SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Examination of the vasomotor function of microvessels in diabetes mellitus

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The Examination takes place at the Department of Pediatrics, Medical and Health Science Center, University of Debrecen
June 5, 2012. 11:00

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The Ph.D. Defense takes place at the Lecture Hall of the 1st Department of Medicine, Institute for Internal Medicine, Medical and Health Science Center, University of Debrecen
June 5, 2012. 13:00

1. INTRODUCTION

Diabetes mellitus (DM) is one of the most significant pandemics in recent years, spreading across the world, and ranking amongst the most common so-called “civilization” diseases. The estimated total number of people suffering with diabetes is projected to rise from 366 million in 2011 to 552 million in 2030. 90 % of diabetes is type 2 diabetes, developing in adulthood and having significant impact worldwide based on the increasing prevalence, incidence and level of the associated cardiovascular complications. In diabetes mellitus the risk of developing cardiovascular complications is elevated compared to the non-diabetic population. There are shocking statistical data suggesting that the risk of development of myocardial infarction in diabetes mellitus is identical to the increase in risk in patients who have previously suffered a myocardial infarction due to another aetiology. Of the atherosclerosis complications developed in diabetes the most significant are coronary, cerebrovascular and lower limb circulatory diseases. Hypertension is another common complication in diabetes; however, the pathomechanism of hypertension differs in the different types of diabetes. In type 1 diabetes (T1DM) hypertension develops on the basis of diabetic nephropathy; in type 2 diabetes (T2DM) hypertension develops in relation to metabolic syndrome. In summary the nephropathy, neuropathy, retinopathy, cerebral-, cardiac-, and lower limb circulatory diseases that develop secondary to microangiopathy contribute to the increased morbidity and mortality in diabetes mellitus.

1.1. The role of L-arginine in cardiovascular diseases

There is abundant evidence that the endothelium plays a crucial role in the maintenance of vascular tone and structure. One of the major endothelium-derived vasoactive mediators is nitric oxide (NO), an endogenous messenger molecule formed in healthy vascular endothelium from the amino acid precursor

L-arginine. Dysfunction of the endothelial L-arginine/nitric oxide pathway is a common mechanism by which several cardiovascular risk factors mediate certain deleterious effects on the vascular wall, such as diabetes mellitus, hypercholesterolemia, hypertension, and vascular inflammation. In the last couple of years studies have focused on L-arginine because of its role in NO synthesis and its beneficial effect in animal models, where its administration stopped or reversed the vascular atherogenesis. Previously, it has been shown that oral or intravenous administration of 5g L-arginine daily increased the NO production in patients with endothelial dysfunction. Recent studies revealed that short- to medium-term administration of L-arginine improved the symptoms of cardiovascular disease. However, a number of studies in animals and humans have found no benefit, or worsening of adverse outcomes, with prolonged administration of supplemental L-arginine. These diverse data raises the possibility that L-arginine supplementation appears not to be beneficial for every patient. The group of patients who will benefit from L-arginine supplementation therefore has to be defined with careful selection. Despite excess L-arginine being present intracellularly, exogenous L-arginine administration increases NO production; this phenomenon termed the “arginine paradox”. In human studies, an initial high dose of L-arginine was administered intravenously (30 mg / 30 min) resulting in vasodilation; furthermore an increased level of pituitary growth hormone (PGH) was also observed. An increased level of NO metabolites (nitrite, nitrate) was observed in the urine, indicating the activity of the NO pathway, but these responses could be partially inhibited with somatostatin, suggesting the growth hormone may contribute to L-arginine-induced vasodilation. These effects were observed with intravenous administration of L-arginine, oral application did not have the same effect, probably because of the lower plasma concentrations.

Despite the observation that the L-arginine concentration was not altered in cardiovascular diseases, increases in arginase activity, the enzyme responsible

for the breakdown of L-arginine, can decrease tissue and cellular L-arginine levels, reducing its availability to eNOS causing the reduction of the NO production. Dexter *et al*. first administered L–arginine clinically in patients with cardiovascular diseases in 1991. During coronarography, ACh and L-arginine administered into the coronary arterioles restored the ACh-induced increase in blood flow in patients with cardiovascular diseases but did not affect coronary blood flow in controls. Based on the research findings, oral administration of L-arginine is effective in patients with impaired endothelial L-arginine-NO metabolism. Possible reasons could be: 1) an L-arginine deficit in nephropathy, 2) upregulated arginase causes increased L-arginine consumption, and/or 3) an elevated concentration of the endogenous NOS inhibitor ADMA. These findings suggest that the oral administration of L-arginine might be an effective treatment of cardiovascular diseases in a specific group of patients.

1.2. The possible role of increased arginase 1 expression in diabetes mellitus

Upregulated arginase activity has been implicated in a number of cardiovascular diseases, such as pulmonary hypertension, erectile dysfunction and coronary diseases. It has been shown that streptozotocin-induced diabetes in rat causes impaired coronary endothelium-dependent vasorelaxation, which is associated with significant increases in arginase 1 protein in both aorta and liver of diabetic rats. It is well known that insulin represses expression of genes for urea synthesis pathways and that insulin signalling is impaired in both type 1 and type 2 diabetes, causing increased arginase activation, as part of the urea cycle, and decreased L-arginine levels. Intravenous insulin administration can significantly decrease the elevated plasma arginase activity, confirming a regulatory effect of insulin in arginase function. Increased arginase activity can decrease the level of L-arginine, reducing its availability to eNOS; this may lead to decreased endothelial NO production. In the case of increased arginase 1 activity, the possible arginase-1 eNOS co-localization can decrease the L-

arginine available for NO production. It is poorly understood how arginase 2 influences eNOS function, but a possible explanation could be that increased mitochondrial L-arginine degradation elevates its transport from the cytoplasm to mitochondria, thereby decreasing the level of the NO synthesis substrate. It has been found that the hyperglycaemia may increase arginase 1 activity in diabetes mellitus. Bovine coronary endothelial cells were incubated for 24 hours in 25 mmol/L glucose solution, which resulted in increased expression and activity of arginase 1 and markedly reduced NO synthesis as compared to the control group. White *et al.* demonstrated the opposite regulation of arginase 1 and eNOS in arginase 1 knock down rat endothelial cells. They demonstrated increased eNOS activation, but no changes in arginase 2 activity suggesting that arginase 1 is the predominant isoform present in the endothelium of rats. They also reported an increased ornithine level, which is an end product of arginase activity, confirming the pathological function of arginase in diabetes mellitus. These findings suggest that influencing the elevated arginase activity or expression may pave a new way in the treatment of cardiovascular diseases.

1.3. Protein O-GlcNAcylation in diabetes mellitus

A specific form of glycosylation is the *O*-linked enzymatic attachment of *N*-acetyl-glucosamine (*O*-GlcNAc) to proteins, which is called *O*-GlcNAcylation and is regulated by the hexosamine biosynthetic pathway (HBP), in which the glucose after transformation via the HBP becomes attached to serine and threonine residues of various proteins. *O*-GlcNAcylated proteins can be found in almost every intracellular compartment, and there are proteins in almost every functional class that are subject to *O*-GlcNAcylation. The key regulatory enzyme of the pathway is the fructose 6-phosphate amidotransferase (GFAT), which converts fructose 6-phosphate to glucosamine 6-phosphate with glutamine as the amino donor. The *O*-GlcNAc competes with the phosphate on the serine and threonine residues on numerous proteins, modifying the function

of the proteins such as kinases, phosphatases, transcription factors, and metabolic enzymes. It is well known that hyperglycaemia is recognised as the primary cause in the pathogenesis of cardiovascular complications in diabetes mellitus. The elevated blood glucose concentration results in increased endothelial intracellular glucose levels. The glucose enters the endothelial cells via the GLUT-1 transporter, driven by a concentration gradient. Earlier studies demonstrated that the elevated flux through the HPB leads to insulin resistance and glucose toxicity. The HBP flux can be increased by addition of exogenous glucose or glucosamine, which enters cells via the glucose transporter system and is phosphorylated to glucosamine-6-phosphate by hexokinase, thereby bypassing the GFAT and elevating the UDP-GlcNAc levels. Recently it has been proposed that in hyperglycaemia similar glycosylation procedures lead to functional changes in eNOS. The increased flux of the hexosamine biosynthetic pathway impairs the IR/IRS/PI3-K/Akt pathway, resulting in disregulation of eNOS activity and consequently endothelial dysfunction. NO does not only contribute towards the regulation of arteriolar diameter, it has an important regulatory role in thrombocyte aggregation, inflammatory mechanisms, remodelling, and atherogenesis inhibition. Adjacent occupancy of a protein region (for example: eNOS) by either *O*-GlcNAc or phosphate can affect protein function either by each modification influencing the cycling of the other, or by their effect on other post-translational modifications. eNOS has different phosphorylation sites; the phosphorylated Thr495 residue inhibits the phosphorylated Ser1177 and activates eNOS. In a recent study, human coronary endothelial cells were incubated in 20 mmol/L glucose or 7.5 mmol/L glucosamine solution for 72 hours. The *O*-GlcNAcylation of eNOS was elevated by 294% compared to the control group, and could be completely blocked with the GFAT enzyme inhibitor azaserine in high glucose conditions, but had no effect in cells incubated with glucosamine. Furthermore, the inhibition of eNOS activation via increased glycolylation could also be demonstrated; however, the

pathophysiological mechanisms of the *O*-GlcNAcylation induced endothelial dysfunction are still not understood.

2. OBJECTIVES

Previous studies using tissue from patients with diabetes mellitus have shown vasomotor dysfunction in conduit vessels. Whether similar changes occur in the human microcirculation, for example in coronary and skeletal muscle arterioles, in diabetes mellitus is not known, and overall the underlying mechanisms are still incompletely understood. Furthermore, the acute effect of hyperglycaemia on smooth muscle and endothelial cell function remains to be evaluated.

Based on these statements I set out the following objectives in my scientific research:

1. To examine the vasomotor function of isolated coronary microvessels obtained from patients undergoing cardiac surgery.
2. To clarify how potential pathologic conditions in diabetes mellitus and increased protein glycosylation affect the vasomotor function of microvessels.
3. Furthermore, to examine how the mechanisms mediated by endothelial and smooth muscle cells underlie the dysfunction.

The results of our experiments, and conclusions from these results may assist in the understanding of the pathophysiological processes of coronary microvessel dysfunction which develop in human diabetes mellitus. Moreover, they may contribute to more precise understanding of pharmacological therapeutic principles, which are aimed to counterbalance altered microvessel function and to decrease cardiac risk associated with this dysfunction.

3. METHODS

3.1. Patient characteristics

All protocols were approved by the Ethical Committee at the University of Debrecen, Medical and Health Science Center and at the New York Medical College. All patients were given written information about the experimental use of human specimens.

Assessment of coronary arteriolar responses was performed in patients (*n*=41) who underwent cardiac surgery. The patients were divided into groups based on the presence (DM(+)) or lack (DM(-)) of documented diabetes mellitus.

3.2. Isolated microvessel technique

We performed our experiments on isolated microvessels using human coronary arterioles (about 100 μm in diameter) and rat gracilis muscle arterioles (about 150 μm in diameter). The coronary arterioles were isolated from a segment of the right atrial appendage removed either during coronary artery bypass surgery or heart valve replacement surgery. The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium and the gracilis muscle was removed. The right atrial appendage and gracilis muscle was fixed with needles in a silicone-based Petri dish containing cold (0-4 degrees Celsius, pH 7.4) Krebs solution (110.0 mmol/L NaCl, 5.0 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1.0 mmol/L $MgSO_4$, 1.0 mmol/L KH_2PO_4, 5.5 mmol/L glucose and 24.0 mmol/L $NaHCO_3$). Then about a 1.5-millimeter segment of the primary coronary or gracilis arteriole was isolated using microsurgery tools and with the assistance of stereomicroscopy (Nikon SMZ 600). The isolated arteriole was first fixed by cannulating at one end (this was to become the proximal end), then blood cells were removed from the lumen by using a perfusion pressure of 20 mmHg. Following cannulation of the distal end of the vessel, it was put into an

organ bath system with constant temperature (37 degrees Celsius, pH 7.4). This process was done after the initial vessel length was preset to an optimal length with the assistance of a microscrew. Continous flow (40 ml/min) with oxygenated (O_2: 10%, CO_2: 5%, N_2: 85%) Krebs solution was ensured in the organ chamber. The intraluminal pressure was raised slowly to 80 mmHg with the assistance of a gravity system, and it was kept at 80 mmHg for about 60 minutes until the condition of the vessel stabilized. The intraluminal pressure was continously measured with a pressure transducer throughout the whole period. Images were recorded with a digital camera (CFW1310, Scion Corp, USA) attached to a microscope (Nikon, Eclipse 80i). The inner diameter of the isolated arteriole was measured using Image J software (NIH Image, MD, USA).

3.3. Examination of arteriole function using vasoactive agents

Spontaneous myogenic tone developed in the isolated coronary arteriole under the effect of applying 80 mmHg intraluminal pressure during the one-hour incubation period. We tested the responses of the isolated coronary microvessels in our experiments evoked by endothelium-dependent and endothelium-independent vasoactive substances, each having well known modes of action. The applied vasoactive substances were administered in adequate finalconcentrations into the perfused bath with a known volume (15 mL) during the protocols. Applying cumulative concentrations the maximal effects of the each individual substance on vessel diameter were continously recorded.

During the first series of experiments changes in diameter were measured first in response to the endothelium-dependent vasodilator **acetylcholine (ACh; 0.1 nmol/L – 0.1 μmol/L)**, then to **sodium nitroprusside (SNP; 0.1 nmol/l – 1 μmol/L)**, an endothelium-independent vasodilator substance acting directly on smooth muscle cells. The individual cumulative concentration-response curves were followed by a wash-out period and an incubation period of 10 minutes, during which the arterioles regained their initial myogenic tone. The same

protocols were repeated in the presence of **N^{ω}-nitro-L-arginine-methyl-ester (L-NAME, 200 μmol/L,** 30 minutes incubation period**)**, an inhibitor of the NO synthase. In separate experiments, coronary arterioles were incubated with ***N^{G}*-hydroxy-L-arginine (L-NOHA, 10 μmol/L,** 30 minutes incubation period**)**, a selective inhibitor of arginase to block the hypothetically upregulated arginase 1 activity; diameter measurements with ACh and SNP were then repeated. Finally, the arterioles were incubated with **L-arginine (3 mmol/L,** 30 minutes incubation period**)** and agonist-induced diameter changes were examined again.

We tested the vasodilatatory function of isolated skeletal muscle arterioles with cumulative concentrations of the endothelium-dependent vasodilator histamine (1 nmol/L - 10 μmol/L), ACh (1 nmol/L – 1 μmol/L) or SNP (1 nmol/L - 1 μmol/L) by measuring changes in diameter after administration. Furthermore, vasoconstrictors that primarily act on smooth muscle cells, such as norepinephrine (NE, 0.3 nmol/L – 1 μm/L) and serotonin (5-HT, 0.1 nmol/L – 1 μmol/L) were also applied to establish their function. In a separate set of experiments the vessels were incubated with 30 mmol/L glucose or 5 mmol/L glucosamine (incubation time: 2 hours) to investigate changes in agonist-induced arteriolar responses. Histamine-induced dilations were also observed in the presence of L-NAME (200 μmol/L, incubation for 20 min) with or without azaserine (20 μmol/L, incubation for 20 min), which is a glutamine analogue and known to irreversibly inhibit fructose-6-phosphate amidotransferase. As an osmotic control, mannitol (30 mmol/L) was used in similar protocols and was found to have no effect on either vasodilator or vasoconstrictor responses (data not shown).

3.4. Immunohistochemistry

In order to visualize individual endothelial cells, after longitudinal cutting, *en face* preparations of coronary arterioles from patients with or without diabetes (*n*=4 in each group) were prepared. Acetone-fixed preparations were

simultaneously immunolabeled with a monoclonal mouse anti-arginase 1 primary antibody (dilution 1:100) and a polyclonal rabbit eNOS primary antibody (dilution 1:100). Subsequent fluorescent labeling was performed with Alexa-488-labeled anti-rabbit or Alexa-597-labeled anti-mouse secondary antibodies. DAPI was used for nuclear staining. To confirm a lack of non-specific binding, the primary antibody was omitted. Using a 100x, oil immersion objective (NA: 1.4) individual endothelial cells were visualized in the en face vascular preparation. Images were collected from multiple endothelial cells (at least 5 cells from 3 different regions from each vessels) with an electron multiplying CCD camera (Luca^{EM}-S, Andor) connected to an Olympus BX61 microscope. Merged RGB images were generated with NIH Image J software and representative images are shown.

3.5. Western immunoblot

Coronary arterioles were dissected from the hearts of diabetic and non-diabetic patients, cleared of connective tissue and briefly rinsed in ice-cold physiological salt solution. Similar-sized, single coronary arterioles were homogenized in 20 μL of RIPA buffer mixed with an equal amount of Laemmli sample buffer. The total homogenate was then loaded into the 10% polyacrylamide gel for electrophoresis (125V, 1 h). After blotting, a goat polyclonal antibody was used for detection of protein expression of arginase 1 (dilution: 1:1000). Membranes were also re-probed with anti-β-actin IgG (dilution: 1:5000) to normalize for loading variations in protein concentrations. Corresponding horseradish peroxidase-labeled secondary antibodies were used and enhanced chemiluminescence was visualized autoradiographically. Optical density of bands was measured and normalized to β-actin.

Branches of femoral arteries were dissected from Wistar rats and cleared of connective tissue. Vessels were exposed to normal and high glucose concentrations or glucosamine and incubated for 2 hours, after which they were

snap frozen in liquid nitrogen. After the addition of 20 µl RIPA buffer (containing protease and phosphatase inhibitors) arteries were homogenized and 20 µl of Laemmli sample buffer was added. Immunoblot analysis was carried out as described before. Primary antibodies were used for detection of *O*-GlcNAc (dilution 1:1000, CTD110.6, Convance, USA) as well as for detection of protein expression of eNOS (anti-eNOS, Transduction, dilution 1:1000) and phospho-eNOS levels (anti-P-eNOS-Ser-1177, dilution 1:500, BD Bioscieses). Anti-β-actin IgG was used as a loading control. Corresponding horseradish peroxidase-labeled secondary antibodies were used and signals were revealed with chemiluminescence and visualized autoradiographically. Optical density of bands were quantified by using Image J software (NIH).

3.6. Statistics

Agonist-induced arteriolar responses and myogenic tone were expressed as changes in arteriolar diameter, as a percentage of the maximal dilation defined as the passive diameter of the vessel at 80 mmHg intraluminal pressure in Ca^{2+}-free medium. Statistical analyses were performed using GraphPad Prism Software (version 5.0 for Macintosh, San Diego, CA). All pertinent risk factors were examined by Fisher exact test, and continuous variables were examined by Student's *t*-test between the two patient groups. Statistical analysis of agonist-induced vascular responses was performed by repeated-measures ANOVA, followed by Tukey's post hoc test. Data are expressed as the mean ± S.E.M.; $p<0.05$ was considered statistically significant.

4. RESULTS

4.1. The effect of diabetes mellitus on vasomotor function of coronary microvessels

The effect of dibetes mellitus on vasomotor function was investigated in coronary arterioles isolated from patients (*n*=41) undergoing coronary bypass or valve replacement surgery. The patients were divided into groups based on the presence (DM(+), *n*=20) or lack (DM(–), *n*=21) of documented diabetes mellitus. In the two groups there was no difference in patient age or gender, or the type of cardiac surgery. To further characterize underlying diseases, heart failure and peripheral vascular disease were observed in the DM(+), but not in the DM(–) group. The two groups were taking similar medication, except the the DM(+) patients also took insulin and various oral antidiabetic drugs.

The spontaneous myogenic tone diameters and passive maximal diameters at 80 mmHg were not significantly different between the arterioles studied from DM(+) and DM(-) patients [active DM(-): 106±9 μm and DM(+): 97±12 μm; passive in Ca^{2+} free solution, DM(-): 131±11μm and DM(+): 133±17 μm], resulting in the same levels of myogenic tone [DM(-): 38 ± 6 %, DM(+): 39 ± 5 %]. First, we found that ACh (0.1 nmol/L – 0.1 μmol/L) only evoked modest dilation and caused significant constrictions in response to the highest (0.1 μM) concentrations of ACh with similar magnitude in patients with or without DM. The NO synthesis inhibitor, L-NAME had no effect on these responses in either group of arterioles. The NO donor, SNP-induced dilations were also similar in magnitude in coronary arterioles isolated from patients with or without DM. These results and the fact that myogenic tone was similar at the beginning, demonstrate intact smooth muscle reactivity in the two groups. Previous studies have shown that diabetes mellitus is associated with reduced or impaired NO production; to reveal the underlying mechanisms, we examined the diameter changes in the presence of selective inhibitor of arginase (L-NOHA, 10 μmol/L,

30 minutes incubation period). We found that in the presence of L-NOHA, ACh elicited significant dilation in coronary arterioles of DM patients, but it had no effect on diminished coronary responses in patients without DM. The upregulated arginase 1 may reduce the level of L-arginine, which is one of the most important substrates for NO synthesis. Similarly, in arterioles from DM patients, application of L-arginine restored vasodilation to ACh, but had no affect on diminished responses of vessels obtained from non-diabetic patients. L-NOHA had no significant effect on the basal diameter of arterioles [after incubation with L-NOHA arteriolar diameter was: 98±10 μm and 91±7 μm in DM(-) and DM(+) patients, respectively], while L-arginine incubation increased basal diameters in both groups [after incubation with L-arginine arteriolar diameter was: 112±9 μm and 113±14 μm in DM(-) and DM(+) patients, respectively].

Using fluorescence microscopy and immunocytochemistry approaches, protein expression of eNOS and arginase 1 was also detected in individual endothelial cells in coronary arterioles of patients with or without DM. Arginase 1 immunostaining was prominent in the coronary endothelium of DM patients, and arginase 1 was less abundantly expressed in those arterioles of non-DM subjects. Importantly, we found that arginase 1 immunostaining was co-localized with eNOS in endothelial cells of coronary arterioles of DM subjects, but not in non-diabetics. Protein expression of arginase 1 was detected by Western blot in coronary arterioles isolated from the atrial appendages of DM and non-DM patients. We found increased arginase 1 expression in coronary arterioles from DM patients as compared to subjects without DM.

In summary, we demonstrated that arginase 1 expression increased in patients with diabetes mellitus, which was likely resulted in greater breakdown of L-arginine. The reduced bioavailability of L-arginine for eNOS causes reduced or diminished NO mediated vasodilatation in human coronary

arterioles. It is likely that these changes play a role in the development of microvascular dysfunction in diabetes mellitus.

4.2. Effect of high glucose and glucosamine on skeletal arteriolar vasomotor responses

The high blood glucose level leads to many vascular complications in diabetes mellitus. We created a high glucose condition to examine the function of the skeletal muscle arterioles. In isolated, pressurized (80 mmHg) gracilis muscle arterioles of the rat, active arteriolar tone developed (~ 30 %) in response to intraluminal pressure without the use of any vasoactive agent. First, we examined the endothelium-dependent ACh- (1 nmol/L – 1 μmol/L) and histamine- **(**1 nmol/L – 10 μmol/L) induced diameter changes in control conditions. Histamine-induced dilations were significantly reduced in the presence of the inhibitor of NO synthase (L-NAME, 200 μmol/L), whereas L-NAME had no effect on ACh-induced arteriolar responses, because ACh induced dilation is mainly via EDHF and not the NO pathway. In comparison with the control responses, histamine-induced dilations were abolished in the high glucose condition (30 mmol/L, 2 h incubation), whereas it had no effect on ACh-induced arteriolar responses. As an osmotic control, mannitol (25 mmol/L, 2 h incubation) was used and found to have no effects on histamine-induced vasodilatation. L-NAME had no effect on histamine- or ACh-induced arteriolar responses in high glucose concentrations. One of the key enzymes of the HBP is the *L*-glutamine-*D*-fructose-6-phosphate amidotransferase (GFAT), which can be irreversibly blocked by azaserine. We found that in the presence of azaserine (20 μmol/L), histamine-induced dilations were restored to the control level, even in the presence of high glucose. In the presence of azaserine, additional administration of the NO synthase inhibitor, L-NAME significantly reduced the magnitude of histamine-induced dilations. The flux through the HBP can be increased by the administration of exogen glucosamine, which enters the cells

via the glucose transporter system, where it is phosphorylated to glucosamine-6-phosphate by hexokinase, thereby bypassing the rate-limiting enzyme GFAT and causing quick elevation of the level of UDP-GlcNAc. In separate experiments arterioles were incubated with glucosamine (5 mmol/L), a direct substrate of the hexosamine-pathway. Glucosamine significantly reduced histamine-induced arteriolar dilations, to a similar extent to that elicited by high glucose exposure, but it was not significantly affected by azaserine treatment. There were no significant differences observed in response to the endothelium-independent vasodilator, sodium nitroprusside; furthermore, no differences were observed in the vasoconstriction to either norepinephrine or serotonin in normal or high glucose buffers.

In order to quantify the level of protein *O*-GlcNAcylation, Western blot analysis was performed in normal and high glucose conditions as well as in arteries exposed to glucosamine. For this assay we used larger femoral arteries for a more efficient and reliable detection of *O*-GlcNAcylation and P-eNOS levels in the vascular wall; therefore, these data could not necessarlily be extrapolated to microvessels and should be interpreted cautiously. We found increases in protein *O*-GlcNAcylation in high glucose and also glucosamine-exposed femoral arteries, when compared with the normal glucose condition. We observed increased protein *O*-GlcNAcylation of various proteins, including those ~ 140 KDa molecular weight proteins that could represent eNOS. We sought to determine the effect of high glucose on the phosphorylation state of eNOS. To this end, arteries were exposed to 5.5 mmol/L and 30 mmol/L glucose concentration and then P-eNOS (Ser-1177) levels were detected by Western immunoblots. Whilst the level of eNOS protein remained unchanged, the level of P-eNOS (Ser-1177) was significantly decreased in high glucose exposed arteries compared to that of normal glucose exposed vessels.

5. DISCUSSION

Despite the current approaches to treat type 1 and type 2 diabetes mellitus, the complications of diabetes still factor amongst the highest mortality rates of diseases. 80 % of the mortality originates from cardiovascular diseases. It has been emphasized that hyperglycaemia causes micro- and macroangiopathy which can be responsible for the high mortality. The UKPDS (United Kingdom Prospective Diabetes Study) revealed that at the diagnosis of type 2 diabetes, microangiopathay was observed in addition to the macroangiopaty seen in 33 % of the patients. While in type 1 diabetes the coronary disease is recognised as a late complication of the long-term metabolic disease, in type 2 diabetes it can be present at the time hyperglycaemia is diagnosed. Although clinical experience clearly shows that in diabetes the coronary diseases are at the top of the high percentage of the morbidity and mortality, unfortunately the underlying mechanisms remain poorly understood. There is even less known about the changes taking place in the resistance vessels.

Therefore the aim of our studies was to investigate the changes in vasomotor function of the human coronary arterioles in diabetes mellitus. We isolated coronary arterioles from the hearts of patients who underwent cardiac surgery, and studied the emergent pathological mechanisms, and the possible reasons for the changes in the endothelium-dependent and smooth muscle-mediated vasomotor function. In addition, we aimed to reveal the role of the underlying mechanisms of vasomotor dysfunction in skeletal muscle vessels, especially the impact of the elevated glucose concentration on the microvessels.

5.1 The effect of diabetes mellitus on vasomotor function of coronary microvessels

NO has an important role in the regulation of the diameter of the vessels in the human body, and therefore in the regulation of coronary arteriolar flow.

Many studies show decreased NO production in the large conduit vessels and resistance arteries in diabetes mellitus. The pathophysiological role of the endothelial dysfunction in the coronary vessels of diabetic patients and the underlying mechanisms are poorly understood. Our observations suggested that the endothelium-dependent vasodilator ACh induced vasoconstriction in coronary arterioles isolated from the right atrial appendage from patients who underwent cardiac surgery, both in the diabetic and non-diabetic group, presumably because of the eNOS dysfunction. In this study we found that inhibition of NO synthesis did not affect ACh-induced coronary vasomotor responses both in patients with or without diabetes. In this context, previously we have found that coronary arterioles obtained from a small number of young individuals (undergoing only valve surgery and had no sign of coronary artery disease, such as patients with Marfan syndrome) develop vasodilation (max: ~60%) in response to ACh (10^{-7} M), a response which was diminished by subsequent inhibition of NO synthesis with L-NAME (Bagi, unpublished observation). Collectively, these aforementioned observations indicate that coronary arterioles from patients involved in the current study lack NO-dependent ACh-induced vasomotor responses and suggest that these patients with various other co-morbidities already exhibit compromised availability of NO, regardless of the presence or absence of diabetes. On the other hand, our finding that the NO donor, SNP, was able to evoke dilation that was similar in the two groups indicates an intact downstream, soluble guanylate cyclase and cGMP-dependent signaling pathway in these arterioles.

It is known that an adequate level of substrates and cofactors for NO synthesis, such as L-arginine and BH_4 is essential for NO synthesis and NO-mediated vasodilation. Diabetes has been shown to interfere with the availability of these cofactors thereby leading to diminished NO synthesis. To provide experimental evidence for this scenario a research group demonstrated that in healthy humans, an oral glucose challenge-induced reduction in forearm blood

flow was restored by pre-treatment with BH_4. Co-infusion of BH_4 and L-arginine into the forearm of diabetic patients prevented ischemia reperfusion-induced endothelial dysfunction in the brachial artery. Oral L-arginine administration alone also improved brachial artery relaxation in women with diabetes. Thus, it seems highly likely that diabetes leads to impaired NO synthesis by interfering with the availability of the eNOS cofactor BH_4. Furthermore, the availability of the NO synthase substrate, L-arginine seems also compromised in diabetes, which has been demonstrated in early investigations in diabetic animals.

Recent studies suggested that arginase, the focal enzyme of the urea cycle, may reduce the L-arginine concentration in close proximity to NO synthase, hence limiting NO synthesis. These observations raise the possible role of arginase in the development of endothelial dysfunction in diabetes. In agreement with this observation and extending those toward human diabetes, in this study we have demonstrated that diabetes is associated with increased expression of arginase 1 in the coronary arteriolar wall. Arginase 1 expression was abundant in endothelial cells and was co-localized with endothelial NO synthase in coronary vessels of diabetic patients, but not in non-diabetics. With Western blot we found increased arginase 1 expression in coronary arterioles of diabetic patients as compared to subjects without. To reveal functional consequence(s) of the upregulated arginase 1 we demonstrated that inhibition of arginase with L-NOHArestored endothelium-dependent agonist-induced dilation in coronary arterioles of diabetic patients; incubation with L-arginine resulted in a similar response. It should be noted that L-NOHA does not have selectivity towards arginase isoforms (arginase 1 versus arginase 2). Thus, the functional role for arginase 2 in the development of coronary vasomotor dysfunction in diabetic patients cannot be entirely excluded. In this regard, a previous study has found that increased expression of arginase 2 may lead to decreased NO synthesis in pulmonary endothelial cells of patients with pulmonary arterial hypertension.

The pathological role of arginase isoforms in diabetes-related coronary microvasular dysfunction has yet to be elucidated.

In this regard, a recent study investigated the effect of the two isoforms of arginase [arginase 1 (AI) and arginase 2 (AII)] in aortas isolated from streptozotocin-induced diabetic mice. The endothelium-dependent vasodilation was significantly enhanced in the aortas isolated from the wild type (WT, 53%) and the $AI^{+/+}AII^{-/-}$ (44%) group, compared to the $AI^{+/-}AII^{-/-}$ (27%), indicating a major involvement of AI in diabetes-induced vascular dysfunction. The mechanisms leading to increased arginase 1 expression in diabetic coronary arterioles are unexplained. It is well known that insulin inhibits the expression of the urea cycle enzymes, and due to the developed insulin resistance (type 2) or lack of insulin (type 1) in diabetes leads to the increased arginase 1 expression. We think that these pathways are involved in diabetes-induced microvascular dysfunction.

5.2 The effect of high glucose on vasomotor function of skeletal muscle microvessels

Previous studies revealed that high blood glucose impairs flow- and agonist-mediated endothelium-dependent vasodilation, thereby contributing to the development of cardiovascular diseases. There are several mechanisms that could be responsible for the effect of high glucose, such as the polyol pathway, protein glycosylation (producing AGE - advanced glycation end product), the HBP pathway, and the activation of the protein kinase C (PKC). The polyol pathway is thought to act by increasing oxidative stress. When the glucose concentration in the cell becomes too high, aldose reductase, converting glucose to sorbitol, depletes NADPH, which is the essential cofactor for regenerating a critical intracellular antioxidant, reduced glutathione, so the steady state is shifted towards the oxidative side. The protein glycosylation non-specifically modifiesintra- and extracellular proteins that directly or indirectly activate

signalling pathways causing dysfunction. Glucose activates PKC by increasing the *de-novo* level of diacylglycerol (DAG). PKC activates numerous mechanisms, including growth factor synthesis (VEGF, TGF-β), and via NADPH oxidase can increase the level of free radicals and decrease eNOS activation. The increased flux through the HBP leads to enhanced *O*-GlcNAcylation on the serine and threonine residues of proteins that are important in the regulation of normal cellular homeostasis. Increased *O*-GlcNAcylation of proteins is considered to be a major contributor to the etiology of various human diseases, such as diabetes mellitus. Therefore, I investigated the effect of high glucose on vasomotor function of skeletal muscle arterioles and the underlying mechanisms in the function of endothelial and smooth muscle cells. Our results indicate that in high glucose conditions the histamine-induced vasodilation was abolished in rat skeletal muscle arterioles, compared to the control cases. We chose to investigate the histamine response as an indicator of the function of eNOS in high glucose conditions, as we previously demonstrated that L-NAME significantly inhibited the histamine-induced vasodilation in arterioles isolated from gracilis muscles, indicating the important role for NO. Furthermore, we investigated the vasodilator effect of ACh, another endothelium-dependent vasodilator, in control cases and in high glucose conditions and we found that high glucose had no effect on the ACh-induced responses. A possible explanation for this phenomenon could be that ACh mostly acts through the EDHF and not the NO pathway. In the presence of the GFAT inhibitor azaserine, histamine-induced dilations were restored to the control level, even in the presence of high glucose. GFAT transforms fructose-6-phosphate into glucosamie-6-phosphate, thereby determining the speed of the HPB pathway, indicating the important role of the O-GlcNAcylation in these mechanisms. Bypassing the rate-limiting enzyme GFAT with glucosamine can cause a quick elevation in the level of UDP-GlcNAc. We found that in the presence of glucosamine, histamine-induced vasodilation was significantly

reduced; however, incubation with azaserine had no effect on the impaired vasodilation compared to the high glucose experiments. The reason for this may be that the flux of the HBP pathway can be increased by administering glucosamine, which is taken up cells via the glucose transport systems and transforms into glucosamine-6-phosphate via hexokinase, thereby increasing the level of the UDP-GlcNAc resulting in increased O-GlcNAcylation. We did not find any difference in the vasodilation induced by endotheliumindependent direct NO donor SNP compared to the control group, leading us to the conclusion that the NO sensitivity of the vascular smooth muscle cells remained unchanged in high glucose conditions. Furthermore, no differences were observed in the vasoconstriction to norepinephrine and serotonin in normal and high glucose. In line with the functional results we found an increased protein *O*-GlcNacylation in skeletal muscle arterioles in high glucose and glucosamine compared to the control group. It is well known that on some proteins, *O*-GlcNAc competes directly with phosphate for serine/threonine residues. The Ser1177 phosphorylation activates the endothelial NO synthase enzyme, but in high glucose conditions the phosphorylation level is reduced compared to the control group by increased *O*-GlcNAcylation.

In summary, in high glucose and glucosamine exposed rat skeletal muscle arterioles the protein *O*-GlcNAcylation is increased, contributing to the decreased endothelial-dependent vasodilation, probably through the diminished phosphorylation of eNOS at Ser1177. Our findings indicate that the increased flux through the HBP has an important role in the development of vasomotor dysfunction in early and established diabetes mellitus. We found that the high glucose and consecutive protein glycosylation leads to impaired endothelium-dependent vasodilation in the early stage of diabetes, whilst the regulatory pathways in the smooth muscles remains unchanged. There remain some unanswered questions, however, such as how the *in vivo* O-GlcNAcylation

influences the function of the microvessels, and whether or not these mechanisms have a role in the increased vascular resistance and in the development of hypertension or other cardiovascular complications in diabetes mellitus.

6. SUMMARY

It is well known that resistance vessel vasomotor function is impared in diabetes mellitus, however, the underlying mechanisms are not completely understood. Therefore, we aimed to study the vasomotor function in isolated human coronary arterioles taken from patients undergoing coronary surgery with and without diabetes mellitus. Furthermore, we examined the effect of high glucose concentration and glucosamine on skeletal muscle arteriolar dilation. Our key observations were the following:

1) In coronary arterioles dissected from the heart of patients with or without diabetes, we found diminished vasodilatation to ACh, which was not affected by the inhibition of NO synthesis.

2) In patients with diabetes, the impaired endothelium-mediated vasodilation was restored by prior incubation with an arginase inhibitor or L-arginine. These findings suggest that in the coronary arterioles of patients with diabetes, arginase 1 is upregulated, which interferes with NO-mediated vasomotor responses.

3) In the subsequent experiments, we found an increased flux through the hexosamine biosythetic pathway, which led to enhanced protein *O*-GlcNAcylation of eNOS under either high glucose or glucosamine conditions, which consequently interfered with NO-dependent arteriolar dilation.

Based on our findings we believe that the increased protein expression of arginase 1 in diabetes mellitus plays a key role in the development of endothelial dysfunction in human coronary arterioles. Furthermore, an increased glucose concentration may lead to augmented glycosylation of various proteins that contribute to the development of diabetic complications. We believe that different inhibitor agents acting on arginase 1 or the hexosamine biosythetic pathway or supplementation with L-arginine may play a potential role in the future treatment of diabetes-associated microvascular dysfunction.

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List of publications related to the dissertation

1. **Beleznai, T.**, Bagi, Z.: Activation of hexosamine pathway impairs nitric oxide (NO)-dependent arteriolar dilations by increased protein O-GlcNAcylation.
   Vasc. Pharmacol. Epub ahead of print (2011)
   DOI: http://dx.doi.org/10.1016/j.vph.2011.11.003
   IF:2.174 (2010)

2. **Beleznai, T.**, Fehér, A., Spielvogel, D., Lansman, S.L., Bagi, Z.: Arginase 1 contributes to diminished coronary arteriolar dilation in patients with diabetes.
   Am. J. Physiol.-Heart Circul. Physiol. 300 (3), H777-H783, 2011.
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List of other publications

3. **Beleznai, T.**, Takano, H., Hamill, C., Yarova, P.L., Douglas, G., Channon, K., Dora, K.A.: Enhanced K+-channel-mediated endothelium-dependent local and conducted dilation of small mesenteric arteries from ApoE-/- mice.
   Cardiovasc. Res. 92 (2), 199-208, 2011.
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4. **Beleznai, T.**, Yarova, P.L., Yuill, K.H., Dora, K.A.: Smooth Muscle Ca2+-Activated and Voltage-Gated K+ Channels Modulate Conducted Dilation in Rat Isolated Small Mesenteric Arteries.
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   Pharmacol. Rep. 61 (1), 99-104, 2009.
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