Thesis for the degree of doctor of Philosophy, Faculty of Medicine

Vascular and metabolic effects of selective PDE-5 inhibition
Clinical and experimental studies

Lovisa Sjögren

Department of Molecular and Clinical Medicine, Institute of Medicine at Sahlgrenska Academy, University of Gothenburg
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To the best family in the world
Claire, Mathilde, Malou and Daniel
ABSTRACT

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Clinical and experimental studies

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Key words: Type 2 diabetes, phosphodiesterase-5 inhibition, inflammation, endothelial cells, HUVEC.

TNFα, ICAM-1, VCAM-1, E-Selectin, JNK, MAPK

Background: Type 2 diabetes (T2D) patients show impaired glucose metabolism, endothelial dysfunction, chronic low-grade inflammation as well as an increased risk of cardiovascular disease. Phosphodiesterase-5 (PDE-5) inhibition amplifies nitric oxide (NO) signaling within the cell and has emerged as a novel treatment option against microvascular insulin resistance and subclinical inflammation. However, very little is known about metabolic effects induced by PDE-5 inhibition in T2D patients.

The overall aim of this thesis was to investigate whether the selective PDE-5 inhibitor tadalafil demonstrate any positive effect on glucose uptake, vascular function and inflammatory markers in T2D patients, and whether any molecular mechanism could be linked to the tadalafil effect in cultured endothelial cells (HUVEC).

Methods and results Paper I-III

Paper I: 17 female T2D patients and healthy controls were recruited and investigated with muscle microdialysis, plethysmography and sampling from an artery and a deep vein in the forearm, to study acute effects of 20 mg tadalafil compared with placebo in a double-blind, randomized controlled trial (RCT). We found that tadalafil treatment resulted in increased capillary recruitment and glucose uptake in forearm muscle.

Paper II: Twenty-six T2D patients of both gender were included in a RCT with parallel groups. Tadalafil 20 mg or placebo were administered before a mixed meal and participants were investigated with muscle microdialysis, plethysmography and blood sampling from an artery and a deep vein in the forearm. In a post hoc analysis we showed positive microvascular, macrovascular and metabolic effects and decreased circulating levels of the vasoconstricting peptide endothelin-1.

Paper III: We studied the effect of tadalafil on inflammatory signaling in HUVEC using Western blot, ELISA and RT-PCR. The results showed that tadalafil reduced gene expression of inflammatory markers and reduced secretion of endothelin-1. Moreover, tadalafil attenuated TNFα-induced phosphorylation of c-Jun N-terminal Kinase (JNK).

Conclusions: Acute administration of the PDE-5 inhibitor tadalafil induced positive metabolic and vascular effects in T2D patients. Furthermore, tadalafil reduced expression of endothelin-1 via a mechanism involving JNK.

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Andelen av befolkningen som har diabetes i världen ökar. Den stora ökningen som sker tros bero på vår allt mer västerländska livsstil med för lite fysisk aktivitet kombinerat med en ökande andel personer i befolkningen som är överviktiga.

Insulin är ett hormon som är viktigt bland annat för att insulin gör att kroppens vävnader kan ta upp det socker som cirkulerar i blodomloppet.

Vid typ 2 diabetes reagerar inte kroppen på det insulin som bukspottskörteln producerar varför den måste producera mycket stora mängder insulin för att vävnaderna ska kunna ta upp socker och näringsämnen som blodet för med sig, vi kallar detta för insulinresistens. Insulin har andra viktiga effekter så som att det kan styrta hur avslappnade och ihopdragna våra blodkärl är. När kärlet slappnar av ökar dess diameter och mer blod passerar genom just det kärlet. Insulin binder till de celler som täcker insidan av kroppens alla kärl, endotelcellerna. När insulinet bundit till sin receptor på endotelcellen frisätter denna cell genen kväveoxid (NO). NO frisätts från endotelcellen och når glattmuskelcellen i käret som slappnar av och kärets diameter ökar och därmed blodflödet genom käret. På så sätt kan insulin styra blodflödet till de vävnader som tar upp socker, framför allt muskler och fettväv.

Patienter med diabetes har en klart ökad risk för att drabbas av hjärtkärlsjukdom och det beror till stor del på en försämrad funktion i just endotelcellerna. När insulinresistens utvecklas i käret slutar endotelcellen att svara på insulins signal att slappna av och käret hålls i stället mer sammandraget. Detta sker främst genom att NO-frisättningen är lägre men också att en kärlsammandragande peptid, Endothelin-1 (ET-1), produceras i större utsträckning.

Det är känt att en ökad inflammatorisk aktivitet driver utvecklingen av diabetes och hjärtkärlsjukdom.

NO har kända antinflammatoriska egenskaper utöver att det slappnar av blodkärlen. ET-1 är andra sidan har inflammatoriska egenskaper.

Tadalafil är ett läkemedel som hårmar NOs egenskaper genom att öka halten av signalsubstansen cGMP inne i cellerna. Detta läkemedel har visat sig ha positiva effekter på endotelcellernas funktion något som är önskvärt hos T2D patienter.

Genom denna avhandling önskade vi söka svar på om tadalaflil kunde påverka sockerupptaget hos patienter i fasta liksom efter att de intagit en måltid. Med hjälp av analysmetoden mikrodialys, som kan mäta socker inne i muskeln kombinerat med blodflödes mätningar kunde vi undersöka tadalaflils effekter på kärlfunktion och sockerupptag i en muskel på patienternas underarm. Vi ville också studera hur tadalaflil eventuellt kunde påverka inflammation genom att studera nivåer av olika inflammatoriska markörer hos patienter efter att de fått tadalaflil behandling. För att fördjupa vår kunskap ytterligare studerade vi odlade endotelceller efter att de fått inflammatorisk stimulering med eller utan förebehandling med tadalaflil.

Vi fann att patienterna fick ett ökat sockerupptag och att blodflödet i de allra minsta blodkärlen, kapillärerna, ökade. Vidare fann vi att tadalaflil sänkte nivåerna av ET-1 hos patienterna men också i de studerade endotelcellerna där även genomtryck av inflammatoriska gener nedreglerades.

Sammanfattningvis visar denna avhandling att tadalaflil kan påverka endotelcellens funktion och att detta troligtvis leder till förbättrat sockerupptag dels genom att öka andelen små kärl som genomblöds samt genom att minska mängden ET-1.
List of papers


II Postprandial effects of the phosphodiesterase-5 inhibitor tadalafil in type 2 diabetes patients – a randomized controlled trial
Lovisa Sjögren, Josefin Olausson, Lena Strindberg, Reza Mobini, Per Fogelstrand, Lillemor Mattsson Hultén, Per-Anders Jansson Submitted to J Clin Endocrinol Metabol

III Tadalafil Decreases Expression of Endothelin-1 In TNF-α-Activated Human Endothelial Cells – Possible Role of The c-Jun N-terminal Kinase Pathway
*Josefin Olausson, *Lovisa Sjögren, Lena Strindberg, Emanuel Fryk, Per Fogelstrand, Reza Mobini, Lillemor Mattsson Hultén, Per-Anders Jansson; * contributed equally. In manuscript
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>ED</td>
<td>Endothelial dysfunction</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>FGU</td>
<td>Forearm glucose uptake</td>
</tr>
<tr>
<td>FBF</td>
<td>Forearm blood flow</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter 4</td>
</tr>
<tr>
<td>GMP</td>
<td>Gyanosine monophosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Gyanosine tri phosphate</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>IAUC</td>
<td>Incremental area under the curve</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IRS1/2</td>
<td>Insulin receptor substrate-1/2</td>
</tr>
<tr>
<td>JNK</td>
<td>C-jun-nh2-terminal kinase</td>
</tr>
<tr>
<td>kDA</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>Erk ½</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa light chain enhancer of activated B-cells</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>PDE-5</td>
<td>Phosphodiesterase-5</td>
</tr>
<tr>
<td>P38 MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PsGlu</td>
<td>Permeability surface area for glucose</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanylate cyclase</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour necrosis factor alpha receptor</td>
</tr>
<tr>
<td>Raf-1</td>
<td>Raf proto-oncogene serine/threonine-protein kinase</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
</tbody>
</table>
Introduction

Type 2 diabetes

Diabetes is a disease with high and rising prevalence worldwide. In fact the number of patients with diabetes in 2008 was estimated to approximately 350 million demonstrating a more than two-fold increase since the 1980s when the reported number of patients were estimated to approximately 150 million (1). The increase in prevalence is believed to be due to physical inactivity as well as an epidemic of obesity which both lead to insulin resistance and type 2 diabetes (T2D) (2). Type 1 diabetes is characterized by an autoimmune mediated destruction of the pancreatic β-cells resulting in insulin deficiency (3). This thesis will however focus on type 2 diabetes that accounts for almost 90% of all diabetes cases and is characterized by insulin resistance, a metabolic state where insulin-mediated whole-body glucose uptake is impaired in combination with insulin deficiency in spite of high plasma levels of insulin. An absolute deficiency of insulin may develop in patients with type 2 diabetes as a result of progressively dysfunctional β-cells in the pancreas. Many tissues are involved in the pathology of type 2 diabetes, the pancreas with impaired insulin release from the β-cells, the liver with increased glucose production due to insulin resistance, the muscles with decreased glucose uptake, the fat with accelerated lipolysis, inflammatory signaling and decreased glucose uptake, the kidneys with increased glucose resorption, the brain with insulin resistance (4, 5) as well as the gastrointestinal tract with impaired incretin function (6) and growing evidence to the importance of changes in gut microbiome (7). However, this thesis will focus on the vascular endothelium and the role it plays in type 2 diabetes. (Fig 1).

Figure 1. Schematic sketch of the underlying pathology of type 2 diabetes.
Type 2 diabetes and cardiovascular complications

A wide range of complications in patients with T2D are due to impaired micro- and macrovascular function, in fact cardiovascular disease is the principal cause of death in this group of patients believed to cause up to 80% of all deaths in type 2 diabetes patients (8, 9). Patients with T2D develop various cardiovascular complications such as retinopathy, nephropathy, erectile dysfunction and accelerated atherosclerosis (10, 11). Atherosclerotic lesions in arteries to the heart, brain, legs and other organs may cause lethal distal ischemia (8, 12, 13).

The endothelium is the biologically active layer of the blood vessels consisting of a single layer of endothelial cells (10) (Fig 2). Through this vessel wall the endothelium controls vascular tone as well as permeability, coagulation, leucocyte adhesion and extravasation and the inflammatory activity in the vessel wall. In the healthy state the endothelium actively decreases vascular tone, provides permeability for nutrients and hormones. Furthermore, it inhibits inflammatory activity (10). The endothelial cells continuously encounter blood-borne factors from the systemic circulation and responds rapidly to insulin stimulation (14). When insulin stimulates the endothelium nitric oxide (NO), a gaseous molecule that plays various important roles in the cardiovascular system will be released (15). This insulin induced NO release will give rise to vasodilation, increased blood flow, capillary recruitment and due to these changes an increase in the delivery of insulin and glucose to the skeletal muscle. NO is not only a vasodilator but is important for it’s regulatory effects on platelets, proliferation, permeability and inflammation (16, 17). Endothelial dysfunction develops well before overt diabetes (18, 19) and is characterized by reduced bioavailability of NO as a result of the insulin resistance described below. In the insulin resistant state and in T2D the endothelial response to insulin is impaired and this endothelial dysfunction is associated to cardiovascular disease (20, 21).

Figure 2. Structure of the human artery showing the inner layer of the vessel lined with endothelial cells.
Type 2 diabetes and endothelial cell signaling

The endothelial cells act as a paracrine organ that regulates cardiovascular physiology and the endothelium is an important target for insulin (10, 22). The effects of insulin are initiated by the binding of insulin to its specific insulin receptor (IR) on the cell surface (23) resulting in tyrosine phosphorylation of the insulin receptor substrate (IRS) by the insulin receptor tyrosine kinase. In endothelial cells IRS-1 and IRS-2 are especially important (24-26). The effects of insulin on vascular tone are mediated through two signaling pathways in the endothelial cell giving rise to the release of NO and the peptide Endothelin-1 (ET-1) following insulin stimulation. Vasodilator actions of insulin are mediated by phosphatidylinositol 3-kinase (PI3-K) dependent insulin signaling pathways in the endothelium. This pathway stimulates production of NO via the PI3-kinase/Akt/endothelial nitric oxide (eNOS) signaling in which Akt phosphorylates eNOS at serine residue 1177 giving rise to increased NO production and vasodilatation (27-29). The mitogen-activated protein kinase (MAPK) dependent insulin signaling pathways regulate secretion of the vasoconstriction peptide ET-1 from the endothelium (30-32). (Fig 3). In the insulin-resistant state only the PI3-kinase dependent insulin signaling pathway is impaired. Thus, insulin signaling in insulin resistant endothelial cells may result in vasoconstriction due to continued ET-1 secretion and reduced levels of NO (33, 34).

Hypophosphorylation of Akt and eNOS is associated with insulin resistance (35) and altered phosphorylation within the insulin receptor substrate signaling is essential for these changes giving rise to a selective PI3-K-pathway specific insulin resistance (33, 36-38). There is evidence from animal models suggesting that hyperinsulinemia might lead to down regulation of IRS-2 levels causing attenuated IRS-2-PI3K- Akt signaling explaining the selective insulin resistance seen in endothelial cells in T2D patients (36, 39, 40). IRS-1 has also been shown to be involved in glucose uptake in a mice model (36). Several kinases induced by inflammation have been demonstrated to inhibit the insulin signal by phosphorylating serine residues of IRS1, including c-jun N-terminal kinase (JNK) (41-43), mitogen-activated kinase (p38 MAPK) (37) and IκB Kinase (IKK) (44). Further, the kinase ERK1/2 has been shown to be increased in endothelial cells harvested from T2D patients (31). Inflammation and its association to T2D and cardiovascular disease will be further revised later in this introduction.

ET-1 production is controlled at the gene level and several transcription factors including the transcription factor activator protein-1 (AP-1) (45) regulate the gene transcription of ET-1. Endothelin converting enzymes (ECEs) produce ET-1 from Big ET. ET-1 is a vasoconstricting peptide that mediates the majority of its effects by binding to two different subtypes of receptors. ETA, localized mainly on smooth muscle cells and mediating vasoconstriction and ETB which mediates vasodilatation when localized on endothelial cells. However, when localized on smooth muscle cells signaling through ETb receptors mediate vasoconstriction (46).

Patients with insulin resistance and patients with type 2 diabetes have been shown to have increased circulating levels of ET-1 (47-49). In contrast one study found no increased levels of ET-1 in patients with T2D and cardiovascular complication (50). Still, microvascular cells harvested from T2D patients have
been shown to have increased ET-1 expression as compared to non-diabetic subjects (31). ET-1 has further been demonstrated to inhibit the IRS-1/PI3K/Akt pathway (51) and can decrease the expression of the insulin receptor as well as IRS-1 suggesting its involvement in insulin resistance (52). Furthermore, ET-1 has been reported to induce the MAPK signaling pathway in endothelial cells thus promoting its own release (31).

To summarize endothelial insulin resistance can be viewed as an imbalance between the production and release of the anti-inflammatory and vasodilatory NO and the pro-inflammatory and vasoconstricting peptide ET-1 in response to insulin signaling (53).

**Figur 3. Insulin signaling and the importance of crosstalk between tissues.**

- **a)** Insulin signaling endothelial cells following binding of insulin to the Insulin Receptor (IR) activates two parallel signaling pathways PI3-K-Akt-eNOS and the Raf-ERK ½-ET-1. Akt kinase phosphorylates eNOS at serine residue 1177 resulting in NO production and vasodilation. The Raf-ERK-1/2 pathway causes vasoconstriction due to ET-1 production from the biologically inactive form, big endothelin. TNFα inhibits the PI3-K signaling pathway resulting in selective insulin resistance through three known kinases p38 MAPK, JNK and IKK. ROS (reactive oxygen species) are produced due to hyperglycemia and can activate MAPK and are involved in the development of endothelial dysfunction and insulin resistance, as are free fatty acids.

- **b)** Insulin signaling in the skeletal muscle and adipose tissue will give rise to glucose uptake by translocation of GLUT4 through the PI3-K-Akt signaling pathway. However, in the insulin resistant state this will be impaired.

- **c)** The balance in vascular tone is regulated by ET-1 and NO resulting in vasoconstriction or vasodilatation mediated by smooth muscle cells.
Type 2 diabetes and inflammation

Chronic low-grade inflammation has been linked to cardiovascular disease and endothelial dysfunction in patients with T2D (54-60). TNFα is an inflammatory cytokine, produced by macrophages, neutrophils and adipocytes that has been reported to be elevated in patients with T2D as well as in patients with obesity and insulin resistance (61-63). Further, TNFα has been shown to impair capillary recruitment as well as glucose uptake (64, 65). In short, inflammatory cytokines alter vasoregulatory responses, increase the vascular permeability, induces pro-coagulant activity and augments the adhesion of leukocytes to the endothelial cells in the vascular wall (10). For this reason, infiltration of the vascular endothelial wall is a key component of the development of atherosclerosis (66). The levels of circulating adhesion molecules are believed to mirror increased levels of adhesion molecules on the cell surface of endothelial cells (67). (Fig 4).

TNFα regulates gene expression, including the genes for the adhesion molecules ICAM-1, VCAM-1, E-Selectin, by activating transcription factors like NF-κB and AP-1 (55). NK-κB consists of a group of transcription factors that regulate the inflammatory responses and function of vascular cells (68). This effect is mediated by mitogen activated protein kinases (MAPK) that are a family of intracellular signaling proteins. These MAPK serve as pathways to relay signals within the cell following extracellular stimuli such as cytokines (69-72).

The c-Jun NH2-terminal kinase (JNK) is a subgroup of MAP-kinases that is activated primarily by cytokines such as TNFα and environmental stress (71). Increased JNK signaling plays an important role in metabolic and cardiovascular diseases (73-75). A major target of the JNK signaling pathway is the transcription factor activator protein-1 (AP-1) which is activated in part by the phosphorylation of JNK (72, 76). It is known that JNK phosphorylates the insulin receptor substrate at the serine 307 residue (77, 78) and JNK has been proposed as a key mediator of insulin resistance (73, 79, 80). Knockout mice lacking JNK1 or the JNK scaffold, JNK interaction protein 1 (JIP1), have been demonstrated to be resistant to the effects of obesity and insulin resistance following high fat feeding in mice (73, 81). In addition, inhibition of JNK in db/db mice improved insulin resistance and glucose tolerance in a previous study (74). The vasodilatory effects of insulin are inhibited by TNFα and this was shown to be dependent of JNK (41).

NO is now known to have anti-inflammatory properties in the vasculature and the decrease in NO content is thought to develop early during the evolvement of insulin resistance and endothelial dysfunction (82-84). In fact, it is known that NO can regulate the expression of adhesion molecules such as ICAM-1 (85) and that an impaired NO-signaling results in increased TNFα levels following nutritive excess (82, 86). NO mediates anti-inflammatory effects in endothelial cells thus, inhibiting leukocyte migration and adhesion by inhibiting the transcription factor NFκB that induces gene expression of adhesion molecules (87). In addition, NO augments the expression of IκBα which itself inhibits NFκB.
Insulin-mediated eNOS phosphorylation with enhanced NO release inhibits ET-1 production in endothelial cells and this effect is mediated by an effect of NO involving NFκB (89).

ET-1 can activate macrophages resulting in increased release of pro-inflammatory cytokines such as TNFα. Further ET-1 has been shown to increase the levels of adhesion molecules on endothelial cells following stimulation with TNFα. Moreover, TNFα and signaling through the NFκB signaling pathway has been reported to induce production of ET-1 (90-92).

**Figure 4.** Regulation of endothelial inflammatory response and its association to insulin resistance. Several adhesion molecules are involved in attracting and activating the leukocyte. First, P-Selectin captures the leukocyte from the bloodstream. This initiates rolling over the E-Selectin and then to adhesion of the leukocyte to ICAM-1 and VCAM-1 and in the end transmigration. In the insulin-resistant state ET-1 levels are higher thus stimulating TNFα release inducing increased gene expression of adhesion molecules. Due to insulin resistance NO bioavailability is lower and in consequence is unable to maintain its anti-inflammatory effects. Perivascular adipocytes and macrophages also contribute to increased inflammatory signaling by releasing cytokines such as TNFα (38).
Postprandial metabolism and vascular response in patients with type 2 diabetes

Patients with insulin resistance and T2D have postprandial hyperglycemia (93) as well as increased postprandial inflammatory response seen as an increase in the levels of TNFα, CRP and adhesion molecules (63, 94-97). These changes are involved in the development of endothelial dysfunction (93, 98). Oxidative stress, a condition where intracellular production of reactive oxygen species (ROS) affects the capacity of the cellular antioxidants defense system is known to be involved in the progression of endothelial dysfunction as well (10, 99, 100). In addition free fatty acids affect glucose transport, NO signaling and the IRS-1-Akt-PI3-K pathway (44, 101) (Fig 3). In tandem, T2D patients have impaired microvascular recruitment following a meal thereby decreasing glucose disposal to the skeletal muscles (102, 103). The glucose uptake of skeletal muscles plays a crucial role in the control of blood glucose concentrations. However in the insulin resistant state this will be disturbed (104). The delivery of insulin to the skeletal muscle is believed to be the rate-limiting step in the insulin induced glucose uptake (105-108). Microvascular changes are known to occur early in the development of diabetes and are believed to be present even before the onset of overt diabetes (18, 19, 109, 110). This impaired microvascular function in patients with T2D has been attributed to a reduced density of capillaries in the muscles (111, 112) as well as decreased blood flow and perfusion of the microvascular capillaries due to decreased insulin signaling. This reduced perfusion of the capillaries has been demonstrated in animal models (113) and human studies (114) and has been shown to depend on the IRS-PI3K-Akt signaling pathway (36, 115). Insulin promotes metabolic actions by promoting glucose disposal but it also has important vascular actions by inducing the production of NO from the endothelial cells (116, 117). However, in the insulin-resistant state the insulin-eNOS signal is impaired why microvascular perfusion due to insulin signaling is decreased resulting in reduced delivery of insulin and glucose to the muscles (36).

The insulin induced microvascular perfusion of skeletal muscle is known to be dependent on endothelial NO (118) and this has been demonstrated in eNOS knockout mice in which insulin induced capillary recruitment in the skeletal muscle was impaired (36, 119). When NO synthase was inhibited by N-monomethyl-L-arginine (L-NMMA) in a rat model the investigators found that the capillary recruitment in response to insulin was blocked (120). These finding were supported by results from a study investigating the effects of inhibition of NOS in humans. The investigators found that inhibiting NOS resulted in diminished insulin induced capillary recruitment in skeletal muscle (121). In summary, insulin can indeed increase the perfusion of the muscular capillaries through NO. Further, NO can in fact self regulate endothelial uptake of insulin and thus the delivery of insulin to the muscles (122).

ET-1 has been shown to impair microvascular function in T2D patients and blocking of the ETₐ receptor improved capillary perfusion (123) In addition, a receptor antagonist of both ETₐ and ETₜ receptor induced improved endothelial function in T2D patients (124). Interestingly, dual Eta/B receptor blockade stimulates glucose uptake in insulin-resistant patients (125).
ET-1 has been proposed to be involved in the impaired glucose uptake seen in type 2 diabetes via effects on peripheral blood flow or directly on insulin signaling (91).

**Phosphodiesterase-5 inhibition**

Once endothelial NO reaches its target cell NO induces intracellular signaling that involves the activation of soluble guanylate cyclase (sGC) thereby increasing the production of cyclic guanosine monophosphate (cGMP) (126) which will activate the threonine kinase cGMP-dependent protein kinase (PKG) resulting in various positive downstream effects due to NO. One of the effects of NO includes relaxation of vascular smooth muscles giving rise to dilatation of the arteries and arterioles with increased blood flow as a consequence. The intracellular level of cGMP are not solely regulated by the activity of soluble guanylate cyclase but is also regulated by phosphodiesterase conversion of cyclic GMP to GMP thereby attenuating the NO induced signal (Fig 5).

There are 11 types of phosphodiesterases (PDEs) (Table 1). However, the main focus here will be on the characteristics of PDE-5 and the developed inhibitors for PDE-5. Pharmacological PDE-5 inhibitors have been developed to sustain and preserve the NO-signaling pathway. (Fig 5).

There are three PDE 5-inhibitors, sildenafil, vardenafil and tadalafil that have been widely used clinically for treatment of erectile dysfunction and for pulmonary hypertension as well as in research (127). Tadalafil differs from the other pharmaceuticals in structure but also in that it; (1) has a selectivity ratio for PDE5:PDE6 of 780:1 making it highly selective and visual side effects rare, (2) a long half time of 17.5 h, (3) a short time to maximum concentration of 2 h and more importantly, (4) tadalafil absorption is not affected by ingestion of a high fat meal (127, 128).

Chronic treatment with tadalafil has been reported to improve endothelial function in patients with erectile dysfunction (129-131). Interestingly, chronic treatment with tadalafil decreased circulating levels of adhesion molecules as well as ET-1 levels (129, 131). However, these findings have not been replicated in all chronic studies of tadalafil (132, 133). These seemingly anti-inflammatory effects of tadalafil in patients have also been observed in animal models of insulin resistance and T2D (134, 135). In a mouse model lacking eNOS the mice developed an increase in inflammatory cytokines and TNFα was significantly increased. However, this increase could be attenuated by PDE-5 inhibition and this was followed by improved insulin sensitivity in the investigated mice (82). Further, tadalafil has been shown to have positive effects on β-cell function following acute and chronic treatment (133, 136).

Finally, PDE-5 inhibition has been demonstrated to improve insulin action in endothelial cells by increased phosphorylation of Akt and eNOS (137).
Figure 5 The intracellular effect of tadalafil treatment. NO signaling pathway involves activation of soluble guanylate cyclase (sGC) giving resulting in synthesis of cyclic guanosine monophosphate (cGMP) thereby activating the cGMP downstream signaling. However, this will be regulated by PDE-5 that will attenuate the NO induced signaling by transforming cGMP to its inactivated form guanosine monophosphate (GMP). Tadalafil sustains the NO signaling by limiting the transformation of cGMP.
<table>
<thead>
<tr>
<th>ISOENZYME</th>
<th>SUBSTRATE</th>
<th>DISTRIBUTION IN TISSUES</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDE1</td>
<td>cAMP</td>
<td>Brain, lung, heart</td>
</tr>
<tr>
<td>PDE2</td>
<td>cAMP/cGMP</td>
<td>Brain, adrenal cortex, liver, goblet cells, olfactory neurones</td>
</tr>
<tr>
<td>PDE3</td>
<td>cAMP</td>
<td>Smooth muscle, platelets, cardiac muscle, liver</td>
</tr>
<tr>
<td>PDE4</td>
<td>cAMP</td>
<td>Wide tissue distribution</td>
</tr>
</tbody>
</table>
| PDE5      | cGMP          | PDE5A1/A2: brain, lung, heart, kidney, bladder, prostate, urethra, penis, uterus, skeletal muscle  
PDE5A3: heart, bladder, prostate, urethra, penis and uterus |
| PDE6      | cGMP          | Rods and cones in the retina                                                          |
| PDE7      | cAMP          | Skeletal muscle, T-lymphocytes                                                        |
| PDE8      | cAMP          | Testis, ovary, gastrointestinal tract, brain                                           |
| PDE9      | cGMP          | Spleen, gastrointestinal tract, brain                                                 |
| PDE10     | cAMP/cGMP     | Brain, testis, thyroid                                                                |
| PDE11     | cAMP/cGMP     | Smooth muscle, cardiac muscle, prostate, pituitary and salivary glands, testis, liver, kidney, skeletal muscle |

Table 1. Characteristics of PDEs modified from Carson and Lue (127).
Aim

The overall aim of this thesis was to investigate the effect of selective PDE-5 inhibition on glucose metabolism, vasculature and on inflammation in T2D patients through a translational approach.

**Paper I** To investigate the acute effect of the PDE-5 inhibitor tadalafil on vascular function and glucose uptake in T2D patients in the fasting state.

**Paper II** To examine the outcome of the PDE-5 inhibitor tadalafil on micro and macrovascular blood flow, glucose uptake and inflammatory response in T2D patients in the postprandial state.

**Paper III** To investigate whether increased cGMP signaling achieved by PDE-5-inhibition may have anti-inflammatory effects in human endothelial cells following TNFα stimulation and to further elucidate the molecular actions propagating these effects.
Patients and Methods

The patients and methods will be described briefly, since the details are reported in the papers.

Paper I–II
Study participants

In total 33 T2D patients and 10 healthy controls were investigated in two different clinical trials. Their characteristics are described in Table 2. The enrolled patients had no significant concomitant metabolic diseases or complications due to their T2D diagnosis. In Paper I and II treatment with oestrogens, nitrates, beta-blockers or glucocorticoids resulted in exclusion. Patients treated with insulin, GLP-1 analogues, DPP-4 inhibitors or glitazones were excluded in Paper II. All women included were postmenopausal. The glucose tolerance of healthy controls was evaluated by an OGGT and controls were included based on their BMI, age and their physical health. Nicotine use resulted in exclusion for patients and controls.

In Paper II four T2D patients on ACE-inhibitors and two T2D patients exhibiting protocol were excluded and the 20 remaining T2D patients were then investigated in a post hoc analysis. All T2D patients and controls gave their written informed consent and protocols were approved by the Ethics Committee at the University of Gothenburg, Sweden.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Subjects</th>
<th>n</th>
<th>M/F</th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
<th>HbA1C (%)</th>
<th>F-Plasma glucose (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>10</td>
<td>7/3</td>
<td>0/10</td>
<td>0/7</td>
<td>60±5</td>
<td>29.5±3.4</td>
</tr>
<tr>
<td></td>
<td>T2D</td>
<td>10</td>
<td>0/10</td>
<td>0/7</td>
<td>61±6</td>
<td>22.7±1.0</td>
<td>30.9±4.2</td>
</tr>
<tr>
<td></td>
<td>T2D-Placebo</td>
<td>12</td>
<td>8/4</td>
<td>62±6</td>
<td>30.9±4.2</td>
<td>30.9±4.2</td>
<td>45±8</td>
</tr>
<tr>
<td></td>
<td>T2D-Tadalafil</td>
<td>14</td>
<td>9/5</td>
<td>60±9</td>
<td>30.9±4.2</td>
<td>30.9±4.2</td>
<td>45±8</td>
</tr>
<tr>
<td>II ITT</td>
<td>T2D-Placebo</td>
<td>10</td>
<td>6/4</td>
<td>63±5</td>
<td>30±4.5</td>
<td>30±4.5</td>
<td>45±7</td>
</tr>
<tr>
<td></td>
<td>T2D-Tadalafil</td>
<td>10</td>
<td>8/2</td>
<td>59±9</td>
<td>30±4.5</td>
<td>30±4.5</td>
<td>45±7</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of controls and T2D patients in Paper I and T2D patients in Paper II. ITT, Intention to treat in Paper II included all 26 investigated T2D patients. In the Post hoc analysis, Phoc, 20 T2D patients were investigated following exclusion of 4 patients on ACE-inhibitors and 2 patients with protocol discrepancies. Reference value a 3.9-5.3 %, b 31-46 mmol/mol. Data presented as means± SD.
Randomization procedures

The randomization was computerized and done 1:1. Furthermore, the randomization was blinded for study participants as well as for the investigators.

Blood flow measurements

All the investigations were performed with the subjects in the supine position and in a calm and quiet laboratory with a controlled temperature kept at 27 °C at the Wallenberg Laboratory, Sahlgrenska University Hospital. To assess forearm blood flow venous occlusion plethysmography was used to enable investigation of the vascular effects of tadalafil treatment. A stretchable tube containing mercury was tied around the third upper part of the right forearm. In short, venous drainage from the arm is briefly interrupted with an inflatable cuff while arterial inflow into the examined forearm remains unaffected; increase in volume is measured (138, 139).

Muscle microdialysis

Intramuscular microdialysis is a minimally invasive technique that makes determination of concentrations of small solutes in the extracellular fluid, possible. This is achieved by inserting a semipermeable hollow fiber connected to an inlet and outlet tubing which is continuously perfused allowing diffusion of the molecule of interest in the chosen muscle. The retrieved levels from the investigated molecule will be dependent on; (1) the concentration of the molecule of interest in the perfusion fluid, (2) the velocity with which the probe is perfused and (3) the size of the cut off of the membrane. In our studies two catheters (16 x 0.5 mm, 20 kDa molecular mass cut off; CMA Microdialysis AB, Stockholm, Sweden) were guided via a steel mandrin of a 20 Gauge cannula into the brachioradialis muscle. A microinjection pump (CMA 100; CMA Microdialysis AB, Stockholm, Sweden) was connected to the probes and isotonic saline supplemented with 1.5 mmol/l glucose and 0.5 mmol/l urea was perfused at a rate of 2.5 µl/min. Urea was used as an internal reference for calibrating the catheters (140).

The forearm model

Arterio-venous (A-V) differences over the forearm permit investigation of local metabolism in the muscle of interest (141, 142). In our investigations one catheter was inserted retrogradely, 6-8 cm, into one of the branches of the antecubital vein draining the forearm muscle and another catheter was inserted into the radial artery for arterial measurements.

Glucose uptake was obtained by using calculation according to Fick: Glucose uptake = (A-V) x Q where A stands for arterial concentration and V for venous concentration and finally Q for blood flow.

Further, capillary recruitment was assessed through the direct measurement of the capillary permeability-surface area product (PS) for glucose. This is made possible by combining blood flow measurements with
recordings of the arterio-venous concentration and arterio-interstitial concentration gradients by means of concomitant arteriovenous and microdialysis measurements (143, 144). Permeability surface area for glucose was determined by the following equation:

\[ V\cdot A = (I - A) \times \left(1 - e^{PS/Q}\right) \]

- \( V \) = Venous plasma concentration
- \( A \) = Arterial plasma concentration
- \( PS \) = Permeability surface area for glucose
- \( Q \) = plasma flow rate which is calculated by multiplying the forearm blood flow by 100 minus the hematocrit as a percentage.

\( e \) is the base of the natural logarithm \((e = 2.71828)\).

**Biochemical analyses**

Circulating glucose, insulin, triglycerides and free fatty acids were measured before and during 5 hours after the meal, and muscle microdialysis monitored interstitial glucose concentrations. Glucose and a Urea concentrations in the dialysate and plasma fractions were determined with a colorimetric (glucose) and UV method (Urea) on a CMA 600 Microdialysis analyser (Microdialysis AB, Stockholm, Sweden). Plasma insulin concentrations were measured by Insulin ELISA (Mercodia AB, Uppsala, Sweden). In addition, serum triglycerides and plasma free fatty acid concentrations were analysed by the Department of Clinical Biochemistry, Sahlgrenska University Hospital, Gothenburg, Sweden.

Arterial serum was used to analyse ET-1 and markers of inflammation such as ICAM-1, VCAM-1 and E-selectin. ET-1 was measured using QuantiGlo ELISA (R&D systems, Minneapolis, MN, USA) using SpectraMAX GeminiXS (MDS Analytical technologies Ltd, Berkshire, UK). Concentrations of VCAM-1, ICAM-1, and E-Selectin were measured in serum with the Human Vascular Injury II Kit and Human E-Selectin kit in a SECTOR 2400 Imager (Meso Scale Discovery, Gaithersburg, MD, USA).

**Study protocols**

**Paper I**

The study was a randomized, placebo-controlled, double-blind, cross-over study where patients and healthy controls were studied after an overnight fast and in the fasting state. Muscle microdialysis and blood flow were measured continuously combined with blood sampling 1h before receiving placebo or 20 mg of tadalafil and 4 hours after. Patients returned to the laboratory after a 4-6 week long washout period to be studied once more while being administered the opposite treatment. (Fig 6).
Figure 6. Study protocol Paper I. A randomized controlled trial (RCT) of acute administration of 20 mg tadalafil. Patients with T2D and controls were randomized to placebo or tadalafil and received the opposite treatment after a 4-6 week long washout period.

Paper II

The study was a randomized placebo controlled trial where T2D patients were studied in parallel groups following treatment with placebo or tadalafil, 20 mg, 30 minutes before they ingested a mixed meal. The meal had a total energy content of 786 kcal (3291 kJ); 46% fat, 48% carbohydrates and 7% protein and consisted of mashed potatoes, meat sauce, hard bread and butter. Muscle microdialysis and forearm blood flow was repeatedly monitored and blood samples were drawn as indicated. (Fig 7)

Figure 7. Study protocol Paper II. A randomized controlled trial (RCT) of acute administration of 20 mg tadalafil. Patients with T2D were randomized to placebo or tadalafil 30 minutes prior to a mixed meal and were investigated in parallel groups with muscle microdialysis, forearm blood flow and blood samples were drawn repeatedly as indicated.
**Paper III**

**Cell culture**

Human umbilical vein endothelial cells (HUVEC) are cells derived from the endothelium of veins from the umbilical cord. HUVECs can be used as a laboratory model system for the study of endothelial cells. (145)

HUVECs were purchased from ATCC (LGC Standards, Borås, Sweden) and cultured in endothelial basal medium (EBM-2) (Lonza, Basel, Switzerland) supplemented with 5% fetal bovine serum (FBS), human epidermal growth factor (hEGF), hydrocortisone, GA-1000 (Gentamicin, Amphotericin-B), vascular endothelial growth factor (VEGF), Arg3 insulin-like growth factor-1 (R3-IGF-1), ascorbic acid and heparin (provided by Lonza) at 27°C in an atmosphere of 5% CO₂.

**Experimental Design**

HUVEC in passage 4-5 were cultured to near confluence and incubated with serum-free media (EBM-2 without supplements) 3 h prior to treatment. HUVEC were treated with either 1µM tadalafil (Lilly, Indianapolis, IN, USA) or left untreated for 1 h before addition of 4 ng/mL TNF-α (Life Technologies, Carlsbad, CA, USA). In experiments using the PKG inhibitor DT-3 (RQIKIWFQNRRMKWKKLRKKKKH) (Merck Millipore, Darmstadt, Germany), HUVEC were pretreated with 1 µM DT-3 for 30 min before addition of tadalafil. Then, HUVEC were stimulated with 10 µM DEA-NONOate (1,1-diethyl-2-hydroxy-2-nitroso-hydrazine sodium, 2-[N,N-diethylamino]-diazenolate 2-oxide sodium salt hydrate) (Sigma-Aldrich, St. Louis, MO, USA) for 15 min before addition of 4 ng/mL TNF-α. In a further set of experiments, HUVEC were preincubated with 1 mM 8-bromo-cGMP (Merck-Millipore, Darmstadt, Germany) for 4 h before addition of 4 ng/mL TNF-α. In addition, experiments using 50 µM of the JNK inhibitor SP600125 (Sigma-Aldrich, St. Louis, MO, USA) or ERK inhibitor PD98059 (Sigma-Aldrich, St. Louis, MO, USA) were conducted. The inhibitors were added 1 hour before addition of 4 ng/mL TNF-α. (Fig 8).
Figure 8. Study protocol Paper III. HUVEC were preincubated with tadalafil 1µM and subsequently stimulated with TNFα 4ng/ml. To establish the molecular mechanisms behind the effect of tadalafil an analog of cGMP, 8 bromo-cGMP, was used. DEA-NONOate was used as a NO donor. Further, for inhibiting kinases different inhibitors were used; SP600125 (JNK), PD98059 (ERK) and DT-3 (PKG).

RNA extraction, synthesis of cDNA and quantitative gene expression analysis

In brief a real-time polymerase chain reaction is a laboratory technique based on the polymerase chain reaction (PCR) which is used to amplify and simultaneously detect or quantify a specific DNA molecule (146).

HUVEC were incubated with TNF-α for 12 h, washed with Dulbecco’s PBS and RTL buffer (Life Technologies, Carlsbad, CA, USA) with addition of 1% β-mercaptoethanol. RNA was extracted using RNEasy plus mini kit (Qiagen Nordic, Sollentuna, Sweden). Single-stranded cDNA was synthesized from 1 µg total RNA using Superscript VILO kit from Life Technologies (Carlsbad, CA, USA). cDNA was amplified using ABIPrism 7900HT and TaqMan reagents (Life Technologies, Carlsbad, CA, USA). 20x Assays (inventoried) used were ET-1 (Hs00174961_m1), E-selectin (Hs00174057_m1), VCAM1 (Hs00365486_m1) and ICAM1 (Hs00164932_m1) (Life Technologies, Carlsbad, CA, USA). Human RNA 18S was used as endogenous control.
Protein measurements in cell medium

Conditioned medium surrounding HUVEC was taken after 12 h incubation with tadalafil and TNF-α followed by centrifugation and were then analysed using ELISA. The assays used in this study included traditional solid-phase sandwich enzyme-linked immunosorbent assays (ELISA) utilizing chemiluminescence detection.

ET-1 QuantiGlo ELISA Kits use a chemiluminescent substrate for analyte detection and require a luminometer for output reading.

Concentrations of ICAM-1, VCAM-1 and E-Selectin were measured with ultrasensitive electrochemiluminescence detection technology in a SECTOR 2400 Imager (Meso Scale Discovery, Gaithersburg, MD, USA).

These kits have a broad dynamic range in comparison to standard colorimetric immunoassays.

Protein extraction and western blot

Briefly, western blot is an analytical technique used to detect specific proteins from a sample. It uses gel electrophoresis to separate denaturated proteins by the length of the polypeptide. The proteins are then transferred to a membrane where they are stained with antibodies specific to the target protein (147, 148)

Our work was carried out as follows: HUVEC were incubated with TNF-α for 15 min, washed with Dulbecco’s PBS (PAA laboratories, Pasching, Austria) and incubated for 15 min at 4 °C with CellLytic M Cell Lysis Reagent (Sigma-Aldrich, St. Louis, MO, USA). Lysates were thereafter centrifuged for 15 min at 16 000 rpm and protein concentration was determined in the supernatant. 40 µg of total protein was separated on NuPAGE Novex 4-12 % Bis-Tris Gels (Life Technologies, Carlsbad, CA, USA) and transferred to 0,45 µm protran BA 85 nitrocellulose membranes (Whatman, Little Chalfont, UK). These membranes were probed with primary antibodies followed by horseradish peroxidase conjugated secondary antibodies. Protein bands were developed using enhanced chemiluminescence (Immobilion, Millipore, Billerica, MA, USA). JNK rabbit mAb, p-JNK (T183/Y185) rabbit mAb, p38 MAPK rabbit mAb, p-p38 MAPK (T180/Y182) rabbit mAb, ERK1/2 rabbit mAb, p-ERK1/2 (T202/Y204) rabbit mAb, IκBα mouse mAb, p-IκBα (S42) rabbit mAb. Secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).
Statistical analysis of data

Paper I

A two-way ANOVA for repeated measures was used for comparing the two groups. The incremental area under the curve (IAUC) was obtained by calculating according to the trapezoid integration method. The correlations between pairs of variables were determined by a simple linear regression analysis, while multivariate relationships were analysed using a general linear model. Data are shown as means ± SD, SEM. A p value of p<0.05 was considered statistically significant. Software from StatView (Abacus Concepts, Berkeley, CA, USA) and SAS Institute (Cary, NC, USA) was used.

Paper II

The incremental area under the curve was calculated in accordance with the method described by Wolever (149). Fisher’s exact test was used for dichotomous variables and Mann-Whitney U-test was used for continuous variables. Data expressed as mean± SD, SEM, a p value of p<0.05 was considered statistically significant. Data was calculated using software from GraphPad Prism 6.0, (GraphPad Software Inc, San Diego, CA, USA).

Paper III

Student’s t-test was performed to compare the statistical differences between the different treatments. Results are reported as per cent decrease/increase compared with TNF-α stimulated cells. Data are expressed as mean±SEM of three or more independent experiments. Differences were considered significant when p < 0.05. Software from GraphPad Prism 6.0a, (GraphPad Software Inc, San Diego, CA, USA) was used.
Summary of results

The aim of this thesis was to assess the acute vascular, metabolic and anti-inflammatory effects of 20 mg tadalafil treatment in T2D patients in the fasting state and in the postprandial state.

For this, muscle microdialysis, venous occlusion plethysmography and blood sampling was combined to determine forearm blood flow, forearm glucose uptake and permeability surface area for glucose.

Circulating levels of glucose, insulin, free fatty acids and triglycerides were obtained through blood sampling. In addition, circulating levels of adhesion molecules (ICAM-1, VCAM-1 and E-selectin) as well as the level of the pro-inflammatory vasoconstricting peptide ET-1 was assessed from blood samples.

We found that a single dose of tadalafil 20 mg, affected the forearm glucose uptake and permeability surface area for glucose in the fasting state when compared with placebo. In the postprandial state forearm glucose uptake, permeability surface area for glucose and forearm blood flow were increased by tadalafil in a post hoc analysis where ACE inhibitors and patients with protocol discrepancies were excluded. However, this was not seen in the Intention to treat group. Arterial stiffness was assessed in the postprandial state and no differences between the groups were observed. Interestingly, postprandial ET-1 levels were significantly decreased in T2D patients receiving tadalafil treatment in the post hoc analysis group. However, these changes were not significant in the Intention to treat group. Circulating levels of adhesion molecules (ICAM-1, VCAM-1 and E-Selectin) were similar for both groups in the postprandial state.

Insulin and glucose levels did not differ between the placebo and tadalafil treated groups in the fasting or postprandial state and no differences were shown for triglycerides or free fatty acids following a meal.

To further understand the molecular actions giving rise to the clinical effects of tadalafil, human umbilical vascular endothelial cells (HUVEC) were studied after inflammatory stimulation by TNFα. We found that HUVEC that were pretreated with tadalafil 1μM had a reduced inflammatory response following TNFα stimulation. The TNFα induced gene expression of the intracellular adhesion molecules (ICAM-1), the vascular cellular adhesion molecules (VCAM-1), E-Selectin and Endothelin-1 were significantly decreased by tadalafil preincubation. Further, the level of ET-1 found in the conditioned media surrounding the cells was diminished by tadalafil treatment. However, ICAM-1, VCAM-1 or E-Selectin levels were not affected by tadalafil treatment.

As previously mentioned TNFα inhibits PI3-Akt-eNOS insulin signaling through known kinases, c-jun-N-terminal kinase (JNK) and IκB kinase (IKK). Therefore, the effect of tadalafil on several kinases were examined. On the protein level we found that cells preincubated with tadalafil showed reduced activation of JNK following TNFα stimulation. In contrast, this was not seen for extra cellular signal-regulated signal ½ (ERK ½), p38 mitogen activated protein kinase (p38 MAPK) or inhibitory kappa B alpha (IκBα) indicating that the JNK-signaling pathway may be involved in the anti-inflammatory effects of tadalafil.

This was further supported by the fact that a blocker of JNK had similar effects on the gene expression of ET-1 as did tadalafil. The fact that 8-bromo cGMP, an agonist of cGMP signaling, mimicked the tadalafil
effect on JNK further supports that tadalafil may mediate its anti-inflammatory effect by affecting JNK by increasing the levels on cGMP. This effect was reversed when cGMP signaling was inhibited by DT-3. Surprisingly there was no effect seen on JNK when a NO donor, DEA-NONOate was used. In conclusion, we found that acute administration of 20 mg of tadalafil can increase capillary recruitment, blood flow and glucose uptake in T2D patients. Tadalafil reduced the gene expression of adhesion molecules ICAM-1, VCAM-1 and E-selectin. In addition, we found that tadalafil decreased the levels of ET-1 *in vivo* and *in vitro* and that these anti-inflammatory effects may be mediated by reduced activation of JNK. As inflammation, development of endothelial dysfunction and insulin resistance are closely associated these effects of tadalafil are interesting and should be further investigated.

**Figure 9.** Main finding from Paper I showing b) significant increase in Incremental AUC for the Permeability surface area for glucose (Ps Glu) and d) Glucose uptake in T2D patients following acute administration of 20mg tadalafil, black circles and boxes, white boxes and circles indicate placebo a) and c) show result from healthy controls. Data presented as means± SEM, * p<0.05
**Figure 10.** Main findings from Paper II. Results from the post hoc analysis investigating acute effects of 20 mg tadalafil in patients with T2D patients demonstrating significant increases in incremental AUC for:

a) forearm blood flow, b) permeability surface area for glucose, c) forearm glucose uptake and d) reduced circulating levels of ET-1. Data presented as means± SEM, *p<0.05
Figure 11. Main findings from Paper III. HUVEC preincubated with 1µM tadalafil prior to TNFα stimulation exhibit reduced mRNA expression of adhesion molecules a) ICAM-1, b) VCAM-1, c) E-Selectin and of d) ET-1. In addition, e) ELISA measurement of ET-1 in the cell medium showed decreased levels following tadalafil preincubation. Furthermore, f) JNK phosphorylation was diminished following tadalafil treatment compared to TNFα stimulation alone. Data presented as means± SEM, * p<0.05, ** p< 0.01, *** p< 0.001.
Discussion

Vascular effects

We wanted to investigate the effect of acute administration of tadalafil on vascular variables and combined muscle microdialysis, venous occlusion plethysmography and blood sampling and found that capillary recruitment was increased in the fasting and in the postprandial state in a post hoc analysis after a single dose of tadalafil 20 mg. This is the first time that tadalafil has been reported to have effect on the microvasculature in the postprandial state. This is an important finding since it has been demonstrated that patients with obesity, insulin resistance and T2D have impaired recruitment of the microvasculature after an OGTT as well as in the postprandial state (102, 103, 150). These results on the microcirculation are in accordance with earlier observations where 10 mg of tadalafil has been shown to increase capillary recruitment acutely in the fasting state (151). However, a previous study found no effect on capillary recruitment when 10 mg of tadalafil was given to obese women with impaired glucose metabolism and diminished capillary recruitment (150). These conflicting results may be explained by the difference in dose of tadalafil but larger studies will have to be carried out to answer this fully.

Forearm blood flow (FBF) was significantly increased by tadalafil in the post hoc analysis. In contrast, this was not seen in the fasting state and no significant acute effects were seen in the markers of Arterial stiffness, Augmentation Index (AIX) or Pulse Wave Velocity (PWV) in the postprandial state.

Our current results with positive effect on the endothelial function in the microvasculature after tadalafil treatment are in agreement with reports of improved endothelial function following chronic treatment with tadalafil (129-132) or sildenafil (152, 153). These finding are further supported by results from a study of chronic administration of tadalafil in a diabetic rat model where improved endothelial function was found (134). In addition, chronic treatment with tadalafil has also been shown to have cardioprotective effects following induced ischemia in a mouse model of T2D (135, 154).

In comparison, one chronic study of tadalafil treatment did not find any vascular effects of chronic tadalafil treatment as ascertained by Endopat and the authors speculate that this may be due to a small sample size as well as the fact that the patients were severely obese (133).

Metabolic effects

We wanted to evaluate the metabolic effects of tadalafil in T2D patients and found that a single dose of tadalafil, 20 mg, could improve forearm glucose uptake in patients with T2D in the fasting state as well as in the T2D patients in the post hoc analysis in the postprandial state. To our knowledge this is the first time increased glucose uptake has been shown following acute administration of a PDE-5 inhibitor in T2D patients. This is of importance since postprandial blood glucose has been shown to predict risk of cardiovascular events and all cause mortality (155).
Previous investigations studied patients after acute administration of 10 mg of tadalafil and found no effect on forearm glucose uptake (150, 151) or insulin sensitivity (133, 136). In comparison, acute administration of sildenafil did not have any metabolic effects in a mouse model of diet-induced obesity and insulin resistance. However, chronic treatment with sildenafil gave rise to improved insulin sensitivity and lower levels of fasting insulin in the same mouse model (156). Thus indicating that the lack of effect on glucose uptake in patients following acute tadalafil treatment in previous studies might be dose-dependent or time-dependent.

Acute and chronic treatments with tadalafil have been shown to induce positive effects on β-cell function (133, 136). However, we observed no significant increase in insulin levels following tadalafil treatment in our studies. Further, no significant differences were seen in circulating levels of plasma glucose or serum insulin in the fasting state nor in the postprandial state. Serum triglycerides and serum free fatty acids did not differ significantly between the placebo or tadalafil treated groups following their meal. The fact that forearm glucose uptake was increased when no effect was seen on the circulating levels of glucose is difficult to explain but might be due to the fact PDE-5 inhibition induces increased perfusion of muscular microcirculation. Thus, increasing the delivery of insulin and glucose to the muscle. However, how PDE-5 affects glucose responses in the adipose tissue (157, 158) and how it affects liver glucose metabolism is yet to be elucidated. Indeed, stimulating the NO-cGMP pathway has been reported to increase hepatic insulin sensitivity (159), but more research in the field is needed. Insulin has also been shown to up regulate the levels of PDE5s in endothelial cells (137) why high levels of insulin might lead to high levels of intracellular PDE-5s counteracting the effects of PDE-5 inhibition.

Figure 12. Main findings from randomized controlled trials investigating the effects of acute administration of tadalafil, 20 mg, in T2D patients in the fasting state, Paper I, and in the postprandial state, Paper II.
Effects on inflammatory response

Finally we were interested in examining the proposed anti-inflammatory effect of tadalafil in T2D patients as well as in cultured endothelial cells. Blood samples were drawn from patients before during and after they ingested a meal to assess circulating levels of ET-1 as well as for the adhesion molecules ICAM-1, VCAM-1 and E-Selectin. We found that ET-1 levels were significantly decreased in the patients in the post hoc analysis in the postprandial state. This finding is in accordance with results from PDE-5 inhibition in animal models of diabetes where levels of ET-1 decreased in a dose-dependent manner following treatment with PDE-5 inhibition (134). More importantly these results are supported by previous observations of decreased ET-1 levels following treatment with tadalafil in patients with cardiovascular disease (129, 131) and T2D patients treated with sildenafil (152). The circulating levels of adhesion molecules did not differ between the placebo group and the tadalafil group and we saw no typical peak in these molecules following ingestion of the meal the patients were served. The effect of tadalafil on adhesion molecules has differed in earlier studies. However, an effect on these molecules would be desirable since a correlation has been found between atherosclerosis and adhesion molecules in T2D patients (160). Treatment with 20 mg of tadalafil on alternate days to patients resulted in attenuated levels of ET-1 as well as decreased levels of VCAM-1 however when patients were treated with on demand treatment with 20 mg of tadalafil or received 2,5 mg or 5 mg of tadalafil no effect on inflammatory markers were seen (129, 131, 132). While yet another investigation reported that both ICAM-1 and VCAM-1 were suppressed by chronic sildenafil treatment in patients with T2D (152). Research revealed that tadalafil mediated its anti-inflammatory effect by affecting the phosphorylation of JNK, a kinase known to mediate insulin resistance. Interestingly, sildenafil treatment in insulin resistant HUVECs restored phosphorylation of Akt and eNOS and subsequently the cells produced and released higher levels of NO after PDE-5-inhibition (137). Insulin stimulation in HUVEC resulted in an increase in PDE-5 expression in a previous study (137) and we observed that the mRNA expression for PDE-5 increased 8 times following TNFα stimulation (unpublished data). Linking these results one could hypothesize that a reduced JNK activity from the PDE-5 inhibition resulted in the positive effect on the Akt-eNOS-NO signaling pathway in the precedent study. However, this needs to be explored further before any conclusions can be drawn.

In addition, JNK has been shown to be involved in the regulation of vascular tone (161) and vasoconstriction induced by ET-1 can be attenuated by inhibiting JNK activation (162). TNFα has been shown to impair the vasodilator but not the vasoconstricting effects of insulin by activating JNK. The vasoconstricting effects were due to continued ET-1 production and release in tandem with abolished Akt-eNOS signaling (41, 163). These results are in agreement with our findings where T2D patients exhibiting increased capillary recruitment also exhibited decreased levels of ET-1. In our experimental setting, JNK was shown to regulate the expression of ET-1.

To summarize: our results show that tadalafil can affect ET-1 levels in vivo in patients with T2D and in vitro in endothelial cells and that this effect may be mediated by modulating JNK activity. Taken together,
the knowledge that ET-1 and JNK have been shown to be involved in inflammation, endothelial dysfunction as well as in impaired glucose homeostasis (61, 70, 73, 79, 91) these findings in cultured endothelial cells may explain our positive results of tadalafil treatment on the metabolism, the vasculature and on inflammation in patients with T2D.

Figure 13. Main findings in experimental work showing that TNFα increased the expression of adhesion molecules as well as ET-1 in HUVEC and that tadalafil attenuated this by reducing the activation of JNK. These results may well explain the improved glucose uptake and capillary recruitment seen in patients due following tadalafil treatment.
Conclusion

Tadalafil treatment induced positive effects on endothelial function seen as increased capillary recruitment in T2D patients in the fasting and postprandial state. Further, decreased circulating levels of the pro-inflammatory and vasoconstricting peptide ET-1 was seen following tadalafil treatment. Tadalafil also increased forearm glucose uptake in T2D patients indicating positive metabolic actions. Investigation of the inflammatory response in HUVECs following TNFα stimulation identified anti-inflammatory effects of tadalafil seen as reduced gene expression of adhesion molecules and ET-1 as well as levels of secreted ET-1. Interestingly, these anti-inflammatory effects of tadalafil were found to be mediated by down regulating the activation of c-Jun NH2-terminal kinase (JNK), a MAPK known to be involved in the development of impaired vascular and metabolic function. These results provide a molecular explanation to the clinical finding observed following acute tadalafil administration.
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