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

©Copyright Juying Han, November 2009. All Rights Reserved.
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
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.


* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
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

1.2.1. Endothelium Properties

1.2.2. Transportation of Glucose in Endothelial Cells

1.2.3. The Concept of Endothelial Dysfunction

1.2.4. Endothelial Dysfunction in Diabetes

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

1.3.1. Definition Proteoglycans

1.3.2. Heparan Sulfate Proteoglycans (HSPGs)

1.3.3. HSPG Structures

1.3.3.1. Syndecans

1.3.3.2. Perlecan

1.3.3.3. Heparin Serglycin

1.3.4. HSPG Biosynthesis

1.3.5. HSPG Biophysiological Functions

1.3.6. Analysis of Heparan Sulfate GAGs

1.3.7. HSPG in Vasculature

1.3.8. HSPG Degradation in Diabetes

1.3.9. HSPG Degradation by Heparanase

1.3.9.1. Heparanase Expression in Cells

1.3.9.2. Heparanase Properties

1.3.9.3. Heparanase Functions

1.3.9.4. Heparanase and Diabetes
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
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, Perlecans and Syndecans.............................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

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE(s)</td>
<td>advanced glycosylation end-product(s)</td>
</tr>
<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>CMF-DPBS</td>
<td>Ca^{2+}-Mg^{2+}-free Dulbecco’s phosphate-buffered saline</td>
</tr>
<tr>
<td>CE–MS</td>
<td>capillary electrophoresis mass spectrometry</td>
</tr>
<tr>
<td>CS</td>
<td>chondroitin sulfate</td>
</tr>
<tr>
<td>CSPG(s)</td>
<td>chondroitin sulfate proteoglycan(s)</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic 3’-5’ guanosine monophosphate</td>
</tr>
<tr>
<td>DS</td>
<td>derman sulphate</td>
</tr>
<tr>
<td>DSPG(s)</td>
<td>derman sulfate proteoglycan(s)</td>
</tr>
<tr>
<td>DM</td>
<td>diabetes mellitus</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DD</td>
<td>double distilled</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EHS</td>
<td>Engelbreth-Holm-Swarm</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin-1</td>
</tr>
<tr>
<td>FAS</td>
<td>fat acid synthase</td>
</tr>
<tr>
<td>GAG(s)</td>
<td>glycosaminoglycan(s)</td>
</tr>
<tr>
<td>Gal</td>
<td>galactose</td>
</tr>
<tr>
<td>GalN</td>
<td>galactosamine</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetylated galactosamine</td>
</tr>
<tr>
<td>GBM</td>
<td>glomerular basement membrane</td>
</tr>
<tr>
<td>GlcA</td>
<td>glucuronic acid</td>
</tr>
<tr>
<td>GlcN</td>
<td>glucosamine</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylated glucosamine</td>
</tr>
<tr>
<td>GlcNS</td>
<td>N-sulfated glucosamine</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosyl-phosphatidylinositol</td>
</tr>
<tr>
<td>GRE</td>
<td>glucose response element</td>
</tr>
<tr>
<td>Glut-1</td>
<td>glucose transporter-1</td>
</tr>
<tr>
<td>Glut-2</td>
<td>glucose transporter-2</td>
</tr>
<tr>
<td>Glut-4</td>
<td>glucose transporter-4</td>
</tr>
<tr>
<td>HAEC(s)</td>
<td>human aortic endothelial cell(s)</td>
</tr>
<tr>
<td>HS</td>
<td>heparan sulfate</td>
</tr>
<tr>
<td>HSPG(s)</td>
<td>heparan sulphate proteoglycan(s)</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
</tbody>
</table>
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<sub>ATP</sub> ATP-sensitive K<sup>+</sup>
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<sub>2</sub> prostaglandin I<sub>2</sub>
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 (http://www.cdc.gov/Features/diabetesfactsheet/). 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 I₂ (PGI₂) 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
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
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.

(Clarke and Lee, 1995)

**Figure 1.2. The Aldose Reductase Pathway of Glucose Metabolism**
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
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-NH₂). 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
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, specifically 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 H₂O, CO₂, 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 mesangial 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-1, 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 bioavailability 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), syndecan-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
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 catalyzed by 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 β1, 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
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.

(Esko and Lindahl, 2001)
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 adhesion. 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 adhesion 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,
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.,
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$_3^-$.
Controversially, ECs are able to bind, internalize, and degrade high-M, 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
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

High Glucose

Heparin and/or Insulin

Heparitinase

Heparan Sulfate Proteoglycans in Endothelial Cells

GAG Chains
Core Proteins of Syndecan and Perlecan

If (-) Release Bioactive Factors from Cell Surface and ECM

Cell Injury or Dysfunction

Diabetic Cardiovascular Complications

Nephropathy, Retinopathy, Neuropathy and Atherosclerosis
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\textsubscript{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\textsuperscript{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 trituated 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 Na₂B₄O₇ in concentrated H₂SO₄) 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 H2O 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) H2O 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 Na3PO4 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 H₂O. The elution was freeze-dried and then dissolved in 15 μl DD H₂O. 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 x 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-cyanoacetamide 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.
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

0.0
0.5
1.0
1.5
2.0
Control
Glucose
Insulin
Glucose+Insulin

24 hours 48 hours 72 hours

B

GAG Concentrations in Medium

0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
Control
Glucose
Insulin
Glucose+Insulin

24 hours 48 hours 72 hours

C

Cell+Medium GAG Concentration (μg/ml)

0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Control
Glucose
Insulin
Glucose+Insulin

24 hours 48 hours 72 hours

abc abc abc
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. $p<0.05$
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 visualized 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

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

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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cell</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>Total HS* (ng)</td>
<td>145.9</td>
<td>39.0</td>
</tr>
<tr>
<td>Total GAGs (ug)</td>
<td>0.97</td>
<td>1.17</td>
</tr>
</tbody>
</table>
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.


* Disaccharides not detected in cells.
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.


* Disaccharide was not detectable 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 disaccharides 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 disaccharides was much higher in control cells than glucose-treated cells both at 24 and 72 hours.

4.4.6. HS Disaccharideess 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 heparinas. 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 heparinas. 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 heparinas. 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 conditions 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% CO₂/95% 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 \( \geq 2.0 \) indicated RNA pure enough to use for cDNA synthesis.

### 5.3.4. Synthesis of cDNA

The AffinityScript\textsuperscript{TM} 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 \( \mu \)g RNA from each sample was used for cDNA synthesis in a 20 \( \mu l \) reaction according to the methods described in the manufacturer’s instruction. The Oligo (dT) primer (3 \( \mu l \) each reaction) was used. The reaction program was: the primer annealing at 25\( ^\circ \)C for 5 minutes, cDNA synthesis at 45\( ^\circ \)C for 15 minutes and the termination of the reaction at 95\( ^\circ \)C for 5 minutes. The newly synthesized cDNAs were stored at -20\( ^\circ \)C for later use.

### 5.3.5. Detection of Gene Expression by Real Time

#### Quantitative PCR

Brilliant\textsuperscript{®} II SYBR\textsuperscript{®} 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’-GGCAAGTATTTTTGGAGCA-3’ and antisense primer 5’-TGGATTGTCAGTGTTTGTGC-3’; perlecan sense primer 5’-TGCCTGAGGACATAGAGACC-3’ and antisense primer 5’-TCGGAATAAACCATCTGGACATAGAGACC-3’;
syndecan-1 sense primer 5’-TGCAGGTGCTTTGCAAGATA-3’ and antisense primer 5’-TTCTGGAGACGTGGGAATA-3’; β-actin (internal control) sense primer 5’-GGCATCCTCACCCCTGAAGTA-3’ and antisense primer 5’-GAAGGTTTCAAACTGATCT-3’. In a 25 µl reagent mixture, assembled according to the manufacture’s instructions, final concentrations for each primer was 200 nM alone with 5 µl 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^{ΔΔC_T} method decribed by Livak et al., (Livak and Schmittgen, 2001). The formula was ΔΔC_T = (C_T - C_{Tactin})_{Treatment} - (C_T - C_{Tactin})_{Control}. Using the 2^{ΔΔC_T} 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, ΔΔC_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^{ΔΔC_T} 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 $2^{-\Delta C_T}$ method, where $\Delta C_T = C_{T,\text{time 0}} - C_{T,\text{time n}}$.

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.
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.

Figure 5.1. Confirmation of Real Time PCR Products
**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 metastatic 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 PKCβ (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 activating 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 degradation 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 functions of insulin and heparin in diabetes.
8. REFERENCES


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


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


173


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


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


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


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


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


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


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


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


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


