Journal of Cell Biology*** 108(4): 1547-1556, 1989

Schachinger V, Britten MB and Zeiher AM: Prognostic Impact of Coronary Vasodilator Dysfunction on Adverse Long-Term Outcome of Coronary Heart Disease. ***Circulation*** 101(16): 1899-1906, 2000

Schmidt AM, Yan SD, Yan SF and Stern DM: The Biology of the Receptor for Advanced Glycation End Products and Its Ligands. ***Biochimica et Biophysica Acta*** 1498(2-3): 99-111, 2000

Scivittaro V, Ganz MB and Weiss MF: AGEs Induce Oxidative Stress and Activate Protein Kinase C-Beta(II) in Neonatal Mesangial Cells. ***The American Journal of Physiology - Renal Physiology*** 278(4): F676-F683, 2000

Segarini PR and Seyedin SM: The High Molecular Weight Receptor to Transforming Growth Factor-Beta Contains Glycosaminoglycan Chains. ***Journal of Biological Chemistry*** 263(17): 8366-8370, 1988

Sensi M, Tanzi P, Bruno MR, Pozzilli P, Mancuso M, Gambardella S and Di Mario U: Nonenzymic Glycation of Isolated Human Glomerular Basement Membrane Changes Its Physicochemical Characteristics and Binding Properties. ***Nephron*** 52(3): 222-226, 1989

Setter SM, Campbell RK and Cahoon CJ: Biochemical Pathways for Microvascular Complications of Diabetes Mellitus. ***The Annuls of Pharmacotherapy*** 37(12): 1858-1866, 2003

Shafat I, Zcharia E, Nisman B, Nadir Y, Nakhoul F, Vlodavsky I and Ilan N: An ELISA Method for the Detection and Quantification of Human Heparanase. ***Biochemical and Biophysical Research Communications*** 341(4): 958-963, 2006

Sharma B, Handler M, Eichstetter I, Whitelock JM, Nugent MA and Iozzo RV: Antisense Targeting of Perlecan Blocks Tumor Growth and Angiogenesis *in vivo*. ***Journal of Clinical Investigation*** 102(8): 1599-1608, 1998

Shichiri M, Hirata Y, Ando K, Emori T, Ohta K, Kimoto S, Ogura M, Inoue A and Marumo F: Plasma Endothelin Levels in Hypertension and Chronic Renal Failure. ***Hypertension*** 15(5): 493-496, 1990

Shih HM and Towle HC: Definition of the Carbohydrate Response Element of the Rat S14 Gene. Evidence for a Common Factor Required for Carbohydrate Regulation of Hepatic Genes. ***Journal of Biological Chemistry*** 267(19): 13222-13228, 1992

Shukla D, Liu J, Blaiklock P, Shworak NW, Bai X, Esko JD, Cohen GH, Eisenberg RJ, Rosenberg RD and Spear PG: A Novel Role for 3-O-Sulfated Heparan Sulfate in Herpes Simplex Virus 1 Entry. ***Cell*** 99(1): 13-22, 1999

Shworak NW, Shirakawa M, Mulligan RC and Rosenberg RD: Characterization of Ryudocan Glycosaminoglycan Acceptor Sites. ***Journal of Biological Chemistry*** 269(33): 21204-21214, 1994

Sibley E, Kastelic T, Kelly TJ and Lane MD: Characterization of the Mouse Insulin Receptor Gene Promoter. ***Proceedings of the National Academy of Sciences of the United States of America*** 86(24): 9732-9736, 1989

Simizu S, Ishida K, Wierzba MK, Sato TA and Osada H: Expression of Heparanase in Human Tumor Cell Lines and Human Head and Neck Tumors. ***Cancer Letters*** 193(1): 83-89, 2003

Singer II, Scott S, Kawka DW and Hassell JR: Extracellular Matrix Fibers Containing Fibronectin and Basement Membrane Heparan Sulfate Proteoglycan Coalign With Focal Contacts and Microfilament Bundles in Stationary Fibroblasts. ***Experimental Cell Research*** 173(2): 558-571, 1987

Smith SE, French MM, Julian J, Paria BC, Dey SK and Carson DD: Expression of Heparan Sulfate Proteoglycan (Perlecan) in the Mouse Blastocyst Is Regulated During Normal and Delayed Implantation. ***Developmental Biology*** 184(1): 38-47, 1997

Snustad DP, Simmons MJ and Jenkins JB. Principles of Genetics. John Wiley & Sons Inc. New York, 1997

Sowers JR: Insulin and Insulin-Like Growth Factor in Normal and Pathological Cardiovascular Physiology. ***Hypertension*** 29(3): 691-699, 1997

Steffes MW, Osterby R, Chavers B and Mauer SM: Mesangial Expansion As a Central Mechanism for Loss of Kidney Function in Diabetic Patients. ***Diabetes*** 38(9): 1077-1081, 1989

Stehouwer, C. In: von Willebrand factor, dysfunction of the vascular endothelium, and the development of renal and vascular complications in diabetes. (4) (Mogensen, C. E. eds.) The kidney and hypertension on diabetes mellitus. Massachusetts USA, Pp. 199-208, 1998

Stehouwer CD, Lambert J, Donker AJ and van Hinsbergh VW: Endothelial Dysfunction and Pathogenesis of Diabetic Angiopathy. ***Cardiovascular Research*** 34(1): 55-68, 1997

Steinberg HO, Chaker H, Leaming R, Johnson A, Brechtel G and Baron AD: Obesity/Insulin Resistance Is Associated With Endothelial Dysfunction. Implications for the Syndrome of Insulin Resistance. ***Journal of Clinical Investigation*** 97(11): 2601-2610, 1996

Stevens RL, Colombo M, Gonzales JJ, Hollander W and Schmid K: The Glycosaminoglycans of the Human Artery and Their Changes in Atherosclerosis. ***Journal of Clinical Investigation*** 58(2): 470-481, 1976

Stipp CS, Litwack ED and Lander AD: Cerebroglycan: an Integral Membrane Heparan Sulfate Proteoglycan That Is Unique to the Developing Nervous System and Expressed Specifically During Neuronal Differentiation. ***Journal of Cell Biology*** 124(1-2): 149-160, 1994

Subramanian SV, Fitzgerald ML and Bernfield M: Regulated Shedding of Syndecan-1 and -4 Ectodomains by Thrombin and Growth Factor Receptor Activation. ***Journal of Biological Chemistry*** 272(23): 14713-14720, 1997

Sugahara K and Kitagawa H: Recent Advances in the Study of the Biosynthesis and Functions of Sulfated Glycosaminoglycans. ***Current Opinion in Structural Biology*** 10(5): 518-527, 2000

Suwaidi JA, Hamasaki S, Higano ST, Nishimura RA, Holmes DR, Jr. and Lerman A: Long-Term Follow-Up of Patients With Mild Coronary Artery Disease and Endothelial Dysfunction. ***Circulation*** 101(9): 948-954, 2000

Svennevig K, Kolset SO and Bangstad HJ: Increased Syndecan-1 in Serum Is Related to Early Nephropathy in Type 1 Diabetes Mellitus Patients. ***Diabetologia*** 49(9): 2214-2216, 2006

Tahiliani AG and McNeill JH: Diabetes-Induced Abnormalities in the Myocardium. ***life Sciences*** 38(11): 959-974, 1986

Tamsma JT, van den BJ, Bruijn JA, Assmann KJ, Weening JJ, Berden JH, Wieslander J, Schrama E, Hermans J and Veerkamp JH: Expression of Glomerular Extracellular Matrix Components in Human Diabetic Nephropathy: Decrease of Heparan Sulphate in the Glomerular Basement Membrane. ***Diabetologia*** 37(3): 313-320, 1994

Teff KL, Mattes RD and Engelman K: Cephalic Phase Insulin Release in Normal Weight Males: Verification and Reliability. ***American Journal of Physiology*** 261(4 Pt 1): E430-E436, 1991

Templeton DM and Fan MY: Posttranscriptional Effects of Glucose on Proteoglycan Expression in Mesangial Cells. ***Metabolism*** 45(9): 1136-1146, 1996

Thompson KS and Towle HC: Localization of the Carbohydrate Response Element of the Rat L-Type Pyruvate Kinase Gene. ***Journal of Biological Chemistry*** 266(14): 8679-8682, 1991

Tilton RG, Chang K, Pugliese G, Eades DM, Province MA, Sherman WR, Kilo C and Williamson JR: Prevention of Hemodynamic and Vascular Albumin Filtration Changes in Diabetic Rats by Aldose Reductase Inhibitors. ***Diabetes*** 38(10): 1258-1270, 1989

Timpl R: Proteoglycans of Basement Membranes. ***Experientia*** 49(5): 417-428, 1993

Ting HH, Timimi FK, Boles KS, Creager SJ, Ganz P and Creager MA: Vitamin C Improves Endothelium-Dependent Vasodilation in Patients With Non-Insulin-

Dependent Diabetes Mellitus. ***Journal of Clinical Investigation*** 97(1): 22-28, 1996

Toyoshima M and Nakajima M: Human Heparanase. Purification, Characterization, Cloning, and Expression. ***Journal of Biological Chemistry*** 274(34): 24153-24160, 1999

Tran PK, Agardh HE, Tran-Lundmark K, Ekstrand J, Roy J, Henderson B, Gabrielsen A, Hansson GK, Swedenborg J, Paulsson-Berne G and Hedin U: Reduced Perlecan Expression and Accumulation in Human Carotid Atherosclerotic Lesions. ***Atherosclerosis*** 190(2): 264-270, 2007

Trial Research Group: The Effect of Intensive Treatment of Diabetes on the Development and Progression of Long-Term Complications in Insulin-Dependent Diabetes Mellitus. The Diabetes Control and Complications Trial Research Group. ***The New England Journal of Medicine*** 329(14): 977-986, 1993

Tsilibary EC, Charonis AS, Reger LA, Wohlhueter RM and Furcht LT: The Effect of Nonenzymatic Glucosylation on the Binding of the Main Noncollagenous NC1 Domain to Type IV Collagen. ***Journal of Biological Chemistry*** 263(9): 4302-4308, 1988

Tsuzuki S, Kojima T, Katsumi A, Yamazaki T, Sugiura I and Saito H: Molecular Cloning, Genomic Organization, Promoter Activity, and Tissue-Specific Expression of the Mouse Ryudocan Gene. ***Journal of Biochemistry*** 122(1): 17-24, 1997

Turnbull JE, Hopwood JJ and Gallagher JT: A Strategy for Rapid Sequencing of Heparan Sulfate and Heparin Saccharides. ***Proceedings of the National Academy of Sciences of the United States of America*** 96(6): 2698-2703, 1999

Uhlin-Hansen L and Yanagishita M: Differential Effect of Brefeldin A on the Biosynthesis of Heparan Sulfate and Chondroitin/Dermatan Sulfate Proteoglycans in Rat Ovarian Granulosa Cells in Culture. ***Journal of Biological Chemistry*** 268(23): 17370-17376, 1993

Ullrich A, Bell JR, Chen EY, Herrera R, Petruzzelli LM, Dull TJ, Gray A, Coussens L, Liao YC, Tsubokawa M and .: Human Insulin Receptor and Its Relationship to the Tyrosine Kinase Family of Oncogenes. ***Nature*** 313(6005): 756-761, 1985

Unger E, Kjellen L and Eriksson UJ: Effect of Insulin on the Altered Production of Proteoglycans in Rib Cartilage of Experimentally Diabetic Rats. ***Archives of Biochemistry and Biophysics*** 285(2): 205-210, 1991

Vaday GG and Lider O: Extracellular Matrix Moieties, Cytokines, and Enzymes: Dynamic Effects on Immune Cell Behavior and Inflammation. ***Journal of Leukocyte Biology*** 67(2): 149-159, 2000

Vainio S, Jalkanen M, Bernfield M and Saxen L: Transient Expression of Syndecan in Mesenchymal Cell Aggregates of the Embryonic Kidney. ***Developmental Biology*** 152(2): 221-232, 1992

Vallance P, Collier J and Moncada S: Effects of Endothelium-Derived Nitric Oxide on Peripheral Arteriolar Tone in Man. ***Lancet*** 2(8670): 997-1000, 1989

van den Hoven MJ, Rops AL, Bakker MA, Aten J, Rutjes N, Roestenberg P, Goldschmeding R, Zcharia E, Vlodavsky I, van der, Vlag J and Berden JH: Increased Expression of Heparanase in Overt Diabetic Nephropathy. ***Kidney International*** 70(12): 2100-2108, 2006

van der Woude FJ and van Det NF: Heparan Sulphate Proteoglycans and Diabetic Nephropathy. ***Nephron Experimental Nephrology*** 5(3): 180-188, 1997

van Det NF, Tamsma JT, van den Born J, Verhagen NA, van den Heuvel LP, Lowik CW, Berden JH, Bruijn JA, Daha MR and van der Woude FJ: Differential Effects of Angiotensin II and Transforming Growth Factor Beta on the Production of Heparan Sulfate Proteoglycan by Mesangial Cells *in vitro*. ***Journal of the American Society of Nephrology*** 7(7): 1015-1023, 1996a

van Det NF, van den Born J, Tamsma JT, Verhagen NA, Berden JH, Bruijn JA, Daha MR and van der Woude FJ: Effects of High Glucose on the Production of Heparan Sulfate Proteoglycan by Mesangial and Epithelial Cells. ***Kidney International*** 49(4): 1079-1089, 1996b

Vaulont S and Kahn A: Transcriptional Control of Metabolic Regulation Genes by Carbohydrates. ***FASEB Journal*** 8(1): 28-35, 1994

Venkataraman G, Shriver Z, Raman R and Sasisekharan R: Sequencing Complex Polysaccharides. ***Science*** 286(5439): 537-542, 1999

Vernier RL, Klein DJ, Sisson SP, Mahan JD, Oegema TR and Brown DM: Heparan Sulfate--Rich Anionic Sites in the Human Glomerular Basement

Membrane. Decreased Concentration in Congenital Nephrotic Syndrome. ***The New England Journal of Medicine*** 309(17): 1001-1009, 1983

Vihinen T, Maatta A, Jaakkola P, Auvinen P and Jalkanen M: Functional Characterization of Mouse Syndecan-1 Promoter. ***Journal of Biological Chemistry*** 271(21): 12532-12541, 1996

Vives RR, Pye DA, Salmivirta M, Hopwood JJ, Lindahl U and Gallagher JT: Sequence Analysis of Heparan Sulphate and Heparin Oligosaccharides. ***Biochemical Journal*** 339 (Pt 3): 767-773, 1999

Vlodavsky, I., Bar-Shavit, R., Korner, G., and Fuks, Z. In: Extracellular matrix bound growth factors, enzymes and plasma proteins. (Rohrbach, D. H. and Timpl, R. eds.) Basement Membrane: Cellular and Molecular Aspects. Academic Press Inc, Orlando, Pp. 327-343, 1993

Vlodavsky I, Eldor A, Haimovitz-Friedman A, Matzner Y, Ishai-Michaeli R, Lider O, Naparstek Y, Cohen IR and Fuks Z: Expression of Heparanase by Platelets and Circulating Cells of the Immune System: Possible Involvement in Diapedesis and Extravasation. ***Invasion Metastasis*** 12(2): 112-127, 1992

Vlodavsky I and Friedmann Y: Molecular Properties and Involvement of Heparanase in Cancer Metastasis and Angiogenesis. ***Journal of Clinical Investigation*** 108(3): 341-347, 2001

Vlodavsky I, Friedmann Y, Elkin M, Aingorn H, Atzmon R, Ishai-Michaeli R, Bitan M, Pappo O, Peretz T, Michal I, Spector L and Pecker I: Mammalian Heparanase: Gene Cloning, Expression and Function in Tumor Progression and Metastasis. ***Nature Medicine*** 5(7): 793-802, 1999

Vlodavsky I, Goldshmidt O, Zcharia E, Atzmon R, Rangini-Guatta Z, Elkin M, Peretz T and Friedmann Y: Mammalian Heparanase: Involvement in Cancer Metastasis, Angiogenesis and Normal Development. ***Seminars in Cancer Biology*** 12(2): 121-129, 2002

Vlodavsky I, Mohsen M, Lider O, Svahn CM, Ekre HP, Vigoda M, Ishai-Michaeli R and Peretz T: Inhibition of Tumor Metastasis by Heparanase Inhibiting Species of Heparin. ***Invasion Metastasis*** 14(1-6): 290-302, 1994

Vogl-Willis CA and Edwards IJ: High Glucose-Induced Alterations in Subendothelial Matrix Perlecan Leads to Increased Monocyte Binding. ***Arteriosclerosis, Thrombosis, and Vascular Biology*** 24(5): 858-863, 2004a

Vogl-Willis CA and Edwards IJ: High-Glucose-Induced Structural Changes in the Heparan Sulfate Proteoglycan, Perlecan, of Cultured Human Aortic Endothelial Cells. ***Biochimica et Biophysica Acta*** 1672(1): 36-45, 2004b

Volk R, Schwartz JJ, Li J, Rosenberg RD and Simons M: The Role of Syndecan Cytoplasmic Domain in Basic Fibroblast Growth Factor-Dependent Signal Transduction. ***Journal of Biological Chemistry*** 274(34): 24417-24424, 1999

Wagner SL, Lau AL and Cunningham DD: Binding of Protease Nexin-1 to the Fibroblast Surface Alters Its Target Proteinase Specificity. ***Journal of Biological Chemistry*** 264(1): 611-615, 1989

Walker, J. D. and Viberti, G. C. In: Pathophysiology of microvascular disease: an overview. (Pickup I. and Williams G. eds.) Textbook of diabetes. Blackwell Scientific, Oxford UK, Pp. 526-533, 1991

Wang L, Fuster M, Sriramarao P and Esko JD: Endothelial Heparan Sulfate Deficiency Impairs L-Selectin- and Chemokine-Mediated Neutrophil Trafficking During Inflammatory Responses. ***Nature Immunology*** 6(9): 902-910, 2005

Wang XL, Zhang L, Youker K, Zhang MX, Wang J, LeMaire SA, Coselli JS and Shen YH: Free Fatty Acids Inhibit Insulin Signaling-Stimulated Endothelial Nitric Oxide Synthase Activation Through Upregulating PTEN or Inhibiting Akt Kinase. ***Diabetes*** 55(8): 2301-2310, 2006

Wardle EN: Heparins for Proliferative Nephritides? Short Review on an Advancing Topic. ***Nephron*** 73(4): 515-519, 1996

Wasty F, Alavi MZ and Moore S: Distribution of Glycosaminoglycans in the Intima of Human Aortas: Changes in Atherosclerosis and Diabetes Mellitus. ***Diabetologia*** 36(4): 316-322, 1993

Wautier MP, Chappey O, Corda S, Stern DM, Schmidt AM and Wautier JL: Activation of NADPH Oxidase by AGE Links Oxidant Stress to Altered Gene Expression Via RAGE. ***The American Journal of Physiology - Endocrinology and Metabolism*** 280(5): E685-E694, 2001

Webb, D. and Cockcroft, J. In: Circulating and tissue renin-angiotensin system: The role of the endothelium. (Warren, J. eds.) An Introduction to current research. Wiley-Liss, New York, Pp. 65-80, 1990

Weigert C, Brodbeck K, Haring HU, Gambaro G and Schleicher ED: Low-Molecular-Weight Heparin Prevents High Glucose- and Phorbol Ester-Induced TGF-Beta 1 Gene Activation. ***Kidney International*** 60(3): 935-943, 2001

Whitelock JM, Graham LD, Melrose J, Murdoch AD, Iozzo RV and Underwood PA: Human Perlecan Immunopurified from Different Endothelial Cell Sources Has Different Adhesive Properties for Vascular Cells. ***Matrix Biology*** 18(2): 163-178, 1999

Wight TN, Kinsella MG and Qwarnstrom EE: The Role of Proteoglycans in Cell Adhesion, Migration and Proliferation. ***Current Opinion in Cell Biology*** 4(5): 793-801, 1992

Wild S, Roglic G, Green A, Sicree R and King H: Global Prevalence of Diabetes: Estimates for the Year 2000 and Projections for 2030. ***Diabetes Care*** 27(5): 1047-1053, 2004

Williamson JR, Tilton RG, Chang K and Kilo C: Basement Membrane Abnormalities in Diabetes Mellitus: Relationship to Clinical Microangiopathy. ***Diabetes/Metabolism Research and Reviews*** 4(4): 339-370, 1988

Wilson VA, Gallagher JT and Merry CL: Heparan Sulfate 2-O-Sulfotransferase (Hs2st) and Mouse Development. ***Glycoconjugate Journal*** 19(4-5): 347-354, 2002

Winegrad AI: Banting Lecture 1986. Does a Common Mechanism Induce the Diverse Complications of Diabetes? ***Diabetes*** 36(3): 396-406, 1987

Wolf G, Chen S and Ziyadeh FN: From the Periphery of the Glomerular Capillary Wall toward the Center of Disease: Podocyte Injury Comes of Age in Diabetic Nephropathy. ***Diabetes*** 54(6): 1626-1634, 2005

Woods A and Couchman JR: Focal Adhesions and Cell-Matrix Interactions. ***Collagen Related Research*** 8(2): 155-182, 1988

Wu C and Dedhar S: Integrin-Linked Kinase (ILK) and Its Interactors: a New Paradigm for the Coupling of Extracellular Matrix to Actin Cytoskeleton and Signaling Complexes. ***Journal of Cell Biology*** 155(4): 505-510, 2001

Wu KK and Thiagarajan P: Role of Endothelium in Thrombosis and Hemostasis. ***Annual Review of Medicine*** 47: 315-331, 1996

Yanagishita M and Hascall VC: Cell Surface Heparan Sulfate Proteoglycans. ***Journal of Biological Chemistry*** 267(14): 9451-9454, 1992

Yang Y, Borset M, Langford JK and Sanderson RD: Heparan Sulfate Regulates Targeting of Syndecan-1 to a Functional Domain on the Cell Surface. ***Journal of Biological Chemistry*** 278(15): 12888-12893, 2003

Yang Y, MacLeod V, Miao HQ, Theus A, Zhan F, Shaughnessy JD, Jr., Sawyer J, Li JP, Zcharia E, Vlodavsky I and Sanderson RD: Heparanase Enhances Syndecan-1 Shedding: a Novel Mechanism for Stimulation of Tumor Growth and Metastasis. ***Journal of Biological Chemistry*** 282(18): 13326-13333, 2007

Yeaman C and Rapraeger AC: Post-Transcriptional Regulation of Syndecan-1 Expression by CAMP in Peritoneal Macrophages. ***Journal of Cell Biology*** 122(4): 941-950, 1993

Yokokawa K, Kohno M, Mandal AK, Tahara H, Yanagisawa M, Takeda T and Kohne M: Heparin Suppresses Endothelin-1 Peptide and MRNA Expression in Cultured Endothelial Cells of Spontaneously Hypertensive Rats. ***Journal of the American Society of Nephrology*** 4(9): 1683-1689, 1994

Yokokawa K, Kohno M, Murakawa K, Yasunari K, Horio T, Inoue T and Takeda T: Acute Effects of Endothelin on Renal Hemodynamics and Blood Pressure in Anesthetized Rats. ***The American Journal of Hypertension*** 2(9): 715-717, 1989

Yokokawa K, Tahara H, Kohno M, Murakawa K, Yasunari K, Nakagawa K, Hamada T, Otani S, Yanagisawa M and Takeda T: Hypertension Associated With Endothelin-Secreting Malignant Hemangioendothelioma. ***Annals of Internal Medicine*** 114(3): 213-215, 1991

Zeng G, Nystrom FH, Ravichandran LV, Cong LN, Kirby M, Mostowski H and Quon MJ: Roles for Insulin Receptor, PI3-Kinase, and Akt in Insulin-Signaling Pathways Related to Production of Nitric Oxide in Human Vascular Endothelial Cells. ***Circulation*** 101(13): 1539-1545, 2000

Zeng G and Quon MJ: Insulin-Stimulated Production of Nitric Oxide Is Inhibited by Wortmannin. Direct Measurement in Vascular Endothelial Cells. ***Journal of Clinical Investigation*** 98(4): 894-898, 1996