Hyperglycemia affects Matrix Metalloproteinase-2 Secretion in Human Umbilical Vein Endothelial Cells *in vitro*

Master thesis by

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Abstract

Goal: The aim of the current thesis was to study the effect of hyperglycemia (HG) on MMP secretion from human primary endothelial cells.

Background: Diabetes Mellitus is characterized by abnormal high levels of glucose in the blood. Endothelial dysfunction is a serious complication of sustained hyperglycemia. Diabetic complications are main reasons for mortality in diabetes. The matrix metalloproteinases (MMPs) and their corresponding inhibitors are the main regulators of the turnover of extracellular matrix (ECM) components, and have been suggested to play an important role in vascular remodelling taking place in diabetes. MMP secretion from endothelial cells may become altered in HG conditions and result in dysregulation of ECM turnover, which may affect vascular functions with relevance to diabetes. HG conditions may also affect intracellular signalling through pathways sensing changes in cellular glucose, such as the hexosamine pathway

Methods: Primary human umbilical vein endothelial cells (HUVEC) were cultured *in vitro* as a model system of the human endothelium. HUVEC were exposed to HG (25 mM) to imitate the diabetic hyperglycemic state and the effect on secretion of MMP-2 and MMP-9 was quantified. MMP secretion was analyzed by gelatine zymography. HUVEC were cultured on traditional plastic wells and on semipermeable filter inserts. The effect of blocking the hexosamine pathway using the inhibitor DON, on MMP secretion was also investigated. The O-GlcNAcylated proteins were analyzed by Western blotting. Bands obtained after gelatine zymography were measured using the programme Quantity-One (Bio-Rad).

Results: Results obtained suggest that there is a weak trend of reduction in MMP-2 secretion in HUVEC grown on plastic. However, variable results were discovered in HUVEC grown on filters. The filter experiments showed that MMP-2 was mostly secreted to the apical side of polarized HUVEC. Finally, exposing HUVEC to DON caused a decrease in MMP-2 secretion both on plastic and on filter. MMP-9 secretion was not detected in any of the current experiments.

Conclusions: The present work showed that HG influences MMP secretion, indicating a reduction in MMP-2 secretion in HUVEC grown on plastic. However, results were not definite and further investigations are needed to study the effects of hyperglycemia on MMP-2 secretion in cultures of primary human endothelial cells and to further investigate the potential role of MMPs as a therapeutic target in diabetes.

Acknowledgments

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Abbreviations

AGE	Advanced glycation end product
AZA	Azaserine
BAEC	Bovine aortic vein endothelial cells
DAG	Diacylglycerol
DON	6-diazo-5-oxo-norleucine
DM	Diabetes Mellitus
EC	Endothelial Cell
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Endothelial growth factor
ER	Endoplasmic reticulum
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
FCS	Foetal calf serum
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GADPH	Glyceraldehyde-3 phosphate dehydrogenase
GDM	Gestational diabetes mellitus
GFAT	Glutamine: fructose-6 phosphate amidotransferase
GlcNAc	*N*-acetylglucosamine
GLUT	Glucose transporter
HG	High glucose
HUVEC	Human Umbilical Vein Endothelial Cell
IL-1β	Interleukin-1β
MMP	Matrix metalloproteinase
NG	Normoglucose
NO	Nitric oxide

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
O-GlcNAcylation	O-linked-*N*-acetylglucosaminylation
OGT	O-GlcNAc transferase
PI3K	Phosphatidylinositol-3-OH kinase
PAI-1	Plasminogen activator inhibitor-1
PKC	Protein kinase C
PUGNAC	O-(2-Acetamido-2-deoxy-D-glucopyranosylidene) amino *N*-phenyl carbamate
RIPA	Radio-Immunoprecipitation assay
ROS	Reactive oxygen species
Sp1	Specificity Protein 1
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α
TIMP	Tissue inhibitor of metalloproteinases
Type 1 DM	Type 1 diabetes mellitus
Type 2 DM	Type 2 diabetes mellitus
UDP	Uridine diphosphate
UDP-GlcNAc	UDP-*N*-acetylglucosamine
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

1 Introduction

1.1 Diabetes

Diabetes Mellitus (DM) is a group of chronic diseases, which arise when the pancreas is unable to produce enough insulin or when the body does not effectively use the insulin it produces. The lack of insulin production or the insensitivity to the hormone, ultimately leads to hyperglycemia, which can be described as an excess of glucose in the blood (WHO, 2011; Thomas & Bishop, 2007). There are as many as 366 million people worldwide who have diabetes and this number is projected to rise to 522 million by 2030 (International Diabetes Federation, 2011). In Norway, approximately 375 000 people have diabetes, in which 350 000 of these are afflicted with type 2 DM (Diabetesforbundet, 2008).

1.2 Types of diabetes

There are several forms of diabetes. Most cases can be categorized into type 1 or type 2 DM. Type 1 DM is characterized by a partial or total failure of insulin production as a result of destruction of the pancreatic beta cells. Although the disease is considered to arise from an autoimmune response, the initializing event for beta cell destruction is currently not known in detail. Type 2 DM is the most prevalent type of diabetes, accounting for at least 75% of diabetes cases (Thomas & Bishop, 2007). The decline in physical activity and rise in obesity rates in the western world are two major reasons behind the epidemic like increase in type 2 DM (Wild *et al*., 2004). The disease occurs either due to a lack of insulin production, or insensitivity to insulin, or both. In addition to being strongly linked to obesity, with 80% of patients with type 2 DM being overweight or obese, type 2 DM is also linked to genetic predispositions (Thomas & Bishop, 2007).

Gestational diabetes mellitus (GDM) is another form of diabetes that occurs in approximately 1-14% of all pregnancies, depending on the population studied. GDB arises due to the mother's inability to produce enough insulin to meet the extra demands of pregnancy. The risk of developing GDB increases with age (> 25 y), and is more prevalent in women with a family history of diabetes, women from an ethnic or racial group with high prevalence of

diabetes, such as Asian, African or Hispanic, or in women who are overweight or obese (American Diabetes Association, 2003). Even though the occurrence of GDB is generally temporarily, it increases the risk of developing type 2 DM in later life (Thomas & Bishop, 2007).

1.2.1 Diagnosis

Diagnosis of diabetes is characterized by a random venous plasma glucose concentration over 11.1 mM or a fasting venous plasma glucose concentration greater than 7 mM. An oral glucose tolerance test (OGTT) 2 h after consuming 75 g of anhydrous glucose is used to confirm diagnosis of diabetes (Thomas & Bishop, 2007).

1.3 Diabetic complications

Diabetic complications are major causes of morbidity and mortality in diabetic patients (Kitada *et al*., 2010). Complications can generally be classified as macrovascular and microvascular. Macrovascular complications are diseases of the large blood vessels, and encompass coronary artery disease, peripheral arterial disease and stroke. Atherosclerosis can lead to a narrowing of arterial walls in the body and is a central mechanism in the development of macrovascular diseases. Microvascular complications are diseases of the smaller blood vessels and include diabetic nephropathy, neuropathy and retinopathy. Lifestyle changes and medical treatment are important for lessening hyperglycemia-induced damages in the vascular tissues and in the prevention of micro- and macrovascular complications (Fowler *et al*., 2008; Thomas & Bishop, 2007).

1.3.1 Diabetic Nephropathy

Diabetic nephropathy is the main cause of renal failure in the United States. It is defined by proteinuria > 500 mg/24 h in the presence of diabetes, but starts with lower levels of proteinuria also referred to as "microalbuminuria". Microalbuminuria refers to an abnormal increase in albumin excretion rate within 30-299 mg in 24 h. Daily albumin excretion rate is in the range of 5-10 mg under normal conditions (Toto, 2004). Diabetic nephropathy is present in 20-30% of patients with type 1 and type 2 diabetes, and the prevalence is as much as 40-50% in type I diabetes patients who have had the disease for more than 20 years (Thrailkill *et al*., 2009). There are a number of risk factors associated with the development of nephropathy including age at diagnosis, race, hypertension, poor glycemic control, genetic predisposition to kidney disease, and dietary composition. However, the specific pathogenic process implicated in the initiation and progression of renal disease is not completely understood. A five-stage process of the development of diabetic nephropathy has been suggested (Thrailkill *et al*., 2009): Renal disease initiates with glomerular hyperfiltration and hypertrophy of the kidneys (stage 1). The glomerular basement thickens (stage 2), leading to microalbuminuria and a decrease in glomerular filtration rate (stage 3), developing to proteinuria and severe hypertension (stage 4), and finally, developing into end stage renal disease (stage 5). The expansion of the kidney mesangium is a result of ECM over-accumulation and is a significant characteristic of renal disease.

During normal matrix remodelling, matrix metalloproteinases (MMPs) produced by the mesangial cells are responsible for approximately 70% of the ECM turnover in the kidney. Thus, an association between intrarenal dysregulation of MMP activity and the progression of diabetic nephropathy has been suggested as many studies have reported a link between MMP dysregulation and diabetic nephropathy (Thrailkill *et al*., 2009; Catania *et al*., 2006). Aberrant MMP activity has also been linked to several other complications of diabetes, such as diabetic retinopathy (Thrailkill *et al*., 1999; Thrailkill *et al*., 2009; Tsilibary, 2003; Lokesh & Veeranjaneyulu, 2003), peripheral arterial disease and acute coronary syndrome (Thrailkill *et al*., 2009). Changes in MMP synthesis, secretion and activities may therefore have vital roles in diabetes pathogenesis. This family of proteases are further described in chapter 1.9.

1.4 The Vascular Endothelium

The vascular endothelium consists of a single sheet of endothelial cells located on the internal lumen of all blood vessels, lining the entire vascular system from the heart to the smallest capillary (**Figure 1.4**) (Singh *et al*., 2010). The endothelium weighs approximately 1 kg, making it one of the largest "organs" in the human body.

The endothelium functions as a semipermeable barrier between the blood stream and the vascular smooth muscle tissue. This barrier allows movement of small solutes in preference to large molecules. The endothelium is implicated in numerous homeostatic processes. The physiological functions of the endothelium include regulation of blood coagulation, vasoconstriction and vasodilation, management of growth and differentiation of vascular smooth muscle cells (VSMC), regulation of the subendothelial matrix and the modulation of inflammatory activity in the vessel wall. Changes in ability of the endothelium to regulate some or all of its functions results in endothelial dysfunction (Sing *et al*., 2010). The endothelium produces a range of substances in order to perform its functions, such as nitric oxide (NO), prostanoids, endothelin-1 (ET-1), Ang-II (angiotensin-II), t-PA (tissue-type-plasminogen activator), von Willebrand factor (vWF), and cytokines (Schalkwijk & Stehouwer, 2005). NO is one of the major vasodilatory agents synthesized by the endothelium, and it also inhibits growth and inflammation. Other inflammatory substances include intracellular adhesion molecule-1, E-selectin and NF-κB (Endemann & Schiffrin, 2004).

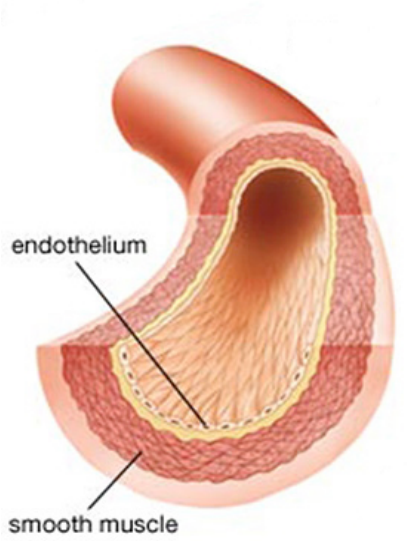

Figure 1.4. The endothelium consists of a monolayer of endothelial cells. The endothelium is involved in numerous homeostatic processes, including vasotone regulation (vasoconstriction and vasodilation), regulation of growth and differentiation of vascular smooth muscle cells (VSMC), and regulation of inflammatory activity (Figure has been adapted from Chelation Health Products, 2008.)

1.4.1 Endothelial dysfunction

The endothelium is versatile and multifunctional, and is distributed in all organ systems. Thus, its dysfunction is not restricted to any specific organ or to the pathogenesis of atherosclerosis (Malyszko, 2010; Feletou & Vanhoutte, 2006). Endothelial dysfunction can be described as the inability of the endothelial cells to control some or all of their functions and can be characterized by reduced vasodilation, a proinflammatory condition, and a prothrombic state. Endothelial dysfunction is commonly associated with a decline in NO bioavailability; due to reduced NO production by the endothelium or an inactivation of NO caused by an increase in reactive oxygen species (ROS) production (Endemann & Schiffrin, 2004). Endothelial dysfunction is a critical complication of sustained hyperglycemia, and is commonly seen in patients with cardiovascular disease, diabetes and hypercholesterolemia (Endemann & Schiffrin, 2004). The pathogenesis of endothelial dysfunction in type 1 and 2 DM has not been established, and the underlying processes resulting in endothelial dysfunction may be different in the two pathologies. However, several common risk factors appear to play an important part in the progression of endothelial dysfunction in both conditions, including hypertension, poor glycemic control, microalbuminuria, dyslipidaemia, poor antioxidant status, and smoking (Singh *et al*., 2010). In diabetes, various factors are considered to give rise to endothelial dysfunction including the level of hyperglycemia, duration of diabetes, accumulation of advanced glycation end products (AGEs), and the

presence of complications such as nephropathy and albuminuria (Feletou & Vanhoutte, 2006). Vascular remodelling is considered to play a crucial role in pathogenesis of arterial and venous disease. Changes to the cellular and ECM components result in structural and functional modifications of the vessel wall. These modifications may lead to atherosclerosis, contributing to plaque initiation and progression, or weakening of the vascular wall causing vessel dilation (Lim *et al*., 2010). Changes in basement membrane synthesis may give rise to altered matrix composition, which can result in arterial stiffening, increased microvascular permeability and vascular tone as seen in diabetes and atherosclerosis (Stehouwer, 2004). The retention hypothesis postulates that proteoglycans are involved in the retention of atherogenic lipoproteins, being an early event in atherogenesis. Retained lipoproteins become modified (oxidized) and can cause lesions and induce increased synthesis of proteoglycans, which may lead to further retention of lipoproteins and aggregation (Skålen *et al*., 2002). One problem with diabetes treatment is that endothelial function may not be improved or restored (Feletou & Vanhoutte, 2006).

The endothelial glycocalyx

The glycocalyx can be regarded as a "gatekeeper" and is located between the blood stream and the endothelial layer (**Figure 1.4.1)**. The glycocalyx consists of proteoglycans and glycoproteins and functions as a protective barrier, regulating blood clotting, blood flow and inflammation (Reitsma *et al*., 2007; Noble *et al*., 2008). Damage to the glycocalyx is considered to arise from enzymatic and pressure-induced shedding. The MMPs are known to take part in the shedding of syndecans (transmembrane domain proteins) from the glycocalyx and are referred to as sheddases (Lipowsky, 2011; Manon-Jensen *et al*., 2010). *In vitro*, it has been shown that the ectodomain of syndecans is continuously shed from cells. This process is accelerated in wound healing and various diseases (Manon-Jensen *et al*., 2010). *In vivo,* studies have shown that shedding of the endothelial glycocalyx occurs under inflammation and hyperglycemia. Dysfunction of the glycocalyx has been proposed to contribute to endothelial dysfunction in hyperglycemic conditions (Reitsma *et al*., 2007). MMPs are capable of modifying the endothelial glycocalyx and facilitate shedding in disease states (Lipowsky, 2011).

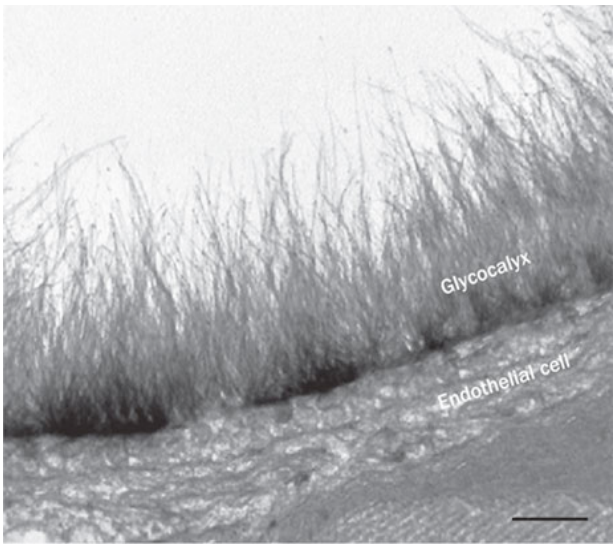

Figure 1.4.1. The image shows the glycocalyx layer above the endothelial cell (Figure taken from Rutledge *et al*., 2010).

1.4.2 Endothelial dysfunction and inflammation

Over time diabetes can lead to a condition of low-grade inflammation in the circulatory system. Causes for inflammation include hyperglycemia, generated advanced glycation endproducts (AGEs) and increased levels of tumour necrosis factor-α (TNF-α). Endothelial dysfunction contributes to vascular inflammation by the generation of vasocontrictor agents, release of adhesion molecules and growth factors (Savoia & Schiffrin, 2007; Stehouwer, 2004). Inflammation is a defensive biological response to a pathological stimulus or tissue injury (Roy *et al*., 2009). It is hypothesized that inflammatory molecules have a key role in the development of diabetes complications in particular diabetic nephropathy, although the mechanisms behind this is not clear (Goldberg, 2009). Proteinuria is an important stage in the development of diabetic nephropathy and is closely correlated with inflammation (Navarro-Gonzalez *et al*., 2011).

An early event of inflammation is the secretion of chemokines from affected tissues including monocyte chemoattractant protein (MCP-1) and macrophage migration inhibition factor (MIF) (Goldberg, 2009). These factors and others induce the secretion of a range of adhesion molecules from the endothelium, including leukocyte adhesion molecules (LAM), intracellular adhesion molecules (ICAM), and vascular cell adhesion molecules (VCAM). The adhesion molecules, which are located on the cell surface, and regulate cell-cell and cell-

matrix interactions, have a regulatory role in inflammation and attract cells implicated in the inflammatory reaction including monocytes, granulocytes and other immune cells (Hadi & Suwaidi, 2007; Aldahi & Hamdy, 2003; Goldberg, 2009).

Adipose tissue is a major contributor to inflammation in overweight patients, which is important as obesity is strongly linked to type 2 diabetes (Goldberg, 2009; Thomas & Bishop, 2007). TNF-α is secreted from adipocytes. Thus, increased adipose tissue can result in higher levels of TNF-α in overweight subjects, which can contribute to inflammation, e.g. through activation of endothelial cell expression of leukocyte adhesion molecules (Aldahi & Hamdy, 2003).

1.5 Hyperglycemia activates the flux through four major pathways

Endothelial dysfunction is a multifaceted disorder and occurs in many different disease processes. Despite this, oxidative stress can be identified as a fundamental common factor, giving rise to endothelial dysfunction (Feletou & Vanhoutte, 2006). Chronic hyperglycemia causes increased oxidative stress and an impaired antioxidant response in endothelial cells of both small and larger vessels (Singh *et al*., 2011). In diabetes, every cell in the body is exposed to unusual high concentrations of glucose. While some cells are able to regulate glucose uptake according to the levels of glucose concentration in the cellular environment, other cells are not able to control the flux of glucose. Vascular endothelial cells are major targets for hyperglycemic damage, as they do not appear to alter the transport of glucose when glucose concentration is increased, ultimately leading to intracellular hyperglycemia (Giacco & Brownlee, 2010). This strongly indicates that the cause for the complications include mechanisms taking place inside the cell, rather than outside. Four pathways have been recognized as being important in the progression of diabetic complications and they all share one single hyperglycema-induced process, which is the overproduction of superoxides by the mitochondrial electron transport chain (**Figure 1.5**) (Brownlee, 2005).

Hyperglycemia causes the activation of four major pathways:

1. An increase in polyol pathway flux
2. Activation of Protein kinase C (PKC) isoforms
3. Increased formation of AGE products
4. Excess shunting through the hexosamine pathway.

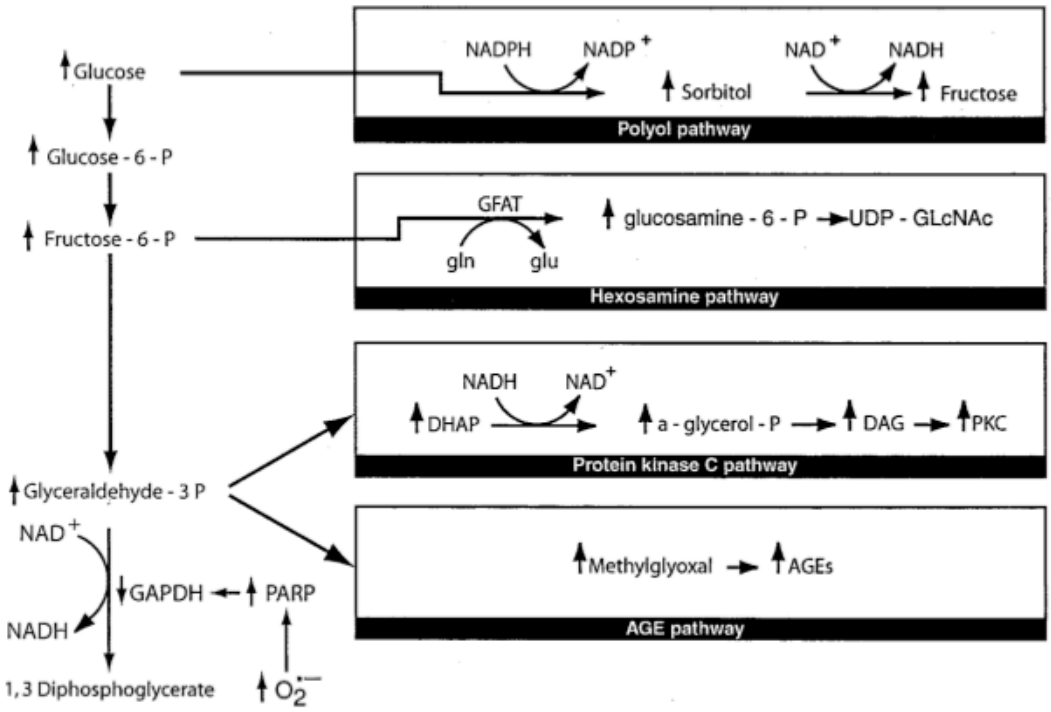

Figure 1.5. Hyperglycemia increases the flux through four major pathways. The four pathways are the polyol pathway, hexosamine pathway, PKC, and AGE pathway (Figure is taken from Brownlee, 2005).

1.5.1 Increased flux through the polyol pathway

In the polyol pathway, intracellular glucose is converted to sorbitol in a rate-limiting step catalyzed by the enzyme aldose reductase (AR), in a NADPH dependent reaction. Next, sorbitol is oxidized to fructose by sorbitol dehydrogenase (SDH). Under normal glucose conditions, only a small fraction of glucose is metabolized through this pathway. In diabetic states however, the increase in intracellular glucose levels may lead to an increased flux of glucose trough AR. NADPH which is used in this reaction, is also a vital cofactor for regenerating an important intracellular antioxidant, reduced glutathione (GSH). If the amount of reduced GSH is decreased, the polyol pathway increases susceptibility to a lower production of NO and leads to an increase in oxidative stress (Brownlee, 2005).

1.5.2 Increased protein kinase C activation

The PKC family is made up of at least eleven isoforms, which are all activated by the lipid messenger diacylglycerol (DAG). Intracellular hyperglycemia increases synthesis of DAG. Moreover, several PKC isoforms are activated by other mechanisms, such as ROS and free fatty acids (FFA). Increased protein kinase C activation has been linked to a variety of changes in gene expression, such as a decrease in endothelial nitric oxide synthase (eNOS) that produces the vasodilator NO, an increase in the expression of endothelin-1, tumor growth factor-β (TGF- β) and the plasminogen activator inhibitor (PAI-1). This may then lead to changes in blood flow, basement membrane thickening, ECM expansion, an increase in vascular permeability, and abnormal angiogenesis (Giacco & Brownlee, 2010).

1.5.3 Increased AGE formation

AGE products are made when glucose reacts with proteins in a non-enzymatic way. A series of chemical rearrangements results in AGEs. In diabetes, AGEs are found to accumulate in the ECM, both in the vasculature and in organs such as the kidneys. There are three general ways in which intracellular production of AGE precursors can harm cells.

1. Intracellular proteins modified by AGEs have changed functions.

2. ECM components can be modified by AGEs, which changes the composition and functions of ECM, signalling between the matrix and the cell, which may contribute to e.g. endothelial dysfunction.

3. Plasma proteins such as albumin can be modified by AGE precursors and bind to AGE receptors (RAGE) on cells such as macrophages, vascular endothelial cells and vascular smooth muscle cells. The binding of plasma proteins to RAGE instigates the generation of ROS, which in turn initiates the transcription factor NF-κB, resulting in many pathological changes in gene expression (Brownlee, 2001).

Studies have shown that serum and tissue AGEs levels are significantly increased in type 1 and type 2 diabetes patients compared to non-diabetics (Berg *et al*., 1997 & Berg *et al*., 1998). Moreover, it has been found that diabetic patients with end-stage renal disease have

approximately twice AGEs in tissue in comparison to diabetic patients with no renal disease (Makita *et al*., 1991). Excess AGEs in kidney is suggested to be responsible for alteration in renal structure and loss of renal function. AGE formation on different types of matrix proteins impairs their breakdown by MMPs, contributing to thickening of the basement membrane and mesangial expansion, which are characteristics of diabetic nephropathy. AGE triggers TGF-β expression, which in turn induces the generation of the important ECM proteins collagen, laminin and fibronectin. AGE-induced TGF-β expression has also been shown to give rise to tubulointerstitial fibrosis in diabetic nephropathy (Yamagashi & Matsui, 2010).

1.6 Increased flux through the hexosamine pathway

There is a strong correlation between type 2 DM and nutrient availability. However, the mechanism by which nutrient excess leads to type 2 DM is not completely understood. Many metabolic and signalling pathways have been hypothesized to play a role in the development of diabetes. One of these is the hexosamine pathway (Wells & Hart, 2003), which has been studied in this thesis in regard to the secretion of MMPs. Excess shunting through the hexosamine pathway caused by hyperglycemia may give rise to changes in both gene expression and protein function, and can play a crucial role in the progression of diabetic complications (Wells & Hart, 2003, Brownlee, 2005, Hart *et al*., 2011).

Glucose can enter cells via glucose transporters (GLUTs) and is metabolized to fructose-6-phosphate (F-6-P) by the enzyme hexokinase and continues into glycolysis (**Figure 1.6**). In a normal glucose state, only a small amount of glucose (2-5%) is metabolized through the hexosamine pathway (Laczy *et al*., 2008; Schleicher & Weigert, 2000). In the presence of chronic hyperglycemia, the flux through this pathway increases. F-6-P is metabolized into glucosamine-6-phosphate (G-6-P) by the rate-limiting enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT). In this process glutamine is amine donor and the activity of GFAT can be blocked using the glutamine analogues 6-diazo-5-oxo-norleucine (DON) or azaserine (AZA). Two different isoforms, GFAT1 and GFAT2, have been identified in humans, sharing 75% identity (Nakaishi *et al*., 2009). A northern blot analysis revealed different expression of the two variants in different tissues. A higher expression of GFAT1 was found in placenta, testis and pancreas, whereas GFAT2 expression was more

abundantly found in heart and central nervous tissue. GFAT activity is strictly regulated by cAMP-dependent phosphorylation. However, phosphorylation of GFAT1 reduces its activity *in vitro*, in contrast to increasing the activity of GFAT2. Different expression and activity of the two isoforms suggest that the enzymes have separate roles in diabetic pathophysiology (Laczy *et al*., 2008). One report studied the expression of both enzymes (using an antibody recognizing the C-terminus of GFAT) in various tissues in healthy human subjects. The immunohistochemical study revealed a high expression of GFAT in adipocytes, skeletal muscle and VSMC. Surprisingly, glomerular cells remained unstained. However, analysis of renal sections from subjects with diabetic nephropathy showed a significant staining of glomerular cells, suggesting that the enzyme is induced in diabetes (Schleicher & Weigert, 2000). Increased activity of human GFAT has been linked to insulin resistance in experimental *in vitro* models (Nakaishi *et al*., 2009).

The major end product in the hexosamine pathway is UDP-*N*-acetylglucosamine (UDP-GlcNAc), which is a substrate for the subsequent O-linked GlcNAc modifications of target proteins at serine and threonine residues. The quantity of UDP-GlcNAc in the cell indicates the flux of glucose through this pathway. UDP-GlcNAc can be considered as a metabolic sensor capable of modifying proteins according to changes in intracellular glucose concentration and availability (Issad *et al*., 2010). UDP-GlcNAc is an allosteric inhibitor of GFAT and regulates the flux of glucose into the hexosamine pathway. From this pathway UDP-GlcNAc can either continue into the endoplasmic reticulum (ER) or Golgi where it used as an essential building block to synthesize glycolipids, proteoglycans or glycoproteins, or it can go through the "signalling" pathway, where it used to modify nuclear and cytosolic proteins by O-GlcNAcylation (**Figure 1.7)**, further described on the following pages (Buse, 2006).

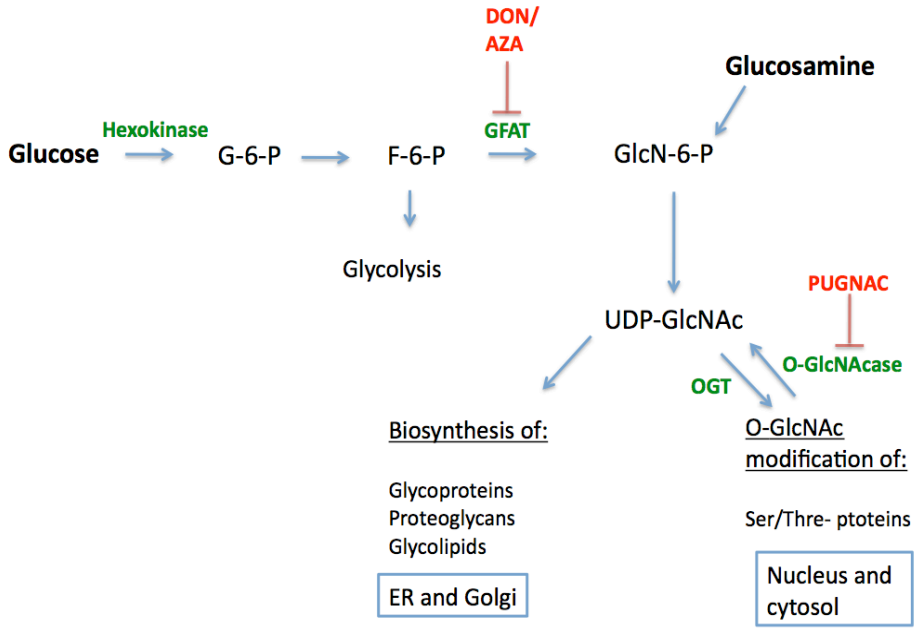

Figure 1.6. Overview of the hexosamine pathway. UDP-GlcNAc can be regarded as a nutrient sensor capable of modifying proteins depending on glucose availability. The nucleotide sugar UDP-GlcNAc can either be used as a precursor for synthesis of proteoglycans, glycolipids and glycoproteins, or it can be used in a translational modification of proteins, described as O-GlcNAcylation: a process in which UDP-GlcNAc is added on to serine and threonine residues in an O-linkage by the enzyme O-GlcNAc-transferase (OGT) and removed by O-GlcNAcase (Figure is adapted from Buse, 2006). Text in green displays the action of enzymes involved in the pathway, while the red lines indicate where the inhibitors act. 6-diazo-oxo-norleucine (DON) and azaserine (AZA) prevent the action of the enzyme GFAT, while PUGNAC (O-(2-Acetamido-2-deoxy-D-glucopyranosylidene) amino *N*-phenyl carbamate) inhibits the action of O-GlcNAcase.

1.7 O-GlcNAcylation

O-linked-*N*-acetylglucosaminylation (O-GlcNAcylation) was initially discovered by G.W. Hart in 1984 (Torres & Hart, 1984) and is described as the addition and removal of N-acetylglucosamine (GlcNAc) on the hydroxyl group of serine or threonine residues on different proteins (**Figure 1.7**). O-GlcNAc modification is a highly dynamic, post-translational modification, similar to phosphorylation that appears to be an essential regulatory mechanism in signalling pathways (Issad *et al*., 2010). However, unlike phosphorylation only two key enzymes regulate the addition and removal of O-GlcNAc on

proteins. O-GlcNAc-transferase (OGT) catalyses the addition of O-GlcNAc to proteins, while O-GlcNAcase catalyses the removal of the sugar molecule (Fülöp *et al*., 2007). O-GlcNAcylation competes with phosphorylation for the same serine or threonine residues, which can then affect the phosphorylation status of a protein (Issad *et al*., 2010).

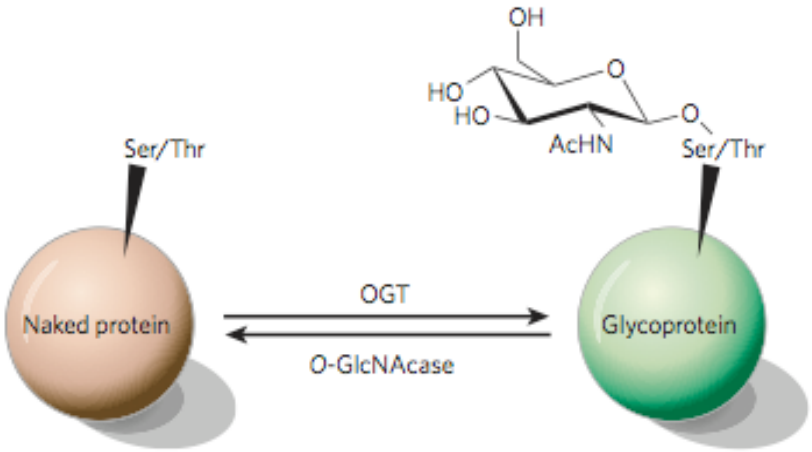

Figure 1.7. The addition and removal of O-GlcNAc from serine and threonine residues is catalyzed by the two enzymes: OGT and O-GlcNAcase (figure is from Hart *et al*., 2007).

1.7.1 The hexosamine pathway and insulin resistance

Several investigations in cell culture and in rodents *in vivo*, have found that an increased flux through the hexosamine pathway can result in insulin resistance (Buse, 2006). Marshall *et al*. (1991) were the first to describe the role of the hexosamine pathway in the development of insulin resistance. They demonstrated that insulin insensitivity was induced in rat adipocytes cultured in medium containing a high-glucose concentration, insulin and glucosamine (Issad *et al*., 2010). The effect of these agents was prevented when blocking the enzyme GFAT using the inhibitors AZA or DON (Zachara & Hart, 2006). In addition to hyperglycemia, insulin and glucosamine treatment, the elevation of free fatty acids and over-expression of GFAT have all shown to increase the flux through the hexosamine pathway and lead to different degrees of insulin resistance (Wells & Hart, 2003). Considering that O-GlcNAc modification is dependent on flux through the hexosamine pathway, it has been suggested to be a potential mediator of insulin resistance (Wells *et al*., 2003). The dysregulation of O-

GlcNAc modification is associated with a number of human diseases, particularly diabetes (Hart *et al*., 2007).

Investigations conducted by Marshall *et al*. (1991) demonstrated that inhibition of GFAT blocks hyperglycemia-induced increases in the transcription of TGF-α, TGF-β1 and plasminogen activator inhibitor-1 (PAI-1) (Nerlich *et al*., 1998). The mechanisms by which increased flux through the hexosamine pathway, lead to hyperglycemia-induced increases in gene transcription has not yet been determined. However, studies have shown that hyperglycemia give rise to a 4-fold increase in O-GlcNAcylation of the transcription factor Specificity Protein 1 (Sp1), inducing activity of TGF-β1 and PAI-1 in arterial endothelial cells (Brownlee, 2001; Kitada *et al*., 2010; Hart *et al*., 2007). O-GlcNAc is postulated to play a central role in modulating insulin signalling (**Figure 1.8**) and in the development of glucose toxicity. Hyperglycemia leads to the conversion of glucose to glucosamine, which in turn results in an increase of the "nutrient sensor" UDP-GlcNAc, ultimately causing a rise in O-GlcNAcylation. The increase in O-GlcNAcylation on insulin receptor substrate (IRS) appears to reduce the interaction with phosphatidylinositol-3-OH kinase (PI3K), blocking insulin signalling at an early point in time (Hart *et al*., 2007). Moreover, many studies have shown that the translocation of insulin-dependent GLUT4 to the membrane is inhibited when the flux through the hexosamine pathway is increased. This has been related to a defect in AKT (also known as protein kinase B) phosphorylation and activation, as a result of hyperglycemia or glucosamine treatment. Thus, it appears that increased movement through this pathway blocks signal transduction at or upstream of AKT (Wells *et al*., 2003).

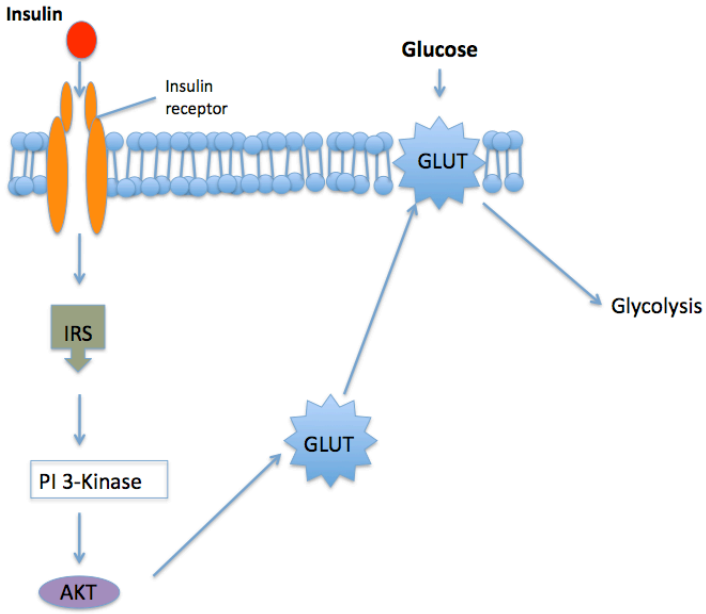

Figure 1.8. Overview of insulin signalling pathway. Binding of insulin to the insulin receptor (IR) leads to a conformational change in the IR, resulting in a signalling cascade inside the cell. Receptor activation initiates phosphorylation of IRS (insulin receptor substrate), triggers the activation of phosphoinositide 3-Kinas (PI3K) and AKT (PKB), causing the translocation of GLUT4 to the cell membrane, and thereby allowing glucose to be taken up into the cell (Rexford *et al.,* 2010) (Figure is adapted from Lebeche, 2008).

1.8 Extracellular Matrix and diabetes

The extracellular matrix (ECM) is a dynamic interactive milieu that critically influences cell functions. The ECM consists of a network of collagens, elastins, structural glycoproteins, proteoglycans and hyaluronan. This network maintains a mechanical support for cells and allows complex interactions to take place between cells, or between cells and the ECM (Ban & Twigg, 2008). There is a continuous synthesis and degradation of the ECM components. The turnover of the ECM is vital for normal growth and development, in addition to cell functions. A disruption of this balance may result in a build-up of ECM molecules. The MMPs and their corresponding tissue inhibitors of metalloproteinases (TIMPS) are main regulators of ECM turnover and homeostasis. An imbalance between MMP levels and their inhibitors may shift the equilibrium of the matrix turnover towards matrix accumulation or increased degradation. Changes in vascular structure contribute to the pathogenesis of vascular complications of diabetes (Lenz *et al*., 2000). Fibrosis is described by a build-up of ECM components and by vascular remodelling. The accumulation of ECM and the thickening of the basement membrane are commonly seen in the major organs affected in diabetes (Ban & Twigg 2008). Increased matrix accumulation in the endothelium also contributes to the accumulation of lipoprotein, through retention, described previously.

1.9 Matrix metalloproteinases

Structure and function of matrix metalloproteinases

MMPs belong to the metzinicin superfamily of metalloproteinases. The enzyme family comprise of 23 zinc and calcium dependent enzymes, which have been found in humans (**Figure 1.9**) (Offersen *et al*., 2010; Hadler- Olsen *et al*., 2011) that share common functional domains. MMPs can be separated into 6 groups based on substrate specificity (Kadoglou *et al*., 2005; Lim *et al*., 2010):

1. Collagenases (MMP-1, -8, -13, -18)

2. Gelatinases (MMP-2, -9)

3. Stromelysins (MMP-3, -10, 11)

4. Matrilysins (MMP-7, -26)

5. Membrane-type MMPs (MT-1, -2, -3, -4, -5)

6. Other MMPs (e.g. MMP-12, -19, -22)

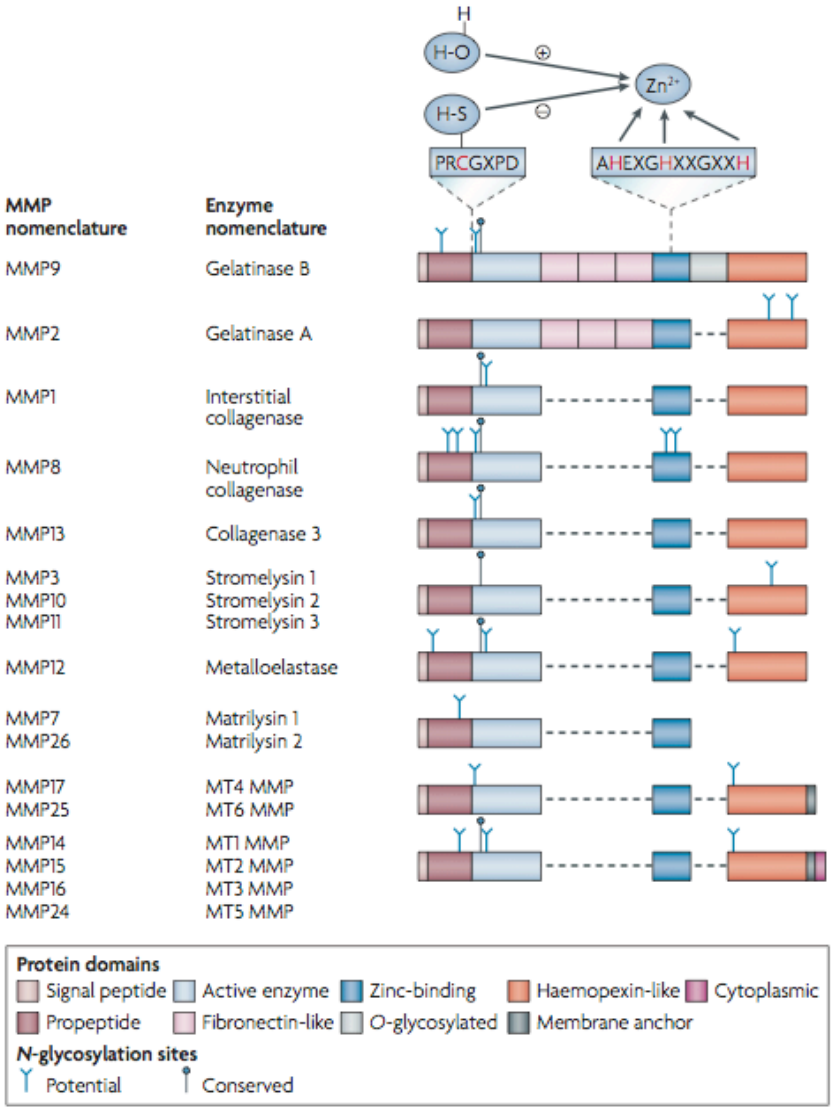

Figure 1.9. Domain structures of matrix metalloproteinases (MMPs). MMPs are multidomain proteases that contain a pro-domain, an active domain, a zinc-binding domain and a heamopexin domain. The characteristic of the MMP family is the catalytic site that contains the zinc-binding domain. MMPs can be divided into 6 different groups depending on their substrate specificity. Gelatinases (MMP-2 and MMP-9) contain a gelatin-binding domain with three fibronectin-like repeats. MMP-9 comprises a serine-, threonine-, and proline rich O-glycosylated domain, and both MMP-2 and MMP-9 can be *N*-glycosylated. The majority of MMPs are secreted in a latent pro-form. The removal of the pro-peptide (about 10 kDa), results in enzyme activation (Figure is from Hu *et al*., 2007).

The substrate specificity of MMPs is broad. MMPs are able to degrade approximately all components of ECM and thus affect the turnover of this compartment. These enzymes are also involved in several other physiological and pathological processes, such as wound

healing, inflammation, development and reproduction, and tumour metastasis (Hadler-Olsen *et al*., 2011; Kadoglou *et al*., 2005; Manon-Jensen *et al*., 2010). It has been suggested that MMPs in blood can be used as potential markers for vascular changes in diabetes (Gharagozlian *et al*., 2009)

1.9.1 Regulation and activation of MMPs

MMP activity is rigidly controlled at 4 different levels, including (1) gene transcription, (2) activation, (3) inhibition by the TIMPs (Kadoglou *et al*., 2005), and (4) compartmentalization (Ra & Parks, 2007; Hadler Olsen *et al*., 2011). A broad range of inflammatory cytokines, growth factors and tumour promoters such as interleukin-6, platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and TNF-α, can induce MMP transcription (Hu *et al*., 2007; Kadoglou *et al*., 2005). All MMPs with the exception of MT-MMPs are zymogens, meaning they are secreted in a latent pro-form (Chakrabarti & Patel, 2005). In this inactive form, a cysteine sulphydryl group in amino-terminal prodomain interacts with a zinc ion and blocks the active site of the enzyme. Activation of the enzymes occurs in extracellular space and is instigated by other proteases such as other MMPs (particularly membrane-type MMPs), elastase or trypsin. Furthermore, MMP activity is also regulated by TIMPs, which act by binding reversibly to latent or active forms of MMPs. The binding of TIMPs blocks the access of substrates to the catalytic site, and thereby inhibits their enzyme activity. There are four known types of TIMPs (TIMP-1-4), each with varying specificity for the different types of MMPs (Hu *et al*., 2007). Compartmentalization describes where and how in the intracellular compartments MMPs are stored and released, and is important for regulating enzyme activity. Based on their motifs and modules, the secreted MMPs are directed to various destinations in the extracellular environment including cell membranes. MMPs can then bind to collagens, laminins, fibronectins, core-proteins and GAG chains of proteoglycans. Compartmentalization regulates MMP activity based on location and by concentrating them close to potential substrates (Ra & Parks, 2007; Hadler-Olsen *et al*., 2011).

The two gelatinases A and B (MMP-2 and MMP-9 respectively) are the most significant MMPs in normal kidneys and are thus believed to play vital roles in basement membrane and mesangial matrix homeostasis. Both enzymes are able to break down basement membrane collagens and gelatines, however, their substrate specificity is not identical. MMP-2 degrades

fibronectin and laminin, and has considerably lower activity against types IV and V collagen than MMP-9 (Hadler-Olsen *et al*., 2011). Moreover, these enzymes differ significantly in their promoter structure and therefore also in their expression pattern. MMP-2 and MMP-9 arrange in complexes with their tissue inhibitors, TIMP-2 and TIMP-1, respectively (Lenz *et al*., 2000). MMP-2 and MMP-9 are secreted as 72 kDa and 92 kDa pro-forms, respectively. They are cleaved into their corresponding active forms: 64 kDa (MMP2) and 83 kDa (MMP-9). MMP-2 is secreted from a variety of cell such as endothelial cells and macrophages, while MMP-9 is mainly produced by neutrophils and eosinophils. However, inflammatory stimulation may induce increased expression of MMP-9 in other cell types such as endothelial cell, macrophages and fibroblasts (Chakrabarti & Patel, 2005).

1.9.2 MMP and diabetic nephropathy

Hyperglycemia has been shown to trigger signalling cascades leading to a disruption of the balance between ECM synthesis and degradation (Ban & Twigg, 2008). The ECM in the basement membrane of the kidney glomeruli is especially significant for filtration properties. Structural modifications in mesangial and basement matrix are associated with proteinuria and hypertension and hence, the development of diabetic nephropathy (Gharagozlian *et al*., 2009). The two enzyme families MMPs and TIMPs play an important role in vascular remodelling of the basement membrane components (Tsilibary, 2003; Lenz *et al*., 2000). Changes in MMP activity is proposed to be a crucial contributing factor in the pathogenesis of diabetic nephropathy (Thrailkil *et al*., 2009; Roy *et al*., 2009). Thickening of the glomerular basement membrane takes places early after the onset of diabetes and may gradually develop into diabetic nephropathy (Tsilibary, 2003; Roy *et al*., 2009). The excess build-up of basement membrane components is a consequence of an up-regulated synthesis and a reduced degradation under hyperglycaemic conditions (Tsilibary, 2003). Thus, the regulation of MMPs is crucial for understanding the different physiological processes and pathogenesis of diseases, along with the development of new specific MMP target drugs, which may be a useful treatment strategy for diabetes and atherosclerosis (Hadler-Olsen *et al*., 2011; Death *et al*., 2003).

2 Aims

The main goal of the current thesis was to study the biological effect of hyperglycemia on human endothelial cells. The intention of my investigations was to study factors that may play a role in matrix changes observed during the development of diabetic nephropathy. MMP-2 and MMP-9 have been identified as the most important metalloproteinases in the kidney, regulating the turnover of the extracellular matrix in the basement membrane. Many of the systemic changes and those seen in the kidneys involve changes in the endothelial cells and in particular the development of endothelial dysfunction. In addition, the hexosamine pathway has been suggested to play a role in the development of endothelial dysfunction in relation to hyperglycemia. To study matrix changes in further detail, primary human endothelial cells were used in this study and grown on traditional plastic wells and on semipermeable filters.

Specific aims:

1. To study whether hyperglycemia will affect synthesis and secretion of MMP in human umbilical vein endothelial cells (HUVEC).

2. To study if the hexosamine pathway is involved in the regulation of MMP secretion in endothelial cells using the inhibitor DON, to interfere with this pathway.

3 Methods

3.1 MCDB Medium

There are different types of MCDB medium, which each have been formulated to provide optimal growth for specific cell lines. MCDB-131 media has been developed specifically to culture human microvascular endothelial cells, including HUVEC (Sigma-Aldrich, 2011).

Preparation of MCDB-131 medium

One container of MCDB-131 powder (11.6 g) was dissolved in 850 ml of MQ-H_2O and 1.18 g of sodium hydrogen carbonate ($NaHCO_3$) was added to the solution. The pH was adjusted to 7.3 while stirring. The final volume was adjusted to 1000 ml by adding MQ-H_2O. The medium was sterile filtered into two 500 ml flasks, using a sterile filter flask with a 0.22 µm pore size. Antibiotics, growth factors and FCS (fetal calf serum) were added to 500 ml of medium, as listed below in **Table 3.1**.

Table 3.1 Components added to the MCDB-131 medium

Components (Concentration)	Amount
FGF (10 µg/ml):	50 µl
Hydrocortisone (10 mg/ml)	50 µl
EGF (10 µg/ ml):	500 µl
Gentamicine (50 µg/ml)	500 µl
Fungizone (250 µg/ ml)	500 µl
FCS (7%) (Heat inactivated)	37.5 ml

3.2. Isolation of Human Umbilical Vein Endothelial Cells (HUVEC)

HUVEC are primary endothelial cells isolated from the vein of the human umbilical cord. The method was first developed by Jaffe and Nachman in 1973 (Jaffe *et al*., 1973). HUVEC is used as a model for endothelial cells for a large community of researchers all around the world (Baudin *et al*., 2007).

HUVEC were obtained fresh from the obstetric and gynaecology division, Rikshospitalet, Oslo. Written consent was acquired from the mothers and ethical approval was obtained from the Human Research Ethical Committee.

Procedure

HUVEC were isolated from the vein of the umbilical cord (**Figure 3.1**) by an adjustment of the method by Jaffe *et al*. (1973). The isolation of the endothelial cells was carried out under sterile conditions. Immediately after delivery, the umbilical cord was placed in a sterile container filled with transport buffer (**Appendix I Table 8.5.0**). The cord was kept in the fridge at 4°C for a maximum of 24 h before isolation. Damaged tissue was removed and the vein was rinsed with phosphate buffered saline (PBS) (**Table 8.5.2)** in order to remove any remaining blood. Thereafter, the cord was incubated with 10 ml of 0.2% collagenase (**Table 8.5.3**) for 10 min, at 37°C in order to isolate the endothelial cells from the vein. The cell solution was centrifuged at 1500 rpm for 10 min. The cell pellet was resuspended in 15 ml of complete MCDB-131-medium, transferred to a 75 cm^2 cell culture flask and incubated at 37°C in a humidified 5% CO_2 atmosphere. The culture medium was changed the following day in order to remove remaining blood cells.

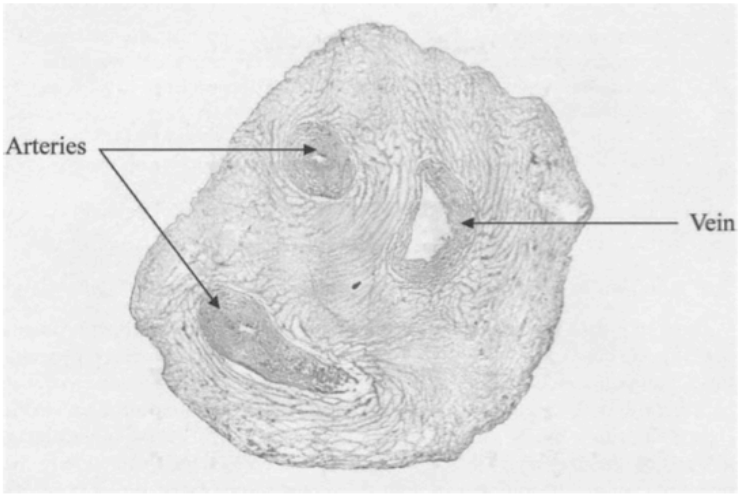

Figure 3.1. HUVEC were isolated from the vein of the umbilical cord under sterile conditions. The figure demonstrates a cross-section of an umbilical cord displaying the vein and its two arteries (Figure is taken from Helgason & Miller, 2005)

3.2.1 Polarization of HUVEC

Polarized HUVEC culture is a useful method for studying endothelial cells, as the cells are able to secrete proteins into both the apical (towards lumen) and the basolateral medium (towards VSMC), in a near *in vivo* condition. To obtain polarization, HUVEC were cultured on 12-well Costar Transwell clear polyester membrane filter inserts, with a 0.4µm pore size (**Figure 3.2**). The filter plates were incubated with MCDB-131 medium containing FCS, 1-16 h in advance of HUVEC culture, in order to coat the membrane with plasma proteins.

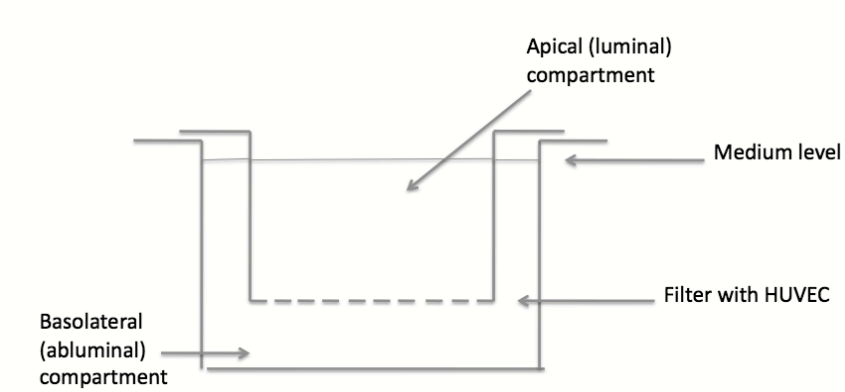

Figure 3.2. Illustration of one well filter inserts for culturing polarized cells. The illustration is based on image from Rosengren *et al*., 1991

3.3 Culture of HUVEC

Cells were cultured at 37°C in 5% CO_2, in complete MCDB-131 medium, containing 7% FCS. Cells were either cultured on traditional plastic plates or on filter plates. The medium was changed every second day, using 15 ml for a 75 cm^2 flask. Only cells from passages 2-4 were used in experiments, due to the risk of changes in phenotype in cells from higher passages (Cheung, 2007). Cells were split when they had reached a confluence of about 80%, which constitute approximately 50-100 000 cells per cm^2 (**Figure 3.3**) Cells were therefore regularly monitored in the microscope. Cells were split approximately 1-2 times per week, depending on their growth. HUVEC were washed with 5 ml of PBS to remove serum, as serum inhibits the action of trypsin. Next, the cells were incubated in 4 ml of trypsin for approximately 2-5 min, in order for the cells to loose surface contact. Detached cells were observed as round shapes as opposed to the long shape of the attached endothelial cell. Fresh complete medium (4 ml) was added in order to deactivate the trypsin. The cells were transferred into a falcon tube and pelleted at 1300 rpm, for 3 min at room temperature and resuspended in a desired volume of complete medium before transferring to flasks or wells. The cells were split in a 1:3 ratio.

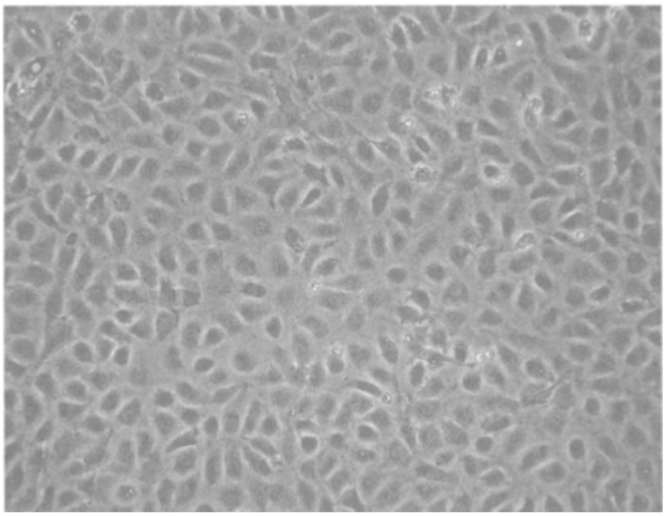

Figure 3.3. The image shows a phase-contrast micrograph of a confluent monolayer of HUVEC (Figure taken from Helgason & Miller, 2005)

3.4 General experimental outline

HUVEC were cultured on conventional plastic plates (6 or 12 wells), in cell culture flasks for Western analysis and on filter plates (12 wells). HUVEC were grown in 2 ml medium for each well of the 6-well plate and in 1 ml of the 12-well plastic plate. In the polarized experiments, 1 ml of medium was loaded in the basolateral well, while 0.5 ml of medium was loaded apically (**Figure 3.2**).

When cells had reached a confluence of more than 80 % on plastic and 100 % on filter, they were exposed to various concentrations of glucose (5, 15, 25, and 40 mM) or DON (50, and 100 µM) for a certain amount of time (usually 24 h). Normoglucose (5 mM, without DON) was used as a control in all experiments. The baseline medium already contained a glucose concentration of 5 mM and therefore had to be taken into account when calculating concentrations. Cells were washed and cultured in serum-free MCDB-medium for the last 24 h, to avoid proteases from the serum to obstruct the results.

3.5 Harvesting of medium

The conditioned medium (1 ml) was transferred to Eppendorf tubes containing 100 µl of 100 mM $CaCl_2$ and 100 µl of 1 M Hepes, while working on ice. Hepes and $CaCl_2$ were added to the samples in order to stabilize the MMP and avoid degradation of the proteins when freezing and thawing the samples. Ca^{2+} stabilizes the active seat of MMP, while Hepes regulates the pH. The samples were spun at 10 000 rpm for 10 minutes at 4°C, to remove cells and allocated to new Eppendorf tubes, and immediately kept in the freezer at -20°C.

3.6 Lysis of HUVEC

Cell lysis is the method by which a cell is broken down as a result of some external force or treatment. The remaining substance consists of the destroyed cells and is referred to as the lysate. The purpose of cell disruption is to release the intracellular proteins. Radio-Immunoprecipitation assay (RIPA) (**Table 3.6**) is used for cell lysis and protein solubilisation while avoiding protein degradation. 250 µl of complete RIPA buffer was added to each well, for the 12 well plastic plate, while 170 µl of RIPA was added to each well when using the semi-permeable 12-well filter plates. The cells were scraped from the wells in ice-cold complete RIPA buffer. Samples were allocated to Eppendorf tubes and placed on ice for 30 min and were tapped approximately every 10 minutes. The whole cell lysates were centrifuged at 10 000 rpm, for 10 min, at 4˚C, in order to remove cell debris. The supernatants were allocated to new Eppendorf tubes and frozen down at -20˚C until protein quantification was carried out.

Table 3.6 Complete RIPA buffer

Components	**Final concentration**
Tris HCl pH 7.4	0.05 M
NaCl	0.15 M
Triton X-100	1%
SDS	0.1 %
Natriumdeoxycholate	0.5 %
EDTA	1 mM
Natriumpyrophosphate	10 mM

1 protease-inhibitor tablet pr 10 ml RIPA added right before use.

3.7 Protein Quantification

Principle

The Bicinchoninic Acid (BCA) assay is a biochemical technique used to determine the protein concentration in a solution. The colour solution indicates the total protein concentration within a sample, as it switches from green to purple. This can be measured by colorimetric methods. The BCA assay is based on two principle reactions. Firstly, the peptide bonds in proteins reduce Cu^{2+} ions from the cupric sulphate to Cu^{1+} (a temperature dependent reaction). The amount of Cu^{2+} reduced is comparable to the quantity of protein in the sample. Secondly, two molecules of bicinchoninic acid chelate with each Cu^{1+} creating a purple coloured product that efficiently absorbs light at a wavelength of 562 nm (Thermo Fisher Scientific, 2011; Olsen & Markwell, 2007).

Procedure for determining protein concentrations

A standard curve was prepared and plotted based on a BSA stock solution (2 mg/ ml). RIPA buffer was added to the standards (20 µl to each well) in order to adjust for potential effects of the RIPA buffer in the samples (**Table 3.7**). In this way, standards and samples were treated as similar as possible.

In each well 20 µl of sample, 30 µl of MQ-H_2O and 200 µl of A + B solution was added. Triplicates were made for each sample. The A+B solution was made from 98% of micro reagent A and 2% of micro reagent B. The microtiter plate was incubated at 37ºC for 30 min. The absorbance was measured at wavelength of 562 nm, using the Titertec Multiscan spectrophotometer. A standard curve was made, plotting the absorbance (Y-axis) versus the known protein concentrations (X-axis). The unknown protein concentrations were calculated using the standard curve, taking dilutions into account.

Table 3.7. Standard samples

Well	BSA (2mg/ml)	MQ-H_2O	Lysis buffer	A+B solution
A	0 µl	30 µl	20 µl	200 µl
B	0 µl	30 µl	20 µl	200 µl
C	3 µl	27 µl	20 µl	200 µl
D	3 µl	27 µl	20 µl	200 µl
E	5 µl	25 µl	20 µl	200 µl
F	5 µl	25 µl	20 µl	200 µl
G	8 µl	22 µl	20 µl	200 µl
H	8 µl	22 µl	20 µl	200 µl

3.8 Gelatin zymography

Principle

Zymography is an electrophoretic technique used for measuring enzyme activity and is widely used for studying MMPs. The standard method is based on sodium dodecyl sulphate (SDS) gels infused with a protein substrate. The substrate used here was gelatine, as MMP-2 and MMP-9 are both able to break down this substrate and can therefore be recognized on gelatine zymograms (Troeberg & Nagaese, 2003). In zymography, the gel traditionally consists of water, buffer, acrylamide, ammonium persulfate (APS) and tetramethylethylenediamine (TEMED). Polymerization is commenced by APS and TEMED. TEMED stimulates the formation of free radicals from persulfate and these in turn catalyze polymerization. TEMED and APS are therefore added right before loading the gel (Menter, 2000). The samples are first loaded into wells of the stacking gel, which has a lower concentration of acrylamide, a lower pH and a different ionic content. This enables the proteins in a loaded sample to be concentrated into tight bands before entering the resolving part of a gel. The separation gel (resolving gel) allows the loaded sample to become fractioned. The gel acts a matrix, separating out molecules according to their charge and size. A higher concentration of acrylamide will ease the separation of small compounds (Thermo Fisher Scientific, 2011). During electrophoresis, SDS causes the MMPs to denature (reducing the protein to its primary structure) and become inactive. Following electrophoresis, the gel is washed in a buffer (**Appendix I Table 8.5.4),** to remove the SDS. The enzymes then partially regain their activity. Subsequently, the gel is activated in 1x incubation buffer (**Table 8.5.5**) over night and the renatured MMPs in the gel will digest the substrate gelatine. Following incubation, the gel is dyed with Coomaisse Blue (**Table 8.5.6**) and then washed in destaining buffer (**Table 8.5.7**) in order to remove unbound dye, where the MMPs have digested the gelatine. Thus, the MMPs are detected as clear bands against a blue background of undegraded substrate (Snoek-van Beurden & Von den Hoff, 2005).

Procedure

Gel caster components were wiped clean with ethanol in order to remove any proteins that could potentially obstruct the results. The separation gel (**Table 8.5.8)** was carefully loaded between the glass plates of the sandwich stack. MQ-H_2O was added in order to obtain a straight line on top of the gel. The gel was left at room temperature for 15-20 min for the gel to solidify. The water was carefully removed from the top of the gel using filter paper, and stacking gel (**Table 8.5.9**) was loaded. The comb was inserted into the stacking gel in order to generate wells. When the gel had stiffened, the gel caster sandwich was removed and placed in the electrophoresis apparatus, and 1x running buffer (**Table 8.6.1**) was poured in. Recombinant standards of pro-MMP-2 and pro-MMP-9, and samples containing equal amounts of protein, were loaded into each well. Electrophoresis was run at 30-40 mA for approximately 2 h when using two gel caster sandwiches, and at 20 mA when only using one. Electrophoresis was continued until the blue colour had driven out of the gel. Next, the gel was washed with 50 ml of washing buffer (**Table 8.5.4**) for approximately 45 min, changing the buffer once. The gel was incubated in incubation buffer (**Table 8.5.5**) overnight at 37ºC. The gel was dyed using 50 ml of staining solution (**Table 8.5.6**) for 30-60 min, followed by 50 ml of destaining solution (**Table 8.5.7**) for another 45-60 min, changing the buffer once. The gel was stored in MQ-H_2O and was photographed under normal light.

Quantification

The intensities of the bands obtained from zymography were quantified using the programme Quantity-One (Bio-Rad). The image (Tiff) was imported into the programme and an equal sized rectangular square was placed around each gelatinase band, in order to measure the intensities (relative units) of the bands.

3.9 SDS-PAGE

In sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), the protein samples are treated with 2-mercaptoethanol to reduce any disulfide bonds. SDS is a powerful anionic detergent, which binds to hydrophobic regions of protein molecules denaturing their structures, causing the unfolding of polypeptide chains. SDS covers the proteins with an overall negative charge. The samples are loaded on a highly cross-linked gel matrix of polyacrylamide. Smaller proteins migrate more easily through the gel than bigger proteins. In this way, a sample consisting of a complex mixture of proteins is separated into a series of distinct protein bands according to size only (Horton *et al*., 2002; Hames & Hooper, 2002).

Procedure

The cell lysates were prepared as indicated earlier, and diluted with RIPA in order to obtain equal concentrations of the proteins. 30 µl of sample was mixed with 10 µl of (4x) loading buffer (**Table 8.6.2**). All samples were boiled for 5 min in a heating block and spun quickly down. Precast gels (Criterion ^{TM} Precast gel, 4-20% Tris HCl) were submerged into the electrophoresis apparatus and running buffer (**Table 8.6.1**) was poured into the container. Samples and protein standards were loaded into the indicated wells. The SDS was run at 120 V for approximately 2 h until the bromphenol blue reached the bottom of the gel.

3.9.1 Western blotting

Western blot is used to identify specific proteins in a given sample. Proteins are separated by size by SDS-PAGE, and are transferred to a membrane. The membrane is blocked in order to prevent any nonspecific binding of antibodies to the surface of the membrane. Thereafter, the membrane is incubated with a primary antibody specific to the protein of interest. The membrane is then incubated with secondary antibody conjugated to horseradish peroxidase (HRP). The secondary antibody recognizes the primary antibody. Enhanced chemiluminescence (ECL) plus is applied to the blot. The ECL solution contains luminol, which is substrate for HRP. Light is produced when luminol is oxidated and the light output can be captured on film.

Procedure

The gel was removed from the electrophoresis apparatus and equilibrated for 15 min in blotting-buffer in order to remove remaining SDS. Polyvinylidene fluoride (PVDF)-membrane and filter papers were cut in the same size as the gel before activating the membrane in methanol for 15 sec. The membrane was then equilibrated for 10 min in blotting buffer (**Appendix I Table 8.6.4**). The sandwich cassette was put together in the following order (red side of the cassette facing up): 1 sponge, 2 filter papers, PVDF membrane, gel, 2 filter papers, and 1 sponge. Air bubbles were carefully removed in order not to prevent the transfer of proteins to the membrane (**Figure 3.9**).

Figure 3.9. Assemble of the cassette

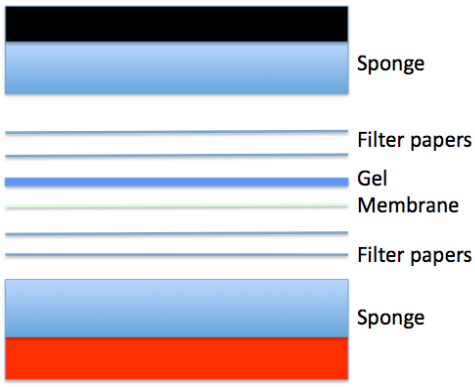

Figure 3.9. Assemble of the cassette. The figure shows that the construction of the components. The sponge was first soaked in TBST and placed on the red side of the cassette, followed by two filter papers, the membrane, the gel, two filter papers and finally a sponge.

The cassette holding the sandwich stack was inserted in the blotting tank as colour indicated, and transfer buffer (**Table 8.6.6**) was poured into the tank to the fill mark. The transfer buffer maintains the conductivity and pH, so that the proteins can easily migrate from the cathode to the anode, thus, transfer of proteins from the gel over to the membrane (blotting). A cooling agent (ice block) and a stirring bar were added to the tank. The blotting was run at 100 V for 60 min.

Blocking non-specific sites

The membrane was blocked with 3% BSA (bovine serum albumin) in TBST (**Table 8.6.8**) prior to immunostaining, in order to prevent non-specific binding of antibodies.

Immunostaining/ Detection

The membrane was incubated with a primary antibody diluted in TBST at 4°C over night at an agitator. The membrane was then rinsed 3 x 5 min in TBST in order to remove any unbound primary antibody. Next, the membrane was incubated with a HRP-conjugated secondary antibody (**Appendix I)** diluted in TBST for 60 min at room temperature, before removing unbound secondary antibodies by washing 3 x 5 min in TBST.

Development

The membrane was incubated with ECL (plus) solution for 5 minutes and placed in a Hypercassette^{TM}. Light produced was captured on a film and the image developed using Hyperprocessor Amersham Pharmacia biotech developer.

4 Results

Endothelial cells are the first cells to be exposed to hyperglycemia in type 1 and type 2 DM. Elevated blood glucose levels may lead to endothelial dysfunction and promote inflammatory reactions in these cells (Brownlee, 2005). To investigate the effects of hyperglycemia on the endothelium we used primary cultures of endothelial cells isolated from human umbilical cords. MMP-2 and MMP-9 were used as targets to investigate possible effects of hyperglycemia, due to the importance of these enzymes in both diabetes related inflammation and fibrosis. HUVEC were exposed to normo- (5 mM) and hyperglycemic (25 mM) conditions and the effects on MMP secretion were studied. In addition, the possible importance of the hexosamine pathway for effects of hyperglycemia on HUVEC was investigated using DON, an inhibitor of the enzyme GFAT.

4.1 Effect of hyperglycemia on protein synthesis

The initial experiments were performed to measure possible effects of hyperglycemia on protein synthesis as a marker for general condition of the cells. HUVEC were cultured in conventional plastic and exposed to four different concentrations of glucose (5 mM, 15 mM, 25 mM, and 40 mM) in serum-free media for 24 h. Serum-free medium was used due to high levels of MMPs in serum. Protein concentration was measured in the cell lysates. Protein synthesis increased slightly with increasing glucose concentrations, up to 25 mM and then decreased with the highest concentration (40 mM), as can be seen in **Figure 4.1**. The same trend has been observed in a second experiment with cells obtained from another umbilical cord (**Appendix II Figure 9.1**).

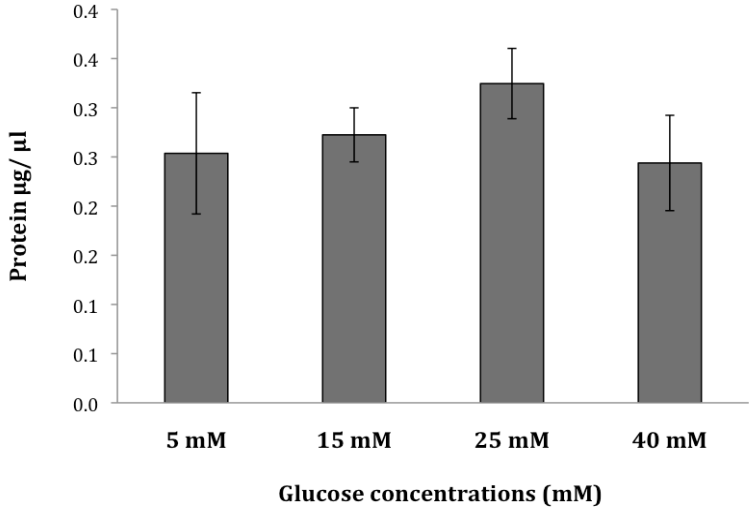

Figure 4.1. Protein synthesis in response to glucose concentrations. HUVEC were cultured with different glucose concentrations (5 mM, 15 mM, 25 mM, and 40 mM) for 24 h in serum-free media. Results presented are from cell fractions originating from three separate wells.

4.2 Effect of hyperglycemia on gelatinase secretion

The purpose of the next experiment was to study the effect of varying glucose concentrations on gelatinase secretion from HUVEC. The cells were obtained from two different donors and incubated with 4 different concentrations of glucose (5 mM, 15 mM, 25 mM and 40 mM) for 24 h. Medium samples with equal protein concentrations were subjected to zymography to measure possible gelatinase activities. The most prominent bands observed, and in most cases the only band observed in the zymography gel, migrated as the pro-MMP-2 (72 kDa) standard, as can be seen in **Figure 4.2A.** There was a greater gelatinase activity obtained from medium of cells from donor 1 cultured under NG conditions (5 mM) compared to HUVEC exposed to higher concentrations. This trend was also seen in medium obtained from cells from donor 2 (**Figure 4.2A**). The intensity of each band was measured (**Figure 4.2B**). These analyses clearly demonstrated that gelatinase activity decreased when increasing the glucose concentration from 5 to 15 mM. Above this concentration there was a variation in effect between the two different HUVEC preparations used. No gelatinase activity was observed that co-migrated with the pro-MMP-9 standard in medium obtained from both donors. To further confirm that HUVEC released MMP-2, conditoned medium was subjected to Western blotting, which showed that bands from both NG and HG media are indeed MMP-2 (**Figure**

4.2C). In addition, a metalloproteinase inhibitor, ethylenediaminetetraacetic acid (EDTA) was used in parallel zymography experiment. Using this chelator showed a complete abolishment of gelatinase activities (not shown), demonstrating that the enzyme activity observed in zymography experiments is of metalloproteinase nature.

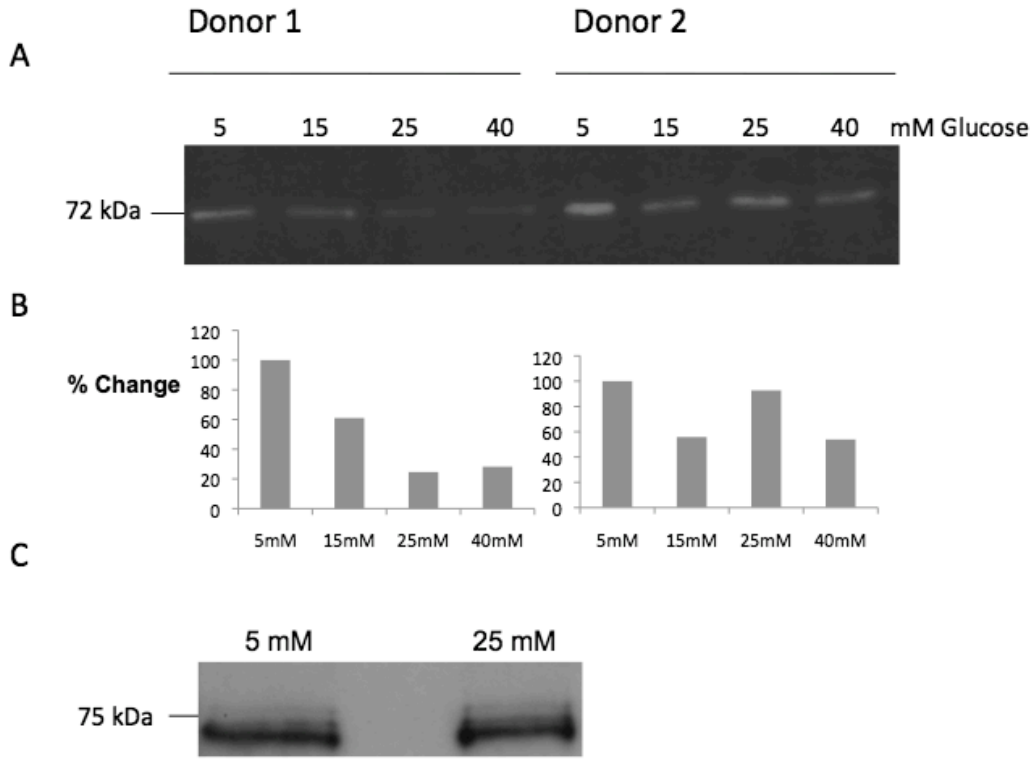

Figure 4.2. A. Gelatinase secretion from HUVEC. Zymography was performed on medium obtained from two HUVEC donors. HUVEC were cultured with different glucose concentrations (5 mM, 15 mM, 25 mM, 40 mM) for 24 h. The medium samples with equal protein concentrations were loaded and separated in a 4-7.5 % SDS-gel. **Figure 4.2.B.** Quantification of bands from the zymography in Figure 2A. Donor 1 shows a higher secretion of pro-MMP-2 in 5 mM compared to 25 mM (306.6 % change). Donor 2 shows a higher secretion of pro-MMP-2 compared to 25 mM (7.9 % change). **Figure 4.2.C.** Immunoblot on medium samples from HUVEC cultured with NG for 24 h using an anti-human MMP-2 polyclonal antibody, which recognized both latent and active forms of MMP-2. In this case, only the pro-form of MMP-2 was visible (72 kDa).

4.3 Effect of hyperglycemia and time of incubation on gelatinase secretion

Furthermore, it was of interest to investigate the effects of hyperglycemia and duration on gelatinase secretion. HUVEC were incubated with 4 different concentrations of glucose (5 mM, 15 mM, 25 mM and 40 mM). Cells were harvested 2, 6 and 24 h after incubation under serum-free conditions and subjected to zymography. The results from the zymography indicate that pro-MMP-2 secretion increased with longer incubation time. In addition, there was a greater activity at NG (5 mM) compared to higher glucose concentrations at 6 h and 24 h. Accordingly there is a decrease in pro-MMP-2 secretion with increasing concentrations of glucose (**Figure 4.3A**). The intensity of the bands was measured (**Figure 4.3B**), which confirmed that the there is a higher activity of pro-MMP-2 at 5 mM after 6 h, compared to the higher concentrations of glucose. After 24 h, the expression of pro-MMP-2 is greater at 5mM compared to 25 mM and 40 mM of glucose, but not 15 mM. Again, no bands were observed with activity migrating as the pro-MMP-9 standard. These results show the same trend as the findings in **Figure 4.2**. Experiments were also performed up to 8 and 14 days in order to investigate the effect of MMP-2 secretion over longer time periods. The results varied, but indicated a greater secretion of pro-MMP-2 in the HG condition opposed to NG, giving contradictory results (**Table 4.1**).

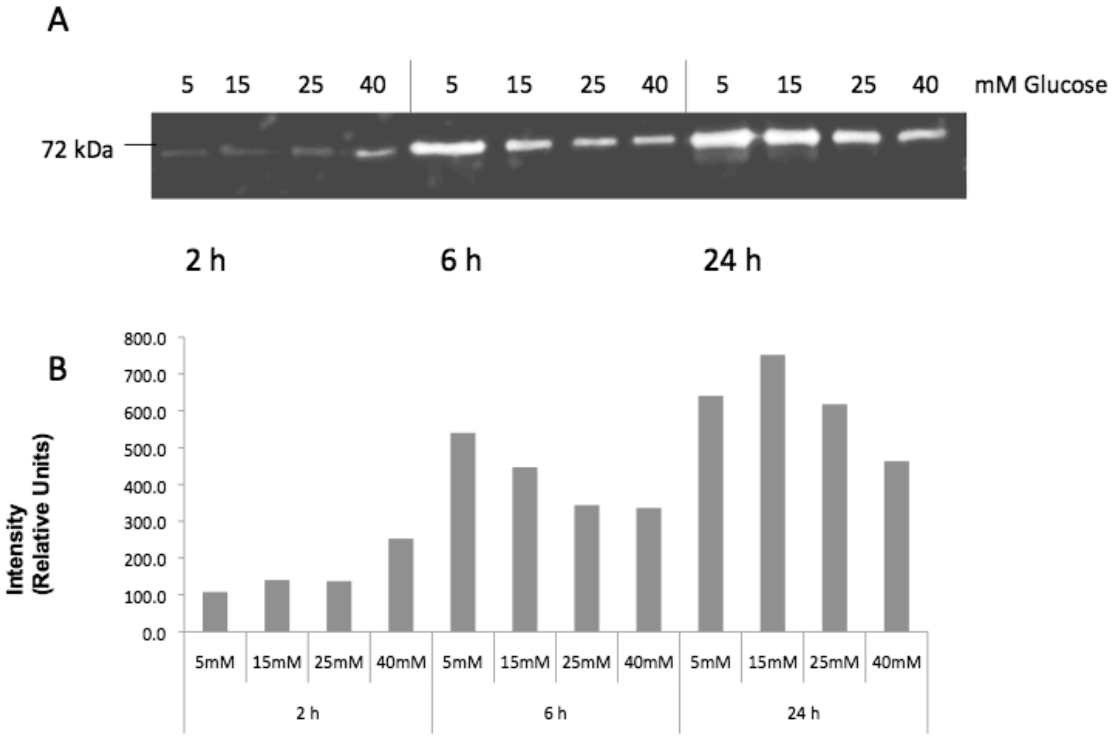

Figure 4.3A. Zymography of medium from HUVEC exposed to various glucose concentrations (5 mM, 15 mM, 25 mM, 40 mM) for 2, 6 and 24 h. Medium samples were adjusted for protein concentrations, and equal concentrations were loaded into each well (Figure 3A). **Figure 4.3B.** Quantification of bands from Figure 4.3A.

A total of six experiments were performed on plastic with HUVEC from different donors. There are conflicting results regarding the secretion of pro-MMP-2 under NG and HG. Four of the experiments showed higher pro-MMP-2 activity under NG, while two other experiments showed increased activity of pro-MMP-2 under HG conditions (**Table 4.1**). All together the experiments on plastic indicate pro-MMP-2 secretion is most potent at NG than at HG.

Table 4.1. Experiments conducted on plastic

Experiment*, 24 h incubation	Demonstrated number of times	% Change increase
Secreted MMP-2 is highest in 5 mM glucose	4	306.6 41.1 7.9 3.7
Secreted MMP-2 is highest in 25 mM glucose	2	24.2 18.4

*The experiments indicated in the table are from different donors.

4.4 Effect of hyperglycemia and incubation time on polarized HUVEC

HUVEC were also grown on semipermeable filters in order to analyze if there was a polarized secretion of MMP-2 and MMP-9 from cultured HUVEC, and to investigate if there was a time-dependent effect on MMP secretion, under NG and HG conditions. Considering that endothelial cells operate as polarized cells, filter experiments are useful supplements to experiments performed on conventional plastic. Such a system allows comparative analyses of secretion to the apical and basolateral side of the endothelium with obvious relevance to the *in vivo* situation.

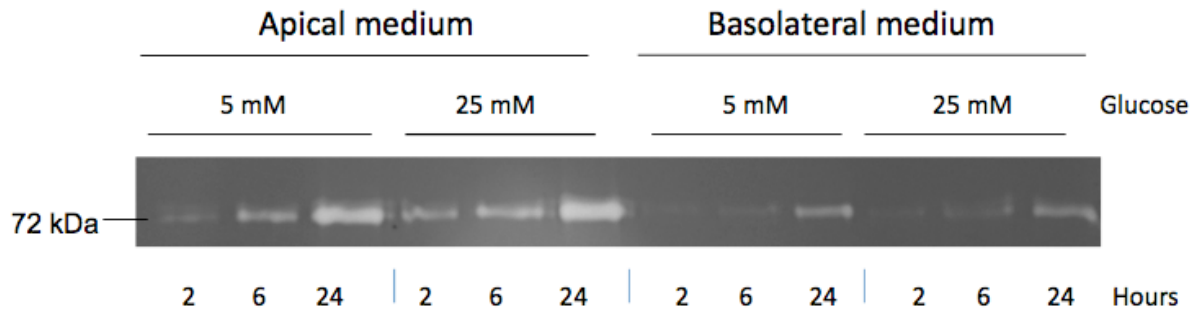

Figure 4.4 Zymography of secreted pro-MMP-2 in apical- and basolateral medium from HUVEC incubated with NG (5 mM) and HG (25 mM), for 2, 6, and 24 h.

Figure 4.4 shows that the secretion of pro-MMP-2 increased in a time dependent manner with highest secretion after 24 h incubation with NG and HG. There is a polarized secretion with more pro-MMP-2 being secreted to the apical side than to the basal side, as the bands detected for the basolateral medium were much weaker than the apical bands. These results were confirmed in a second experiment using HUVEC from another umbilical cord (results not shown).

Furthermore, the results suggest that there is a stronger secretion of pro-MMP-2 from cells incubated with HG compared to NG in the apical medium, when comparing secretion after 2 and 6 h. However, the secretion of pro-MMP-2 appears to level off after 24 h of incubation, showing no great difference between the NG and HG. However, the quantification analyses indicated a greater secretion under NG conditions, with a 4.5 % increase in pro-MMP-2 secretion in the NG state. This increase is probably neglible. No bands co-migrating with the pro-MMP-9 standard could be detected. All together, four experiments were performed on filter, using HUVEC from different donors, in which no clear trend was found (**Table 4.2**). Two experiments showed a clear increase in pro-MMP-2 activity under HG conditions in the apical medium, whereas two experiments showed a higher secretion under NG conditions (in the apical medium).

Table 4.2 Experiments conducted on filter

Experiment*, 24 h incubation	**Demonstrated number of times**	**% Change increase**
Secreted MMP-2 is highest in 5 mM glucose	2	140.1 3.3
Secreted MMP-2 is highest in 25 mM glucose	2	212.5 33.1

*The experiments are from different donors.

4.5 Effect of DON on MMP secretion

HUVEC were isolated and cultured on plastic plates. Cells were exposed to NG or HG using different concentrations of DON (50 and 100 µM). Cells and medium frations were harvested after 24 h. A zymography was performed in order to analyze the enzyme activity of MMP-2 and MMP-9. Under NG conditions, increased DON concentration, (from 0 to 100 µM) caused a small reduction in pro-MMP-2 secretion (**Figure 4.5A**). (The intensity measurements are presented in **Appendix II Figure 9.2**). The same trend was observed for cells cultured under HG conditions. No gelatinase activities corresponding to pro-MMP-9 were observed in any of these experiments. Western blot was performed in order to investigate the effects of DON on the level of O-GlcNAcylated proteins. Whole cell extracts were analyzed by Western blotting using the monoclonal antibody CTD 110.6. The immunoblot shows that the level of O-GlcNAcylated proteins is increased when HUVEC was exposed to 100 µM DON under NG. (**Figure 4.5B**). However, the glycosylation appears to be decreased under HG conditions (the experiment has been reproduced with HUVEC from another umbilical cord, not shown). Moreover, the Western blot also indicates that the level of O-GlcNAcylated protein is increased from NG to HG in the control (0 µM DON).

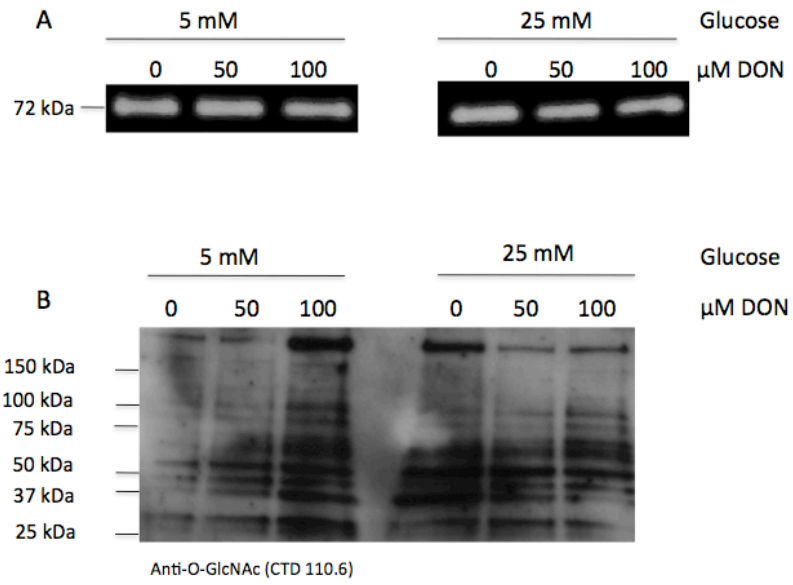

Figure 4.5A. Zymography of secreted pro-MMP-2 in medium from HUVEC cultured with NG (5 mM) and HG (25 mM) together with different concentrations of DON (0, 50, and 100 µM). **Figure 4.5B.** Western blot of whole cell extracts from HUVEC stimulated with NG (5 mM) and HG (25 mM)

and different DON concentrations (0 µM, 50 µM, 100 µM). The monoclonal antibody CTD 110.6 was used to detect O-GlcNAcylated proteins.

4.6 Effect of DON on polarized cells

Experiments using DON as an inhibitor were also carried out on polarized cells, in order to observe if DON treatment gave a similar effect on filter as on plastic. Cells were exposed to 50 and 100 µM of DON and were incubated with either NG or HG for 24 h. MMP activity for the apical medium fractions was analyzed by zymography. No bands were detected for pro-MMP-9 (**Figure. 4.6**). Intensities of the bands have been quantified (**Appendix II Figure 9.3**).

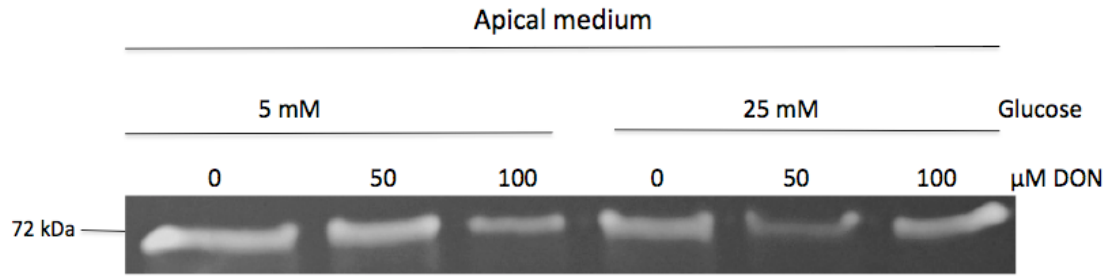

Figure 4.6. Zymography of secreted pro-MMP-2 in apical medium from HUVEC incubated with NG and HG and various concentrations of DON (50 and 100 µM) for 24 h.

There was a polarized secretion from endothelial cells grown on filter. The secretion of pro-MMP-2 was strongest to the apical side of medium. The bands in the basolateral medium were too weak to be quantified. Under NG conditions, pro-MMP-2 secretion is decreased with higher concentrations of DON (50 and 100 µM). In the HG state, pro-MMP-2 secretion is also decreased when exposed to 50 µM of DON. However, pro-MMP-2 seems to increase with 100 µM DON under HG conditions, suggesting that the highest concentration of DON does not give consistent results.

5 Discussion

In the present thesis, the effect of HG on MMP secretion from HUVEC was investigated. Secondly, the involvement of the hexosamine pathway on MMP secretion was studied. HUVEC were exposed to HG (25 mM) to mimic diabetes *in vitro*. NG (5 mM) was used as a control. These concentrations were chosen as they have been widely used in previous experimental short-term diabetes studies *in vitro* (Garaghozlian *et al.*, 2006; Death *et al*., 2003). 5 mM is near the normal glucose range (3.6 -5.8 mM) kept in healthy, non-diabetic humans. Although 15 mM is closer to the diagnostic value of diabetes (> 11 mM after intake of 75 g of anhydrous glucose), a greater difference in MMP expression was seen between 5 and 25 mM in the experiments presented in this thesis (**Figure 4.2** and **4.3**). Furthermore, **Figure 4.1** depicts that there is greater difference in protein synthesis between 5 and 25 mM, rather than 5 and 15 mM.

5.1 Major findings: Effects of hyperglycemia on MMP secretion on plastic and filter

In the current investigation, a total of six experiments were carried out on plastic exposing HUVEC to NG and HG for 24 h. Four of these experiments demonstrated that secretion of MMP-2 was reduced in HUVEC exposed to HG. However, the opposite was found in two other experiments, in which MMP-2 secretion was increased in HG compared to NG (**Table 4.1**). Overall, these investigations indicate a weak trend showing that MMP-2 secretion is decreased when exposed to HG.

Tram Thu Vuong, a member of the lab (personal communication), also studied the effect of HG on MMP-2 secretion under the same conditions. In these four experiments carried out the secretion of MMP-2 was lower at HG-compared to NG (unpublished data). This is in agreement with the weak trend in my findings. Available literature regarding MMP-2 secretion from endothelial cells is conflicting. Similar to my findings on plastic, Gharagozlian *et al*. (2006) found that MMP-2 secretion was reduced in cultured HUVEC exposed to HG (25 mM) conditions compared to controls (5 mM). In contrast, Death *et al*. (2003) showed that there was a significant increase in expression of MMP-2 in HUVEC exposed to HG (25

mM). Likewise, Ho e*t al*. (2007) discovered that MMP-2 was elevated in HUVEC culture after treatment with HG (33 mM). To my best knowledge, there are very few reports regarding the secretion of MMP-2 from HUVEC under HG conditions. Other studies that have addressed MMP secretion under HG, involve different cell types. McLannen *et al.* (2000) found that HG (25 mM) decreased MMP-2 activity in isolated human mesangial cells, suggesting that HG reduced breakdown of the mesangium matrix, due to a down regulation of MMP activities. Anderson *et al*. (1996) studied the effect of HG (25 mM) on MMP-2 secretion in mesangial cells. He also found that the level of MMP-2 secretion was reduced under HG conditions compared to NG (5 mM).

In the present study, HUVEC were also cultured in a polarized fashion, exposing cells to NG and HG for 24 h. Culture of polarized cells is a useful method for studying HUVEC, as the cells secrete proteins into the luminal and basal side of the filter, mimicking the *in vivo* state (Cole-Parmer, 2011). Variable results were found in the four experiments conducted on filter (**Table 4.2**). More experiments were undertaken, but the gelatinase activities were too weak to be measured and these experiments were therefore not included in this thesis. Two experiments showed a higher secretion of MMP-2 under NG conditions compared to HG (**Figure 4.4** and **4.6**), which is consistent with the weak trend observed for experiments conducted on plastic plates. However, the opposite was found in two other experiments, demonstrating a greater secretion of MMP-2 at HG compared to NG (results are not shown). The culture of polarized HUVEC is not a widely established method and there are a limited number of studies using this system. No trend was discovered in the filter experiments conducted by other members of the research group (Vuoung, unpublished data). More experiments on filter are needed in order to conclude on the effects of HG on polarized HUVEC.

The experiments using polarized HUVEC revealed a greater secretion of MMP-2 to the apical compartment (**Figure 4.4** and **4.6**). Other reports have also found MMP secretion to be greater to the apical side of the cells compared to the basal side (Martin *et al*., 2000). The reason behind the predominantly apical secretion is not known. A higher secretion to the apical side may be conflicting with the hypothesis that basement membrane components are primary targets of MMP in HUVEC. However, the secretion to the apical side may potentially target the MT-MMPs. MT-MMPs form a heterotrimeric complex with MMP-2 and TIMP-2, resulting in the cleavage of the 72-kDa pro-MMP-2, to 67-kDa its active form (Martin *et al*.,

2000). In the current study the majority of MMP-2 was released in its inactive pro-form. Secretion of MMPs to the apical side of the cells has also been proposed to be involved in the degradation of tight junction of cells, which may result in impaired endothelial barrier function (Leone *et al*., 2007) and vascular functions, such as platelet aggregation and vasoconstriction (Sawicki *et al*., 2000). MMP can also be involved in inflammatory reactions through shedding of components of the glycocalyx such as cell surface proteoglycans of the syndecan type (Manon-Jensen, 2010).

There are a limited number of studies that have been conducted regarding MMP secretion from vascular cells *in vitro*. Other reports focus on plasma MMP levels in diabetics, in which the majority of these studies point to an increase in MMP-2 levels in plasma in diabetic subjects. A study conducted by Gharagozlian *et al*. (2009) analyzed serum MMP-2 and MMP-9 from diabetic patients and healthy controls. The results showed significant higher serum levels of both MMP-2 and MMP-9 in diabetic patients compared to healthy controls. Thrailkill *et al*. (2007) found that urine and plasma levels of MMP-2 were significantly increased in type 1 diabetic patients compared with healthy control subjects. Similarly, Lee *et al*. (2005) found that levels of MMP-2 concentrations were increased in type 2 diabetic patients. Derosa *et al*. (2007) also found significantly increased plasma levels of MMP-2 and MMP-9 in type 2 diabetic patients compared to healthy subjects. Out of all studies found conducted on humans, an increase in MMP-2 was demonstrated in patients with diabetes. However, it is uncertain whether plasma concentrations of MMP and TIMP indicate their corresponding enzyme activity in the vascular wall (Kadoglou *et al*., 2005). Considering that MMPs are important effectors and regulators of inflammation, it is possible that increased levels of MMP-2 and MMP-9 in serum of diabetic patients arise due to a higher secretion of MMPs from immune cells into the blood. Inflammatory cells including macrophages, neutrophils, mast cells and T-lymphocytes are major sources of MMPs and MMP activator cells. These inflammatory cells have shown to increase the production of MMP-2 and MMP-9 from other cells (Singh and Rabbani, 2005; Morgan *et al*., 2004). In addition to these inflammatory mediators, oxidized lipoproteins can promote the expression and activation of MMPs (Gharagozlian *et al*., 2006). Moreover, one must take into the account the effect of confounding factors such as diabetes medications, as insulin has shown to increase MMP-2 activity in rat mesangial cells (Thrailkill *et al*., 2009).

Several studies on rodents have found a reduced expression of MMP-2 activity in renal tissues including a higher activity of its inhibitor TIMP-2. However, conflicting results exist as both increases and decreases have been demonstrated when rodent mesangial cells have been cultured and exposed to hyperglycemic conditions (Thrailkill *et al*., 2009). An *in vitro* study by Uemura *et al*. (2001) investigated the effect of HG on bovine aortic endothelial cells (BAEC), and found that MMP-9 was increased in HG (25.5) compared to NG (5.5 mM). Moreover, they demonstrated that oxidative stress was an important influence on MMP-9 expression, as enhanced MMP-9 activity was significantly reduced with antioxidant treatment.

In the current study, MMP-9 was either not detected or in few cases the bands were too weak to be quantified. Similarly, Gharagozlian *et al*. (2006) did not observe MMP-9 activity from HUVEC. Endothelial cells primarily secrete MMP-1, MMP-2, and MMP-3, while MMP-9 is predominantly secreted from macrophages (Death *et al*., 2003). However, when exposed to inflammatory stimulation, secretion of MMP-9 is increased in other cell types, including endothelial cells (Chakrabarti & Patel, 2005; Min *et al*., 2009). Diabetes is described as a low inflammatory condition (Stehouwer, 2004). Therefore, it would be interesting to expose HUVEC to inflammatory stimulation such as Interleukin-1beta (IL-1β) in addition to HG, in a future study to observe the effects on MMP-9 secretion.

Considering that thickening of basement membrane occurs in HG conditions, a potential mechanism underlying this effect is reduced degradation of matrix components, caused by a decrease in MMP secretion from endothelial cells or due to an increased expression of TIMPs. The findings in the current investigation indicate a lower expression of MMP-2 under HG, in HUVEC cultured on plastic, but does not take into the account the effect of TIMP. These natural occurring inhibitors may become up-regulated under HG, causing a decreased activity of MMP-2 (Thrailkill *et al.*, 2009). Gharagozlian *et al*. (2006) did not find an altered expression in TIMP-1 and -2 by Western blotting, in HUVEC exposed to HG. In a future scenario, reverse zymography could possibly reveal if TIMP-2 secretion is increased, corresponding to a lower secretion of MMP-2. Another important mechanism in MMP-2 activation is TGF- β, which has been shown to induce MMP-2 activity (Lenz *et al*., 2000). HG has been shown to increase TGF- β in cell culture, animal models and humans (Zhu *et al*., 2007), and may also be a potential focus point for future research.

In diabetes, hyperglycemia is strongly linked to accumulation of matrix components, which may result in endothelial dysfunction and reduced filtration properties in the kidneys (Garagozlian *et al.,* 2006). Previous reports (Thrailkill *et al*., 2007; Kadoglou *et al*., 2005) indicate a possible correlation between MMP-2 dysregulation and accumulation of ECM in diabetes, which may be an instigating factor in complications such as diabetic nephropathy. However, evidence appears contradictory (Thrailkill *et al*., 2007). Thus, a clear role of MMP in the progression of diabetes complications and its involvement in the regulation of ECM is yet to be established.

5.2 Major findings: Effect of DON and hyperglycemia on MMP secretion on plastic and filter

The hexosamine pathway is considered to play a significant role in the pathogenesis of diabetic complications. In the present work, the involvement of this pathway under HG conditions was studied, by blocking the rate-limiting enzymatic step in this pathway with the inhibitor DON. HUVEC were exposed to different concentrations of DON (50 and 100 μM) for 24 h, to study the effect of DON on MMP secretion. HUVEC cultured on plastic showed that MMP-2 secretion was attenuated under NG when exposed to 100 μM of DON compared to control (0 μM DON) (**Figure 4.5A**). Under HG conditions a reduction in MMP-2 was observed when exposed to both 50 μM and 100 μM of DON compared to control (0 μM DON). The same trend was discovered on filter. A reduction in MMP-2 activity was found in HUVEC exposed to NG, 50 and 100 μM of DON compared to control (0 μM DON). Under HG conditions there was also a reduction in MMP-2 secretion in HUVEC exposed to 50 μM but not 100 μM DON (**Figure 4.6**).

Overall, the current investigation shows that there is a reduction in MMP-2 secretion in both the plastic and filter experiments, when exposing HUVEC to DON. There is no available literature regarding the hexosamine pathway and MMP-2 secretion. More experiments are needed in order to conclude on the importance of the hexosamine pathway in regulating MMP-2 secretion under HG conditions. The mechanism by which blocking GFAT in the hexosamine pathway may lead to decreased MMP-2 secretion is currently not known. The inhibition of GFAT has been shown to block hyperglycemia-induced increases in the

transcription of growth factors central to diabetes (Giacco & Brownlee, 2010). The hexosamine pathway may play a crucial role in the pathogenesis of diabetes as it may influence cell signalling and MMP secretion.

The exposure of HUVEC to DON may influence MMP-2 secretion in several ways (**Figure 1.6**), as the inhibition of GFAT may alter the biosynthesis pathway (synthesis of proteoglycans and glycoproteins) or it may change the expression of MMP-2 via the signalling pathway (O-GlcNAcylation) either indirectly by acting on transcription factors, or by directly acting on MMP-2. A YinOYang prediction was performed to investigate potential O-GlcNAcylated sites of MMP-2. The prediction results showed 4 potential sites for O-GlcNAcylation attachment sites (**Appendix Figure 9.4**), indicating MMP-2 as a possible O-GlcNAcylated target. However, the MMP-2 sequence contains a signal peptide, meaning that it is unlikely to contain an O-GlcNAc site. Furthermore, it appears that DON is relatively unspecific as it may target several different enzymes and not only GFAT (Pinkus, 1977). Therefore one must not exclude the possibility that DON influence MMP secretion through other pathways.

5.3 Limitations

The experiments in this study were all carried out using HUVEC from different donors. Individual variation between the donors may explain the variation observed in my results (Skliankina *et al*., 2011). Thus, an increased number of donors are needed in order to establish a clear trend. The HUVEC that was received in this project originates from healthy mothers, but does not take into account complications during delivery such as caesarean section. Trauma during delivery may have affected my results. Further studies could profit from comparing endothelial cells with other cells in circulatory system such as monocytes.

One drawback is the low number of experiments included in this study, although many experiments were performed. Unfortunately, many of the experiments conducted on filter failed to produce conclusive results, as the bands were too weak to be analyzed and quantified. The reason for this observations in several of the polarized HUVEC experiments is not known. Perhaps increasing duration of exposure would increase secretion of MMP-2 in HUVEC grown on filter, as experiments conducted on plastic with exposure time up to 14

days showed a considerable increase in MMP-2 activity (results not shown). A further limitation is that the results from the plastic and filter experiments originate from different donors as HUVEC were initially grown on plastic and subsequently on filter. Considering the great variation observed in the donors, a different experimental design using the same HUVEC donor in parallel experiments conducted on plastic and on filter, may bring clearer findings for the effects of HG on MMP secretion in polarized cells. Finally, it is important to note the potential restrictions of the programme Quantity-One, seeing that some of measurements were not entirely consistent with the visual bands on the images.

6 Conclusions

The present study indicates that HG influence MMP-2 secretion in HUVEC. In the experiments conducted on plastic a weak trend was found, showing a reduction in MMP-2 when exposing HUVEC to HG. Other members of the group have also confirmed this in parallel studies. However, some experiments demonstrated the opposite effect, with an increase in MMP-2. No trend was found on polarized cells, due to the limited number of conclusive experiments. In future experiments it would be interesting to perform experiments where comparisons are made between HUVEC obtained from the same donor and cultured both on plastic and on filter. This could potentially explain the variations observed in this thesis.

The current work also indicates that MMP-2 secretion in HUVEC grown on plastic is attenuated when exposing cells to different concentrations of DON, inhibiting the enzymatic step of GFAT. However, more experimental data are needed for clear conclusions. Results on secretion of MMP-2 from polarized HUVEC exposed to different concentrations of DON are still inconclusive. Before firm conclusions can be drawn, more extensive investigations are necessary, particularly studies regarding polarized cells, as this is closer to the *in vivo* state.

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8 Appendix I:

8.1 Materials and Equipment

Amersham Hyperfilm TM ECL	GE Healthcare, UK
Cell culture plastic plates (6, 12 wells)	BD Labware, USA
Cell scraper	BD Labware, USA
Criterion Precast gel (4-20%), 12, 18 wells	Bio-Rad, USA
ELISA microplate (96 wells plate)	Greiner bio-one, Germany
Eppendorf tubes (1.5 ml)	Sarstedt, Germany
Falcon tubes (5ml, 15 ml)	BD Labware, USA
Falcon Pipettes (5, 10, 25 ml)	BD Labware
Hypercasette ^{TM} autoradiography cassettes	Amersham Biotech, UK
Immobilon ^{TM} Transfer Membrane (PVDF)	Millipore, USA
Messoft Compresses	Tendra, Sweden
Metal Cannulas	Technical Department, UIO
Multiple Gel caster	Amersham Biosciences
Omnifix single use syringes (10, 50 ml)	Braun, Germany
Parafilm ® “M”, Laboratory Film	American National Can^{TM}, USA
Pipetboy	Integra Biosciences AG, Switzerland
Single use scalpel	Paragon, UK
Sterican sterile needle	Regent, USA
Sterile culture cell flask (75 cm^2)	BD Labware, USA
Sterile filter flasks	TPP, Switzerland
Sterile surgical gloves	Regent, USA
Whatman Filter paper	Schleider & Schwell

8.2 Instruments

Cell counter, Countess ®	Invitrogen
Celloshaker variospeed	Vialta, Italy
Centrifuge, Biofuge, Heraeus, Fresco	DJB labcare Ltd, UK
Electronic vibrofix	Janke & Kunkel
Kodak X-OMAT, 1000 processor	KODAK
Mettler Toledo Model, Delta range scale	Mettler, Norway
Microscope Olympus	Olympus Corps., Japan
pH- meter PHM210	Meterlab, France
Rotating device	Labinco, Netherlands
Titertek Multiskan PLUS	Eflab, Finland

8.3 Antibodies

Antibody	**Dilution**	
Primary antibody, mouse anti-human MMP-2	1:1000	Biosciences Inc., San Diego
Secondary antibody, sheep anti-mouse	1: 3000	Amersham Biosciences, UK
Monoclonal antibody CTD 110.6	1: 5000	Covance, UK, MMS-248R
Secondary antibody, goat anti-mouse, IgM	1: 5000	Sigma Aldrich Co, USA, Cat. NO: 8786

8.4 Chemicals

Acrylamide	Sigma Aldrich, Germany
Ammonium persulfate (APS)	Bio-Rad, USA
BC Assay protein quantification kit	Uptima Interchim, France
2-mercaptoethanol	Sigma Aldrich, Norway
Bovine Serum Albumin (BSA)	Sigma Aldrich, Germany
Bromphenol Blue	Bio-Rad, USA
Collagenase (0.2%)	Sigma Aldrich, Germany
Coomassie Brilliant Blue	Sigma Aldrich, Germany
Complete Mini EDTA-free Protease inhibitor, cocktail tablets	Roche, Germany
DON (6-diazo-5-oxo-norleucine)	Sigma Aldrich, Germany
D (+) Glucose	Sigma Aldrich, Germany
ECL ^{TM} PLUS Western Blotting detection reagent	Amersham, UK
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, Germany
Epidermal growth factor (EGF)	R&D systems, UK
Foetal bovine serum (FBS)	Sigma Aldrich, Germany
Fibroblast growth factor (FGF)	R&D systems, UK
Fungisone	GIBCO Invitrogen, UK
Gelatin (Type A: From porcine skin, kat. G 2500)	Sigma Aldrich, Germany
Glycerol	Sigma Aldrich, Germany
Glycine	Sigma Aldrich, Germany
Hepes	Sigma Aldrich, Germany
Hydrocortisone	Sigma Aldrich, Germany
Hydrochloric acid (HCl)	Merck, Germany

MCDB-131 basis powder	Sigma Aldrich, Germany
Methanol	Merck, Germany
Precision Plus protein standards (all Blue)	Bio-Rad, USA
Precision Plus protein standards (Dual Colour)	Bio-Rad, USA
SDS (sodium dodecyl sulfat)	Sigma-Aldrich, Norway
TEMED	Bio-Rad, USA
Tris-Base	Calbiochem, USA
Triton X-100	Merck, Germany
Trypan blue solution	Sigma Aldrich, Germany
Tween ® 20	Sigma Aldrich, Germany

8.5 Solutions

Table 8.5.0. Cord buffer

Components	Volume	Final Concentration
Streptomycin	0.5 g in 1 ml sterile MQ-H_2O	50 mg/ l
1X PBS (sterile)	1000 ml	

Stored at 4°C

Table 8.5.1. 10x PBS pH 7.2-7.4

Components	Volume
25 mM NaH_2PO_4 x H_2O	3.9 g
8 mM NaH_2PO_4	14.1 g
NaCl	85.2 g
MQ-H_2O	Up to 1000 ml

The pH was adjusted to 7.2-7.4 by adding 1 M NaOH or 1 M HCl

Table 8.5.2. 1x PBS

Components	Volume	Final Concentration
10x PBS	100 ml	1x
MQ-H_2O	900 ml	

Table 8.5.3. Collagenase (0.2%)

Components	Volume	Final Concentration
Collagenase	200 mg	0.2%
1X PBS	100 ml	

Table 8.5.4. Washing buffer

Component	Volume
Triton X-100	2.5 ml
MQ-H_2O	97.5 ml

Table 8.5.5. 10x Incubation buffer

Component	Grams
10 mM Tris base	12.1 g
40 mM Tris HCl	63.0 g
200 mM $NaCl_2$	117.0 g
5 mM $CaCl_2$ * H_2O	7.4 g
0.02% Brij 35	6.66 g 30% (w/v) Brij 35 Sigma

Mixed in MQ-H_2O giving a total volume of 1000 ml

Table 8.5.6. Staining solution (2x)

Component	Grams/ ml
Coomassie Brilliant Blue R-250	0.4g/ 1 tablet
MQ-H_2O	80 ml
Methanol	120 ml

The solution is ready for use after it has been mixed with 1 part acetic acid.

Table 8.5.7. Destaining buffer

Component	Amount
Methanol	120 ml
Acetic acid, glacial	40 ml
MQ-H_2O	240 ml

Table 8.5.8. Separation gel

Components	Volume
1.5 M Tris pH 8.8	2.5 ml
30 % Acrylamid solution	2.5 ml
2% Gelatine	0.5 ml
MQ-H_2O	4.5 ml
10% SDS	100 µl
10% APS	50 µl
TEMED	5 µl

Table 8.5.9. Stacking gel

Components	Volume
0.5 M Tris pH 6.8	1.3 ml
30% acrylamid solution	0.7 ml
MQ-H_2O	3.25 ml
10% SDS	50 µl
10% APS	25 µl
TEMED	5 µl

Table 8.6.0. Running buffer pH 8.3 (10x)

Components	Volume
Tris Base	29.0 g
Glycin	144.0 g
SDS	10.0 g
MQ-H_2O	Up to 1000 ml

Table 8.6.1. Running buffer pH 8.3 (1x)

Components	Volume	Final concentrations
10x Running buffer	100 ml	1x
MQ-H_2O	900 ml	

Table 8.6.2. Loading buffer (4x)

Components	Volume	Final concentration
0.5 M Tris buffer pH 6.8	1.0 ml	0.05 M
Glycerol	4.0 ml	40 %
SDS	0.8g	0.08 %
Bromphenol Blue	8.0 mg	8 %
2-mercaptoethanol	1.0 ml	10 %
MQ-H_2O	Up to 10 ml	

Table 8.6.3. Blotting buffer (10x)

Components	Final Concentration
Trizma base	0.25 M
Glycine	1.92 M

Table 8.6.4. Blotting buffer (1x)

Components	Final Concentration
Methanol	10-20 %
10x blottingbuffer	1x

Table 8.6.5. Transfer buffer (10x) pH 7.4

Components	Volume
Tris-HCl	39.4 g
Glycine	143.0 g
MQ-H_2O	Up to 1000 ml

Table 8.6.6. Transfer buffer (1x)

Components	**Volume**	**Final Concentration**
Methanol	1000 ml	10 %
10x Transfer buffer	100 ml	1x
MQ-H_2O	800 ml	

Table 8.6.7. TBS (10x)

Components	**Final Concentration**
Trizma HCl	0.2 M
NaCl	1.37 M
Adjust pH to 7.6 with HCl	

Table 8.6.8. TBST (1x)

Components	**Final Concentration**
10x TBST	1x
Tween 20	0.1 %

Table 8.6.9. 1.0 M Glucose

Components	Volume	Final Concentration
D-(+)-Glucose	2.703 g	1.0 M
MQ-H_2O	15 ml	

The solution was sterile filtered

Table 8.7.0. 10 M NaOH

Components	Volume	Final Concentration
NaOH	40 g	10 M
MQ-H_2O	Up to 1000 ml	

Table 8.7.1. 1.0 M NaOH

Components	Volume	Final Concentration
NaOH	10 ml	1.0 M
MQ-H_2O	90 ml	

Table 8.7.2. 10 M HCl

Components	Volume	Final Concentration
HCl concentrated (12.1 M)	30 ml	10 M
MQ-H_2O	Up to 100 ml	

Table 8.7.3. 100 ml 1.0 M HCl

Components	Volume	Final Concentration
10 M HCl	10 ml	1.0 M
MQ-H_2O	90 ml	

Table 8.7.4. 0.5 M Tris pH 6.8

Components	Grams/ Volume
Tris-Base	12 g
MQ-H_2O	Up to 400 ml

pH is adjusted to 6.8 with HCl

Table 8.7.5. 1.5 M Tris pH 8.8

Components	Grams/ Volume
Tris-Base	72.6 g
MQ-H_2O	Up to 400 ml

pH is adjusted to 8.8 with HCl

9 Appendix II

Endothelial cells are from a different HUVEC donor, and indicate the same trend as in **Figure 4.1.**

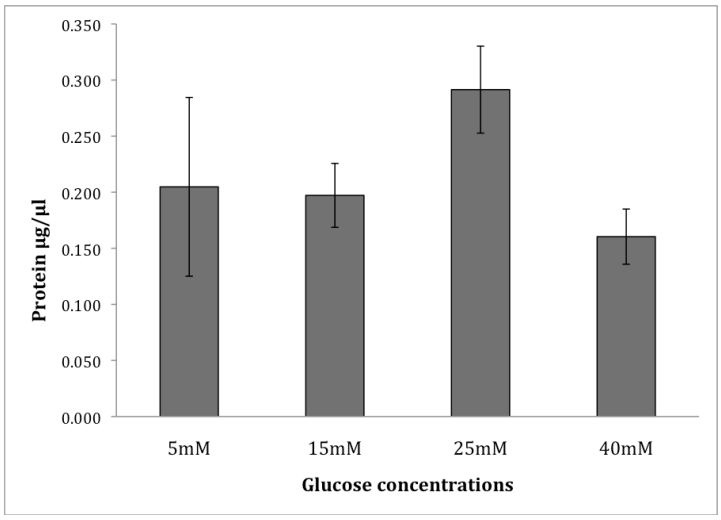

Figure 9.1 Protein synthesis in response to glucose concentrations. HUVEC were cultured with different glucose concentrations (5 mM, 15 mM, 25 mM, and 40 mM) for 24 h in serum-free media. The results presented are from cell fractions originating from three separate wells.

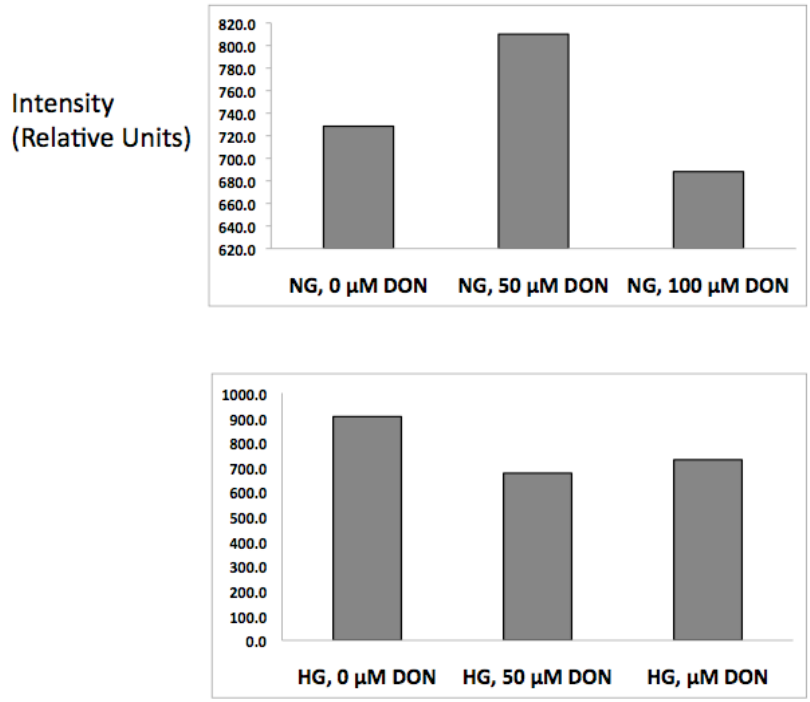

Figure 9.2 Quantification of zymography bands from Figure 4.5A. Y-axis: Intensities (relative units).

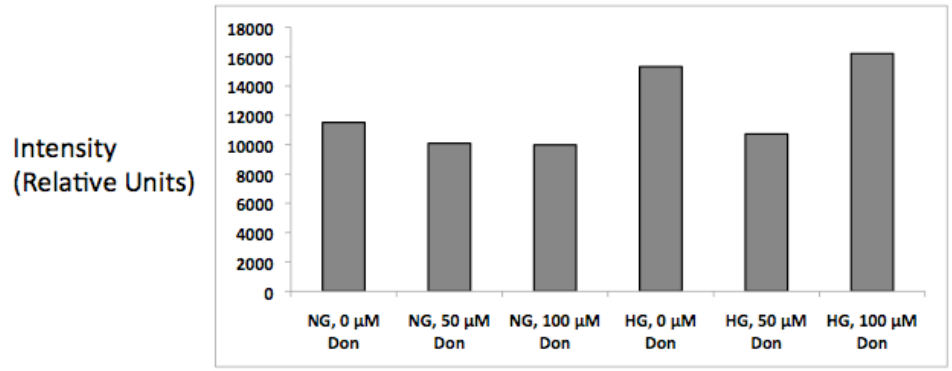

Figure 9.3 Quantification of zymography bands from Figure 4.6

"This sequence seems to contain a signal peptide!!
Proteins with signal peptides are most probably secreted
and are unlikely to contain an O-(beta)-GlcNAc site".

SeqName	Residue	O-GlcNAc result	Potential (o-glcnac)	Thresh. (1)	Thresh. (2)	NetPhos potential (Thresh=0.5)	YinOYang?
Sequence	199	T	+	0.4638	0.4387	0.5417	
Sequence	300	T*	+	0.4689	0.4581	0.5678	0.926 *
Sequence	307	T*	+	0.4429	0.4272	0.5262	0.804 *
Sequence	365	S*	+	0.4626	0.4370	0.5395	0.952 *

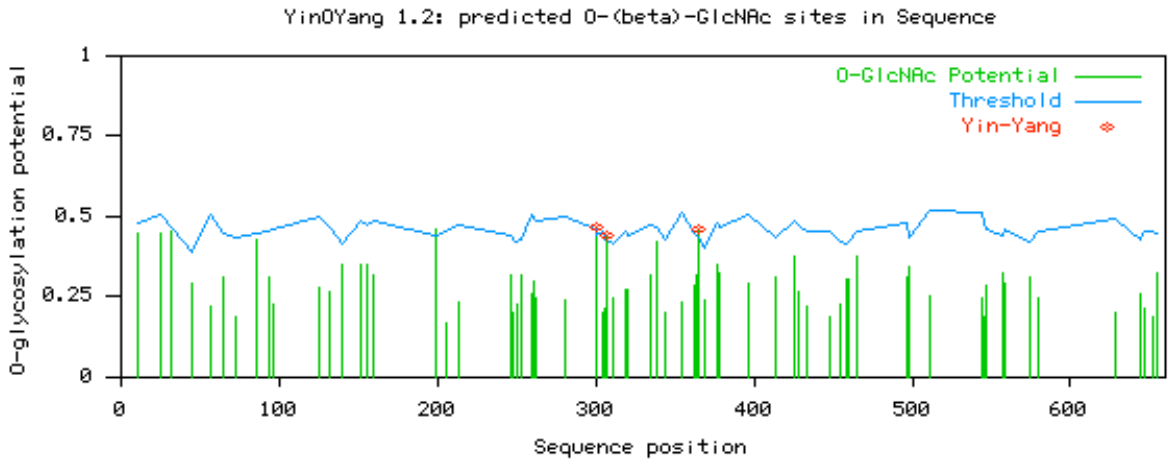

Figure 9.4 show four potential O-GlcNAcylated sites at MMP-2 protein (+).

YinOYang 1.2 output format

```
Name:  HXA3_HUMAN       Length:  443
(sequence)
MQKATYYDSSAIYGGYPYQAANGFAYNANQQPYPASAALGADGEYHRPACSLQSPSSAGGHPKAHELSEACLRTL
SAPPS      80
QPPSLGEPPLHPPPPQAAPPAPQPPQPAPQPPAPTPAAPPPPSSASPPQNASNNPTPANAAKSPLLNSPTVAKQI
FPWMK      160
ESRQNTKQKTSSSSSGESCAGDKSPPGQASSKRARTAYTSAQLVELEKEFHFNRYLCRPRRVEMANLLNLTERQI
KIWFQ      240
NRRMKYKKDQKGKGMLTSSGGQSPSRSPVPPGAGGYLNSMHSLVNSVPYEPQSPPPFSKPPQGTYGLPPASYPAS
LPSCA      320
PPPPPQKRYTAAGAGAGGTPDYDPHAHGLQGNGSYGTPHIQGSPVFVGGSYVEPMSNSGPALFGLTHLPHAASGA
MDYGG      400
AGPLGSGHHHGPGPGEPHPTYTDLTGHHPSQGRIQEAPKLTHL
(annotation line, G=O-GlcNAc, Y=YinYang)
.....................................G...................GY.................
G...G      80
.....................................Y.......GG.Y.....G...G.................
.....      160
.....Y...GYYY..........Y.....Y.....G..G.....................................
.....      240
.................G.Y...Y.G............................Y....G.....G......G...G
..G..      320
.....................................................................G..
.....      400
.........................................G..

-------------------------------------------------------------------------
----
SeqName     Residue  O-GlcNAc  Potential  Thresh.  Thresh.   NetPhos
YinOYang?
                      result   (o-glcnac)   (1)      (2)    potential
                                                          (Thresh=0.5)
-------------------------------------------------------------------------
----
HXA3_HUMAN      5  T    -        0.4047    0.4125   0.5064     <-- A
negative site
HXA3_HUMAN      9  S    -        0.4267    0.4631   0.5746
HXA3_HUMAN     10  S    -        0.3548    0.4653   0.5776
HXA3_HUMAN     36  S    ++       0.5781    0.4421   0.5463     <-- O-GlcNAc
predicted (++)
HXA3_HUMAN     51  S    -        0.4089    0.4299   0.5298
HXA3_HUMAN     54  S    -        0.3183    0.3966   0.4849     0.783  <-
Phos,No-GlcNAc
HXA3_HUMAN     56  S    +        0.4483    0.3792   0.4615
HXA3_HUMAN     57  S*   +        0.4357    0.3856   0.4702     0.967       *
<- YinYang
HXA3_HUMAN     68  S    -        0.2631    0.4479   0.5542
HXA3_HUMAN     74  T    -        0.3126    0.4422   0.5464
HXA3_HUMAN     76  S    ++       0.5023    0.3922   0.4791
HXA3_HUMAN     80  S    +++      0.6007    0.3667   0.4447
HXA3_HUMAN     84  S    -        0.2640    0.3769   0.4584     0.991
HXA3_HUMAN    115  T*   +++      0.6261    0.3591   0.4344     0.770       *
<- YinYang
```

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.
.

O-GlcNAc **Result**
This can be one of 5 possibles:-

- No O-GlcNAc predicted

O-GlcNAc predicted: *(different strengths)*
`+` `Potential > Thresh-1`
`++` `Potential > Thresh-2` (Thresh-2 is a threshold based on more stringent surface measures)
`+++` `Potential > (Thresh-2 + 0.1)`
`++++` `Potential > (Thresh-2 + 0.1) AND Potential >= 0.75`

NetPhos
These potentials are displayed if 'YinYang' output was selected and if the NetPhos potential crosses the NetPhos (fixed) threshold.
Predictions are run in parallel from the NetPhos server.

YinOYang
Ser/Thr residues which are predicted to be O-GlcNAcylated as well as phosphorylated are marked by an asterisk (*) in both the *residue* and the *YinOYang* column. Such sites may be reversibly and dynamically modified by O-GlcNAc or Phosphate groups at different times in the cell.

SignalP
With all predictions, the SignalP server is run in parallel. If a sequence is predicted to contain a signal peptide, a warning is displayed. Such sequences are unlikely to be intracellular, and hence unlikely to be O-GlcNAcylated.

Graph
The figure illustrates O-GlcNAc and NetPhos predictions across the length of the sequence. The x-axis represents the sequence from N-terminal to C-terminal. Vertical impulses (green) are O-GlcNAc potentials. For predicted O-GlcNAcylated sites, these potentials would cross the threshold (blue wavy horizontal) line.
Small red marks/circles on the green impulses indicate YinYang sites.