Microvascular effects of insulin in the skin

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Linköping University Medical Dissertations
No. 1788

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Linköping 2021

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Printed by LiU-Tryck AB,
Linköping, Sweden, 2021

ISBN 978-91-7929-009-2
ISSN 0345-0082

To my family!

*"The only limit to the height of your achievements is the reach
of your dreams and your willingness to work hard for them"*

Michelle Obama

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Abstract

The microcirculation in the skin is essential for skin homeostasis. In instances of altered microvascular function, that may be the result of insulin resistance, tissue morbidity may ensue. The underlying mechanisms are however complex and not fully understood. By studying the physiological effects of insulin in the skin, the understanding of the complex interplay between glucose metabolism and skin microcirculation can be improved.

The general aim of this thesis was to develop an experimental *in vivo* model to study metabolic and microvascular responses to insulin in the skin in healthy subjects. Microdialysis is a suiting technique as it allows for both local delivery of drugs and simultaneous monitoring of the local metabolic and vascular effects in the very same tissue compartment.

The effects of local and systemic insulin provocation on skin blood flow and metabolism were investigated using microdialysis urea clearance and laser speckle contrast imaging (paper I). An insulin dependent increase in skin blood flow was observed, presumably induced through the nitric oxide pathway (paper II). Investigating the protein expression during an oral glucose provocation using proteomic approaches however indicates interactions with other pathways, such as the renin-angiotensin system and the kallikrein-kinin system (paper IV). Paper III also investigated methodological concerns regarding the sampling of insulin using microdialysis.

This *in vivo* model can, in the future, be applied to assess the microvascular effects of insulin in the skin in different patient groups, including those with microvascular dysfunction due to, for instance, insulin resistance.

Svensk sammanfattning

Insulin är ett hormon som produceras i bukspottskörteln som svar på höga blodsockernivåer efter matintag. Via blodet når insulinet alla kroppens vävnader och möjliggör upptag av socker till cellerna. Dessa effekter på ämnesomsättning är välkända. Senaste årtionden har dock intresset för att kartlägga insulinets effekter på blodcirkulationen ökat.

Målet med denna avhandling var att skapa en modell för att studera insulinets effekt på blodcirkulationen i huden på friska försökspersoner. Detta har gjorts med mikrodialys teknik. En mikrodialys kateter är en tunn plastslang som spolats igenom med en vätska i mycket långsam takt ($\mu\text{L}/\text{minut}$). I toppen av katetern sitter ett genomsläppligt membran med många små hål, genom vilket molekyler kan vandra ut och in från kateterslangen tills jämvikt uppstår. Funktionen liknar ett konstgjort blodkärl. På så sätt fås ett mer vävnadsspecifikt prov från just den vävnad man sätter in mikrodialys katetern i, jämfört med ett blodprov.

Genom att tillföra insulin lokalt till huden (via mikrodialys katetern) och systemiskt via kroppsegen insulinproduktion (stimulerat av sockerintag) sågs att insulin ökar både blodflödet och ämnesomsättningen av socker lokalt i huden (delarbete I). Detta regleras av kväveoxid (delarbete II), som produceras lokalt i kärlväggen vid insulinstimulering. I delarbete III utfördes olika experiment för att undersöka svårigheter till att få ut insulin via mikrodialys katetern, såsom att insulin klibbar fast mot själva membranet. Till sist analyserades mikrodialysvätskan för att identifiera proteiner involverade i andra signalvägar som också är viktiga för blodcirkulationen vid en insulinstimulering (delarbete IV).

I framtiden kan denna modell appliceras på olika patientgrupper för att se hur effekterna av insulin på blodflöde och ämnesomsättning skiljer sig hos vid insulinresistens, såsom vid övervikt eller diabetes.

List of papers

This thesis is based on the following papers, which will be referred to by their Roman numerals in the text:

- I. F. Iredahl, **A. Högstedt**, J. Henricson, F. Sjöberg, E. Tesselaar, S. Farnebo
Skin glucose metabolism and microvascular blood flow during local insulin delivery and after an oral glucose load
Microcirculation (2016) 23:597–605
- II. **A. Högstedt**, F. Iredahl, E. Tesselaar, S. Farnebo
Effect of NG-monomethyl L-arginine on microvascular blood flow and glucose metabolism after an oral glucose load
Microcirculation (2019) 00:1–9
- III. **A. Högstedt**, B. Ghafouri, E. Tesselaar, S. Farnebo
Measuring insulin in different tissue compartments using microdialysis: methodological aspects
Scientific Reports (2020) 10:21948
- IV. **A. Högstedt**, B. Ghafouri, E. Tesselaar, S. Farnebo
Investigation of proteins important for microcirculation using *in vivo* microdialysis after glucose provocation – a proteomic study
Scientific Reports (2021) 11:19093

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Abbreviations

ANOVA	analysis of variance
BMI	body mass index
ELISA	enzyme-linked immunosorbent assay
HOMA	homeostasis model assessment
<i>In vitro</i>	Latin: within the glass. Experimental testing outside an organism.
<i>In vivo</i>	Latin: within the living. Experimental testing inside an organism.
IR	insulin resistance
L-NMMA	NG-monomethyl L-arginine
LSCI	laser speckle contrast imaging
MD	microdialysis
MVDA	multivariate data analysis
nLC-MS/MS	nano-liquid chromatography tandem mass spectrometry
NO	nitric oxide
NOS	nitric oxide synthase
OGTT	oral glucose tolerance test
OPLS-DA	orthogonal partial least square discriminant analysis
PCA	principal component analysis
PU	perfusion units
SD	standard deviation
SEM	standard error of the mean
STRING	search tool for retrieval of interacting genes/proteins
VIP	variable importance for the projection

Introduction

Function and anatomy of the skin

The human skin is a complex organ with several vital functions. Its main role is to act as a barrier to physical agents and mechanical injury in order to protect the underlying tissue. It is also an important immunological organ, plays a role in vitamin D production, reduces the penetration of UV radiation, regulates body temperature and acts as a sensory organ [1, 2].

Making up about 16% of the body weight, the skin is one of the largest organs in the human body. It is composed of three different layers: the epidermis, the dermis and the subcutis. The outermost layer, the epidermis, is a stratified squamous epithelium and consists mainly of keratinocytes. The keratinocytes proliferate in the basal membrane connecting the epidermis to the dermis, and then differentiate and migrate upwards to the surface of the epidermis. The epidermis is generally about 0.1 mm thick, but it varies considerably between different locations on the body. For instance, the thickness of epidermis on the palms and soles can be up to 1.4 mm.

The middle layer of the skin, the dermis, is a tough supportive connective tissue matrix. It is mainly composed of collagen fibers, contributing to toughness and strength, but also elastin fibers, which provide elasticity to the skin. The dermis is further divided into the superficial papillary dermis and the deeper reticular dermis. The subcutis (also referred to as subcutaneous fat or hypodermis) is the third and innermost layer of the skin and consists mainly of adipocytes and loose connective tissue. This layer primarily acts as insulation but does also provide shock absorption and store nutrients and energy. Skin appendages, such as hair follicles, sweat glands, nerves and lymphatic vessels, is found mainly in the dermis but extends occasionally down into the subcutaneous fat [1-3].

The microcirculation in the skin

The microcirculation generally encompasses blood vessels $<150\text{ }\mu\text{m}$ in diameter, including arterioles, capillaries and venules [4]. The microcirculation in the skin was historically believed to be a randomly anastomosing network. Since the 1970s, research on the field of cutaneous microcirculation has discovered that the microcirculation in the skin is divided into two different vascular plexuses [3, 5, 6] (Figure 1). The arterioles and venules in the dermis form 1) an upper horizontal plexus in the papillary dermis, from which the nutritive capillary loops of the dermal papillae arise, and 2) a lower horizontal network in the junction between the reticular dermis and the subcutaneous tissue. Arteriovenous shunts connect the two plexuses and have an important role in thermoregulation [6, 7].

Figure 1. Schematic illustration of the microcirculation in the skin. The capillary loops are supplied by arterial blood (from superficial arterial plexus, SAP) while the venous blood is drained by two parallel veins (upper superficial venous plexus, USVP, and deep superficial venous plexus, DSVP). Illustration designed by Johan Thorfinn.

The balance between the local metabolic demand and the microvascular perfusion in a tissue is regulated by the arteriolar diameter. What underlying mechanisms

orchestrating this is not completely understood. The vascular bed is dynamic and blood flow is redirected from nutritive to non-nutritive tissues depending on local factors, such as availability to oxygen and glucose [8]. In this way, the tissues in

need of more nutrients due to increased metabolic activity get more perfused. This phenomenon is called capillary recruitment. It is believed that the smooth muscle cells in arterioles, and the pericytes surrounding terminal arterioles and capillaries, regulate this contractile dynamic [9]. No living cell is farther away from a capillary than it can be reached by diffusion by oxygen and nutrients, a model known as the Krogh length [10-12].

There are two branches of sympathetic nerves that, to some extent, control skin blood flow: noradrenergic for vasoconstriction and cholinergic for vasodilation [13]. In addition, the endothelium itself affects the vascular tone. The endothelium is the innermost, single layer of endothelial cells lining all blood vessels in the human body. By virtue of its direct contact with the circulating blood, the endothelium provides a critical interface between the blood and the tissues. The endothelium does not only react to vasoactive substances released upstream in the circulation but does also produce various vasoactive factors itself. Endothelial production and release of nitric oxide (NO) and endothelium derived hyperpolarizing factors (EDHF) induce relaxation of the vessels, whereas prostanoids and endothelin-1 (ET-1) cause vasoconstriction [14, 15].

Endothelial dysfunction

Endothelial dysfunction is characterized by an impaired ability of the vascular endothelium to adequately maintain vascular homeostasis (balance between vasodilation and vasoconstriction). This is commonly observed in patients with obesity, type 2 diabetes mellitus and cardiovascular disease [15, 16], which are all conditions associated with insulin resistance and disturbed glucose homeostasis. Endothelial dysfunction is believed to contribute to impaired insulin action, both by inhibiting the transcapillary passage of insulin [16] and because of a reduction in NO-dependent vasodilation, and hence, impaired delivery of substances, including insulin, to target tissues. This has been shown in numerous experiments where the use of pharmacological blockade of NO synthesis induces an abnormal vascular response [17, 18], similar to the one observed in insulin resistant patients.

Assessment of the microcirculation in the skin

A great advantage when studying the microcirculation and microvascular responses to provocations in the skin is that the skin as a tissue is easily accessible. Non-invasive, optical methods are therefore suiting and commonly used to assess the microcirculation in the skin. Laser Doppler flowmetry (LDF) was invented in the 1970s as the first method to non-invasively measure cutaneous perfusion. LDF uses the Doppler effect to estimate the velocity and concentration of red blood cells in the tissue [19-21]. The measurement area is however small (a single point area of 1 x 1 mm), and the technique is sensitive to motion artifacts [22].

Based on the LDF technique, the laser Doppler perfusion imaging (LDPI) was later invented in the 1990s [23-25]. LDPI instead uses a single beam laser to scan the tissue of interest, thence providing a perfusion image rather than a single value in a single point as the LDF does. LDPI therefore has better spatial resolution compared to LDF. Clinically this is important, as it allows assessment of regional differences within a flap or body part that is monitored for signs of morbidity. The temporal resolution in LDPI is however substantially lower compared to LDF, as the scanning is time-consuming (minutes compared to milliseconds).

The refined and further developed laser-based imaging techniques for blood flow measurements were then introduced. Laser speckle contrast imaging (LSCI) is a camera-based technique that, instead of using the Doppler effect, uses a laser beam to illuminate a larger area (up to 20 x 20 cm) on the target tissue. The reflected light creates a speckle pattern on the camera sensor, the dynamics of which depend on the movement of red blood cells. The LSCI thereby provides high resolution (0.2 mm/pixel at 25 cm distance), real-time images visualizing the cutaneous microcirculation [26-28]. As the LSCI can capture 21 images per second in a large area, LSCI is less sensitive to motion artifacts and enables studying temporal variations. Compared to LDF, LSCI also has better reproducibility for repeated measurements [29, 30].

In addition to the non-invasive optical techniques, the microcirculation in the skin can also be assessed using different clearance techniques. Two commonly

used techniques in cutaneous microdialysis (described later) studies are ethanol clearance [31] and urea clearance [32, 33]. These techniques are based on the addition of a marker, with known concentration, to the perfusate of the microdialysis catheter. When the marker reaches the semipermeable membrane, it will diffuse into the interstitium. The rate of diffusion depends on the local blood flow surrounding the catheter. The dialysate concentration of the marker is therefore inversely proportional to local blood flow.

In this thesis, the microcirculation in the skin has been assessed using both laser speckle contrast imaging (paper I-II) and urea clearance (paper I-III), further described in the *Methods* section.

Insulin

The active human insulin molecule comprises 51 amino acids and has a molecular weight of 5,8 kDa. It is synthesized as a single polypeptide, preproinsulin, in the pancreatic beta cells. Preproinsulin is enzymatically cleaved into proinsulin and finally into the active and mature insulin molecule, comprising an alpha and a beta chain [34, 35]. Insulin plays a crucial role in glucose homeostasis, where the insulin dependent GLUT4 transporter, mainly present in adipose tissue and muscle, facilitates glucose uptake from the blood stream into the cells [36]. In this way, plasma glucose concentration in healthy humans is tightly regulated between 4-6 mM [37]. In addition to these well-known metabolic effects, it has become increasingly evident that insulin also has important vascular actions.

Vascular effects of insulin

The general view is that insulin has the ability to induce both vasodilation and vasoconstriction in various tissues, but with a net effect usually favoring vasodilation [38-41]. In particular, insulin enhances microvascular perfusion in skeletal muscle [42], adipose tissue [43], skin [44, 45], brain [46, 47] and cardiac muscle [48], and this effect is strongly linked to vasodilation mediated by nitric oxide (NO). The vasoconstrictive effect of insulin is mainly mediated through endothelin-1 (ET-1) [39, 49].

Insulin acts directly on the vascular endothelial cell insulin receptors, thereby activating the phosphatidylinositol-3-kinase (PI3K) signaling cascade [39, 50] (Figure 2). This cascade enhances the activity of endothelial nitric oxide synthase (eNOS), subsequently increasing the production of NO. Nitric oxide thereafter diffuses to vascular smooth muscle cells (SMC) in adjacent terminal arterioles, where it activates guanylate cyclase leading to SMC relaxation. Since the eNOS activity is dependent on oxygen, ischemia results in vasoconstriction because the action of endothelin is unopposed. In addition to insulin, the nitric oxide pathway can be activated through other well-known signaling pathways, for instance the renin-angiotensin-system and the kallikrein-kinin system, further described in the discussion.

Figure 2. An illustration of the pathways by which insulin induce vasodilation and increased glucose uptake. Illustration made by Simon Farnebo. IR = insulin receptor. NO = nitric oxide. eNOS = endothelial nitric oxide synthase. P-eNOS = phosphorylated endothelial nitric oxide synthase. cGMP = cyclic guanosine monophosphate. GLUT4 = glucose transporter type 4.

Insulin resistance

Insulin resistance is typically defined as an impaired biological response to insulin stimulation in its target tissues, primarily the liver, muscle, and adipose tissue [51]. This consequently results in compensatory increased insulin production and thereby hyperinsulinemia. Insulin resistance is a prominent risk factor for type 2 diabetes mellitus, cardiovascular disease, hypertension, chronic kidney disease and obesity [52-55].

Measuring insulin resistance

Measuring insulin resistance *in vivo* is challenging and typically done through indirect measurements. To date, the hyperinsulinemic-euglycemic glucose clamp technique is gold standard [56]. With this technique hyperinsulinemia is

maintained by continuous infusion of insulin, while plasma glucose concentration is held constant (clamped) at euglycemic (5 mM) level by a variable glucose infusion. Repeated blood samples are collected (usually every fifth minute) to measure plasma glucose concentration. When a steady state is achieved, the glucose infusion rate equals glucose uptake by all the tissues in the body, which is a measure of overall insulin sensitivity. In this way, insulin resistance can be determined.

The applicability of the glucose clamp is however clinically limited. A number of surrogate methods to measure insulin resistance is therefore clinically used, such as HOMA-IR, serum triglycerides, oral glucose tolerance test and mixed-meal tolerance test.

The HOMA-IR model uses a mathematical equation ($[\text{fasting insulin } (\mu\text{U/mL}) \times \text{fasting glucose (mmol/L)}] / 22.5$) to estimate insulin resistance [57]. The oral glucose tolerance test (OGTT) provides information about insulin secretion and action but does not directly yield a measure of insulin sensitivity. The test induces hyperglycemia by ingestion of 75 g glucose mixed in 250 ml water. Blood samples are collected in fasting and two hours after glucose intake and analyzed for concentration of glucose and insulin [58]. In a mixed meal tolerance test (MMTT) a standardized meal composed of protein, fat and carbohydrates is ingested and blood samples drawn in fasting and repeatedly the following three hours after meal intake. MMTT is equivalent to both OGTT and gold standard glucose clamp regarding ability to estimate beta cell function, however more time consuming and also requires additional blood samples compared to OGTT [59, 60].

All of these methods are based on systemic provocations and thereby offer an estimate of the systemic insulin resistance. These methods are in themselves however not suited to assess the responses to insulin in specific target tissues. A method to measure the local effects of insulin in a specific target tissue, such as the skin, is therefore of interest. For this purpose, microdialysis is a suitable technique.

Microdialysis

Microdialysis is a technique for continuous sampling of small molecular weight, water-soluble substances in the interstitial space of living tissue. The technique was first invented by Bito *et al* in 1966 [61] and Delgado *et al* in 1972 [62], but the method was later refined and introduced in its present form by Ungerstedt *et al* in 1974 [63].

The basic principle of the microdialysis technique is to mimic the passive function of a capillary blood vessel [64]. The microdialysis system comprises a small double lumen catheter with a semi-permeable membrane at the tip, which is inserted into the tissue of interest (Figure 3). A physiological solution (perfusate) is pumped through the inner lumen of the catheter to the semi-permeable membrane, where it equilibrates with the extracellular fluid through passive diffusion. The microdialysis sample (now referred to as dialysate) is then transported through the outer lumen and collected in a small vial (Figure 4).

Figure 3. Microdialysis catheter, sampling vial and pump.

With courtesy of M Dialysis AB.

The dialysate concentration of an analyte of interest is dependent on the degree of equilibration between the perfusate and the sampling medium. This is referred to as relative recovery, while the absolute recovery is defined as the total amount (mass) of an analyte recovered during a defined time period. The recovery depends on several factors, including perfusion flow rate, diffusion rate, properties of the catheter membrane (pore size, surface area and material), physicochemical

properties of the analyte (charge, molecular weight and hydrophobicity) and composition of the perfusate [65-67]. Local characteristics of the sampling medium (such as viscosity and temperature) and the local blood flow surrounding the catheter also affects recovery [66, 68].

Microdialysis has been used in humans to monitor substances such as inflammatory markers [69, 70], energy metabolites [71, 72] and hormones [73, 74]. Due to the membrane being semipermeable, substances can however diffuse in both directions, depending on the concentration gradient of the interstitial fluid and the perfusate. Microdialysis therefore enables both monitoring of local metabolic conditions in the interstitium of the target tissue (sampling), and simultaneously delivery of substances or drugs to the very same tissue compartment (provoking). In this thesis, insulin and L-NMMA were used for local provocation through the microdialysis system, described in the method section.

Figure 4. Illustration of the microdialysis system.

Designed by Simon Farnebo.

A great advantage is that microdialysis provides an opportunity to continuously monitor metabolic trends, where the temporal resolution depends on how long intervals are between vial exchange. This makes it possible to dynamically assess physiological processes and also direct responses to systemic or local provocations.

There are a number of limitations to the microdialysis technique. Microdialysis is usually referred to as a minimally invasive procedure, due to the small size of the catheter and therefore minimal discomfort to patients during insertion. Nevertheless, insertion of the microdialysis catheter inevitably causes some degree of damage in the tissue, initiating an inflammatory response that could interfere with the results [75].

Sampling larger molecules like insulin is challenging, presumably both due to low recovery and due to adsorption to the microdialysis membrane and tubing. This will be further discussed in the discussion.

Proteomics and data analysis

The field of proteomics has rapidly grown since the end of the 20th century and is now a well-established field of research used in biomarker research. Proteomics is usually defined as the science and the methodology of investigating the proteome [76]. The proteome, in turn, is defined as the complete set of proteins that can be expressed by a genome, cell, tissue or organism at a certain time [77]. The human proteome organization (HUPO) has found 18 357 proteins (March 2021), which represents 92.8% of the predicted total human proteome [78]. Mapping the human proteome could provide important information about biological and molecular functions and thereby advance diagnoses and treatment of diseases.

The gold standard tool for protein identification is the use of a mass spectrometry (MS), which determines a protein's mass and amino acid sequence in order to identify the protein. Before identification in MS, complex protein mixtures must be separated into individual components. Many techniques can be used for this.

The analysis technique requires high resolution (multidimensional separation into simple mixtures of proteins or peptides), high throughput, and compatibility with downstream analysis by mass spectrometry, as this is the major technology platform for high-throughput protein identification. The two groups of analysis techniques therefore dominating the proteomic field is two-dimensional gel electrophoresis (2DGE) and liquid chromatography (LC) [79].

In 2DGE, denaturated proteins are separated according to charge (pI) using isoelectric focusing (IEF) and molecular weight. This is a top-down proteomic approach, i.e. proteins are separated before identification in MS. Combining liquid chromatography with tandem mass spectrometry (LC-MS/MS) is called shotgun, or bottom-up, proteomics. This involves proteolytical digestion of proteins, commonly using trypsin, before multidimensional peptide separation using LC and finally identification in MS [80]. The term bottom-up implies that information about the constituent proteins is reconstructed from individually identified fragment peptides. The generated data from mass spectrometry are searched against the protein database UniProt. The UniProt database is an online protein database providing detailed information regarding protein name, protein accession number, amino acid sequence, genes and involvement in biological functions and processes [81].

Regardless of method (2DGE or LC-MS/MS), all proteomic methods usually generate a large set of data, sometimes consisting of hundreds of independent variables (e.g. proteins). Analysis using univariate and bivariate techniques is in these cases inappropriate, as it might generate misleading results [82]. Multivariate data analysis (MVDA) is therefore a suiting and well-established method for analysis of large sets of variables (proteins) but simultaneously few observations (subjects).

Bioinformatics is the application of computational technology to obtain, store and interpret large quantities of biological data and to make sense of the data. There are multiple different bioinformatic tools available today. There are also several open-source databases for analysis and visualization of complex protein

interaction networks linked to the biological functions of specific proteins, e.g. Search Tool for Retrieval of Interacting Genes/Proteins (STRING) [83].

Aims

The overall aim of the papers described in this thesis was to develop an experimental *in vivo* model to investigate the microvascular and metabolic effects of insulin in the human skin.

The specific aims for the separate papers were:

Paper I

To study the effects of insulin on local blood flow and metabolism in the skin when it is endogenously produced and delivered to the skin via the systemic circulation or directly delivered to the interstitium using microdialysis.

Paper II

To investigate whether the effects on local blood flow and metabolic changes observed in the skin after an endogenous systemic increase in insulin are mediated by the endothelial NO pathway.

Paper III

To (1) measure the concentration of insulin in three different compartments (intracutaneous, subcutaneous and intravenous) during an oral glucose provocation and (2) to study how much insulin is adsorbed to the microdialysis membranes and tubing during a typical microdialysis experiment, both *in vivo* and *in vitro*.

Paper IV

To examine the protein expression in microdialysis samples, in order to identify protein biomarkers that are related to microvascular changes in different tissue compartments during an oral glucose provocation.

Methods

Subjects

All subjects were healthy volunteers, consecutively recruited in Linköping city through advertising on social media. All subjects were informed and gave their written consent before participating in the studies. Demographic data of the subjects is presented in Table 1. Exclusion criteria for all four studies included conditions potentially impairing the microvascular function in the skin and endothelial dysfunction, such as smoking [84] and hypertension [85]. None of the subjects used any regular medication, except for oral contraceptives.

Experiments took place in a temperature-controlled room (20-23 °C) at Linköping University Hospital. On the day of the experiment, the subjects arrived in the morning after an overnight fast and were only allowed to drink water during the experiments. Drinks containing caffeine were especially not allowed since caffeine is known to affect microvascular function in healthy individuals [86, 87]. Subjects were positioned in a comfortable reclined position.

All studies were done according to the Helsinki declaration and were approved by the regional ethical review board of Linköping.

Table 1. Overview of the four papers included in this thesis.

	Paper I	Paper II	Paper III	Paper IV
	<i>In Vivo</i>	<i>In Vivo</i>	<i>In Vivo + In Vitro</i>	<i>In Vivo</i>
Compartment	Skin	Skin	Skin, adipose tissue, blood	Skin, adipose tissue, blood
Number of subjects (female)	N = 14 (7)	N = 12 (6)	N = 16 (7)	N = 8 (4)
Number of microdialysis catheters	N = 28	N = 24	N = 31 (<i>in vivo</i>), 11 (<i>in vitro</i>)	N = 28
Demographics:				
- Age (years)	- 23.0 (5.1)	- 23.6 (2.0)	- 23.9 (2.3)	- 24.9 (1.6)
- BMI (kg/m ²)	- 22.6 (2.2)	- 23.3 (3.5)	- 22.5 (1.5)	- 22.3 (1.3)
Provocation:				
- Systemic	- OGTT	- OGTT	- OGTT	- OGTT
- Local	- Insulin	- L-NMMA		
Microdialysate analysis method	CMA600, ELISA	CMA600, ELISA	ISCUSflex, ELISA	ISCUSflex, ELISA, LC-MS/MS
Blood flow measurement	UC, LSCI	UC, LSCI	UC	-

Experimental microdialysis model

An experimental microdialysis model was designed and used in all four studies. Flow charts for the experimental model, and the specific adjustments for each paper, is presented in the section *Review of the studies*.

Microdialysis catheters were inserted in the dermis (intracutaneous), subcutis (subcutaneous) and in a peripheral vein (intravenous) (Figure 5). The intracutaneous catheters were inserted at the volar side of the non-dominant forearm (paper I-IV). When dual intracutaneous catheters were used (paper I-II) they were inserted parallel to each other (to facilitate interpretation of LSCI measurements) and positioned at least 3 cm apart (to separate the control catheter area from the area surrounding the provocation catheter). The placement of the intracutaneous catheters have previously been shown, using ultrasound, to be at a depth of approximately 0.8 mm, i.e. at the border of dermis and subcutaneous fat [88].

The subcutaneous catheters (paper III-IV) were inserted in the periumbilical subcutaneous fat. The intravenous catheters (paper III-IV) were inserted in a peripheral vein in the cubital fossa. Both the intracutaneous and subcutaneous catheters were 100 kDa (10 mm membrane length, CMA71, M dialysis AB, Stockholm, Sweden), while a 20 kDa cut off microdialysis catheter was used intravenously (10 mm membrane length, CMA 67, M dialysis AB, Stockholm, Sweden).

Briefly, the microdialysis catheter insertion was made by advancing an 18-Gauge cannula (BD Venflon Pro, Becton Dickinson Infusion Therapy AB, Helsingborg, Sweden) through the skin surface. The steel mandrin from the cannula was then removed and replaced with the microdialysis catheter. When the microdialysis catheter was in place, the plastic part of the cannula was withdrawn and the catheter was connected to a microinjection pump (CMA 107, M dialysis AB, Stockholm, Sweden). Perfusate flow rate was 1.0 $\mu\text{L}/\text{min}$ in all papers.

The microdialysate perfusate consisted of a Ringer's acetate solution with addition of 2.5% albumin and 30 mmol/L urea (APL AB, Umeå Sweden). Different additives were then added to the perfusate in the different studies to give local provocations. In the intravenous catheters (paper III-IV), heparin (Heparin LEO, Malmö, Sweden) was added in the perfusate as an anticoagulant, to enable adequate sampling.

Figure 5. Photos of the microdialysis catheters inserted in the intracutaneous (left) and subcutaneous (right) compartments. The red laser crosshair (left) indicates the measuring area for LSCI.

Provocations

To study physiological processes and mechanisms, specific provocations need to be done to change the blood flow and metabolism from their basal states. Provocations of the microcirculation can be made systemically or locally. An oral glucose tolerance test (OGTT) was used as a systemic provocation to induce endogenous hyperinsulinemia [58].

In paper I and II, pretreatment of the skin before and during oral glucose provocation was done using local delivery by either insulin (paper I) or L-NMMA (paper II) through the microdialysis catheter. In paper I, insulin aspart (Novorapid, Novo Nordisk A/S, Bagsværd, Denmark) was added in the provocation catheter while insulin diluting medium (Insulin Diluting Medium for Novorapid and Levemir, Novo Nordisk A/S, Bagsværd, Denmark), which is identical to insulin aspart apart from insulin, was added in the control catheter. In paper II, the NO synthase inhibitor L-NMMA (Bachem Distribution Services, Germany) was added in the provocation catheter to blunt the vasodilator effects of insulin. In all experiments, subjects were blinded to which substances were administered at which site.

Blood flow measurements

LSCI

Laser speckle contrast imaging (LSCI) is a non-invasive, camera-based imaging technique suited for measurement of microvascular perfusion in the human skin (Figure 6). The measurement is based on illumination of the skin with a divergent laser beam and analysis of the moving speckle pattern of the reflected light, which is related to the movement of red blood cells in the tissue. A strength of LSCI, compared to laser-Doppler-based techniques, is that it can create a blood perfusion map of an area of skin up

to 20×20 cm in a few seconds, while a high spatial resolution is maintained (4 pixels/mm² at 25 cm distance) [27, 89, 90]. Perfusion is expressed in perfusion units (PU), which are proportional to the mean velocity and the average concentration of the red blood cells in the measurement volume.

Figure 6. The LSCI device used in paper I-II.

Urea clearance

Urea clearance is based on the principle that urea, when added to the perfusate, diffuses freely through the semipermeable membrane of the microdialysis catheter and further into the interstitium. The rate of diffusion depends on the local blood flow surrounding the catheter. With increasing blood flow, urea is removed from the tissue around the catheter at a higher rate because of an increased concentration gradient between the perfusate and the interstitium. The urea dialysate concentration is therefore considered inversely proportional to local blood flow [32, 33].

Biochemical analyses

Blood samples

Blood samples in paper I-III were collected from the median cubital vein from the opposite arm in which microdialysis catheters were inserted. They were analyzed directly by the clinical laboratory at Linköping University hospital. Plasma glucose was analyzed using hexokinase enzymatic method while serum insulin was analyzed by electrochemiluminescence.

Additional blood samples for *in vitro* microdialysis experiments (paper III) were collected and centrifuged directly (1800 g for 10 minutes). The serum was then extracted and frozen awaiting further analysis.

Capillary blood samples in paper I-III were collected alternately from the index, middle and ring finger using a lancet, and then directly analyzed using a handheld spectrophotometer (Glucose 201+ system, HemoCue AB, Ängelholm, Sweden or Accu Chek Inform II, Cobas, Switzerland).

Microdialysate

The dialysate concentration of glucose, lactate, pyruvate and urea was enzymatically analyzed by either the CMA 600 Microdialysis Analyzer (paper I-II) or the ISCUSflex Microdialysis Analyzer (paper III; M dialysis, Stockholm, Sweden). Absorbance measurements were made with a filter photometer, with the wavelength of 546 nm (for CMA600, 530 nm for ISCUSflex) for glucose, lactate and pyruvate, while the wavelength for urea was 365 nm (for CMA600, 375 for ISCUSflex).

ELISA

Insulin concentration was analyzed by an enzyme-linked immunosorbent assay (Ultrasensitive ELISA, Mercodia, Uppsala, Sweden). Since the minimum analysis volume for ELISA is 25 μ L, dialysate from consecutive microvials were pooled hourly in order to obtain a higher temporal resolution of the other analytes. All analysis results under the detection limit (0.15 mU/L) were set to zero.

Proteomics

Proteomic analyzes (paper IV) were done on microdialysate from three tissue compartments: intracutaneous, subcutaneous and intravenous. All samples were blinded before sample preparation.

Albumin was added to the perfusate as a colloid. Since high concentrations of albumin could interfere with the protein quantification and mask the identification of other proteins in LC-MS/MS, each sample was depleted of albumin using an albumin and IgG depletion column (Thermo Scientific, Rockford, USA). Briefly, each dialysate sample was added to the resin slurry in each column, incubated in room temperature on a shaker for 30 minutes and then centrifuged for 2 minutes at 1000 g to finally collect the depleted sample. The samples were then desalted and concentrated using 3 kDa Amicon spin-filter (Merck Millipore, Darmstadt, Germany). The concentrated samples were dried by speed vacuum concentrator (SpeedvacTM, ThermoFisher Scientific, Waltham, USA) and re-dissolved in 40 μ L of urea buffer solution (8 M urea in 25 mM ammonium bicarbonate) to denature the proteins and incubated in room temperature for 2 hours. The proteins were reduced with dithiothreitol (DTT) (25 mM) and incubated for 15 minutes, and then alkylated with iodoacetamide (75 mM) for an additional 15 minutes. Finally, the samples were diluted with 25 mM ammonium bicarbonate up to 250 μ L and desalted.

Protein concentration was then measured using 2D quant kit (GE Healthcare, Uppsala, Sweden) before trypsinization. A total amount of 10 μ g protein was removed to a new tube and incubated with trypsin (0.2 μ g/ μ L) in a ratio of 1:25 w/w trypsin/protein at 37 °C overnight. The tryptic peptides were dried in speed vacuum concentrator and stored at -20 °C until analysis. During the sample preparation all the samples were stored on ice to prevent protease activation.

LC-MS/MS

On the day of the analysis, the frozen samples were first dissolved in 100 μ l formic acid (0.1%) and then was 10 μ l applied to the liquid chromatography tandem mass spectrometry (EASY-nLC-MS/MS, Thermo Scientific, Waltham, USA). Peptides were separated by reverse phase chromatography on a C18 pre column (20 mm $\times$ 100 μ m) followed by a C18 column (100 mm $\times$ 75 μ m) with particle size 5 μ m (NanoSeparatoons, Nieuwkoop, Netherlands) at a flow rate of 300 nL/min. The gradient buffers contained 0.1% formic acid in water (buffer A) and 0.1% formic acid in acetonitrile (buffer B), and a linear gradient from 0% to 100% of buffer B was used for 90 minutes to separate the peptides. Automated online analyzes were done with a LTQ Orbitrap Velos Pro hybrid mass spectrometer (Thermo Scientific). Raw files were searched using MaxQuant v. 1.5.8.3 (Max Planck Institute of Biochemistry, Martinsried, Germany) against Uniprot Human database (downloaded 2019).

Statistics

Univariate statistics

All univariate statistical calculations were done using GraphPad Prism version 5.0, 6.0 or 8.0 (GraphPad Software, San Diego, California, USA). The alpha level for statistical significance was ≤ 0.05 for all tests. Baseline was defined as a mean of the first four measurements.

Paired Student's *t* tests were used when comparing blood pressure before and after the experiment, as well as differences in volume recovery between the catheters. Changes in insulin concentration and skin temperature during the course of the experiment were analyzed using one-way analysis of variance (ANOVA) for repeated measures, followed by multiple comparisons using Sidak's correction (paper I-III). One-way ANOVA for repeated measures was also used for comparison between insulin adsorbed to the microdialysis membranes *in vivo* (paper III).

Two-way ANOVA for repeated measures, followed by multiple comparisons using Sidak's correction, was used to analyze 1) dynamic changes during the experiment and 2) differences between different catheters (provocation catheter and control catheter), both regarding concentration of analytes in the microdialysate (glucose, lactate, pyruvate, urea; paper I-III) as well as skin blood perfusion measured using LSCI (paper I-II).

Regression analysis was done on the data of insulin concentration measured in serum using different methods (electrochemiluminescence and ELISA; paper III).

Multivariate data analysis

The proteomic data (paper IV) was processed using multivariate data analysis (MVDA), using the multivariate software tool SIMCA P⁺ (version 15.0; Sartorius Stedim Biotech, Umeå, Sweden). The unsupervised model Principal Component Analysis (PCA) was applied to detect potential multivariate outliers using score

plots in combination with Hotelling's T^2 and distance to model in X-space (DModX). The supervised model Orthogonal Partial Least Square discriminant analysis (OPLS-DA) was then applied to investigate 1) differences in the proteome from the different tissue compartments and 2) changes over time (before and after glucose provocation) in the proteome from the same tissue compartment. The variables (proteins) with variable influence on projection (VIP) > 1 were considered important. VIP indicates the relevance of each X-variable pooled over all dimensions and Y-variables (the group of variables that best explain Y). R^2 describes the goodness of fit (the fraction of sum of squares of all the variables explained by a principal component), and Q^2 describes the goodness of prediction (the fraction of the total variation of the variables that can be predicted using principal component cross validation methods) [91]. The more components the model consists of the higher the risk for "noise" contamination. More components would result in a higher R^2 but only at the expense of a lower prediction score of the Q^2 . A difference between R^2 and Q^2 greater than 0.3 implies overfitting, meaning that the robustness of the model is poor. The validity of the model was estimated using cross validated analysis of variance (CV-ANOVA). A p value < 0.05 for CV-ANOVA indicate that the multivariate regressions were significant.

Bioinformatics

In paper IV, the bioinformatic tool STRING (Search Tool for Retrieval of Interacting Genes/Proteins) was used to analyze the protein-protein association network of the proteins identified in the three different tissue compartments. Protein accession numbers (as described in UniProt) were entered in the search engine (multiple proteins) and the organism *Homo sapiens* was selected. The highest confidence (0.900) for interaction scores were applied. A false discovery rate (FDR) ≤ 0.05 was used when classifying the Biological Process (GO) of each protein). PPI enrichment p value ≤ 0.01 was obtained for each protein network.

Review of the papers

Paper I – Skin glucose metabolism and microvascular blood flow during local insulin delivery and after an oral glucose load

This study investigated the metabolic and microvascular effects of insulin in the human skin when insulin was directly delivered to the interstitium using microdialysis or endogenously produced and delivered to the skin via the systemic circulation.

Experimental design

Microdialysis catheters ($n = 28$) were inserted intracutaneously in 14 healthy, fasting subjects (Figure 7). Microdialysis was used both to deliver insulin locally to the skin and to measure changes in tissue concentration of metabolic markers (glucose, lactate and pyruvate) and insulin. A systemic physiological increase in insulin was induced by an oral glucose tolerance test (OGTT). Microvascular blood flow in the tissue surrounding the microdialysis catheters was simultaneously assessed by urea clearance technique and by LSCI. The catheter insertion causes a local trauma in the skin. In a control experiment, six subjects did not receive any oral glucose load. This was to assess the importance of the insertion trauma on microvascular effects in the results.

Results and discussion

Control subjects (no oral glucose load): No changes in glucose ($p = 0.37$), lactate ($p = 0.69$) or pyruvate ($p = 0.39$) were observed during the experiment. A tendency of decreased urea concentration (i.e. increased local blood flow) was observed over time, however this effect was not statistically significant ($p = 0.43$). LSCI detected a significant increase in local perfusion in the tissue surrounding the microdialysis catheter membranes compared to a non-affected remote skin site, probably due to catheter insertion trauma.

Figure 7. Experimental setup in paper I.

Experimental subjects (received oral glucose load): During local insulin delivery, an increase in the concentrations of lactate ($p = 0.04$) and pyruvate ($p = 0.01$), but no difference in glucose concentration ($p = 0.97$), could be observed when comparing the insulin and control catheter. Urea clearance increased more in the insulin catheter than in the control catheter, as a result of increased local blood flow ($p = 0.047$). This suggests that local insulin delivery, in itself, increased tissue blood flow and thereby also the local delivery of more insulin and glucose to the tissue. In turn, this increased tissue metabolism, seen as increased concentrations of lactate and pyruvate.

Figure 8. Changes in dialysate concentration of glucose (circles) and insulin (diamonds) in the skin during continuous intradermal delivery of insulin (grey circles) or control substance (black circles) during an oral glucose provocation. A separate control group (pink circles) received neither intradermal insulin delivery nor an oral glucose load. Error bars represent mean $\pm$ SEM.

The oral glucose load induced an adequate physiological increase of the concentrations of glucose, lactate or pyruvate in both the insulin catheter and the control catheter, but without significant differences between the catheters. Interstitial glucose concentration peaked approximately 68 minutes after intake of the oral glucose load (Figure 8).

During the course of the experiment, urea clearance increased in both catheters, while LSCI perfusion increased significantly 1 and 2 hours after oral glucose intake compared to baseline ($p < 0.001$), see Figure 9. Both indicating increased local blood flow caused by the glucose provocation and systemic hyperinsulinemia.

Figure 9. Changes in local skin blood flow during continuous intradermal delivery of insulin (grey circles) or control substance (black circles) and during an oral glucose provocation, measured using the urea clearance technique (left) and LSCI (right). A separate control group (pink circles) received neither intradermal insulin delivery nor an oral glucose load. PU = perfusion units. * indicates a significant difference between the insulin and control catheter (left), while encircled symbols indicate a significant change from baseline (right). Error bars represent mean $\pm$ SEM.

Conclusions

- Local delivery of insulin caused an immediate increase in microvascular blood flow and increased glucose metabolism in the human skin.
- Increased urea clearance after oral glucose intake, compared to control subjects not receiving oral glucose, indicates an insulin dependent increase in blood flow in the skin.

Paper II – Effect of NG-monomethyl L-arginine on microvascular blood flow and glucose metabolism after an oral glucose load

Previous research using transdermal iontophoresis and systemic clamps have shown that the vascular actions of insulin most likely are due to production of endothelial nitric oxide (NO). This study was designed to investigate whether the effects on local blood flow and metabolic changes observed in the skin after an endogenous systemic increase in insulin using microdialysis technique (paper I) were mediated by the endothelial nitric oxide pathway.

Experimental design

Microdialysis catheters (n = 26) were inserted intracutaneous in 12 healthy, fasting subjects (Figure 10). One catheter was used for local delivery of L-NMMA, a NO synthase inhibitor, in order to inhibit NO-mediated vasodilation induced by endogenously released insulin. The other catheter served as control. Subjects ingested an oral glucose load to induce endogenous, systemic hyperinsulinemia. Microvascular blood flow was monitored using urea clearance technique and LSCI. Metabolic markers (glucose, lactate and pyruvate) were measured in the microdialysate to assess changes in the local metabolism.

Results and discussion

Provocation with oral glucose induced an increase of glucose concentration in the microdialysate from both catheters ($p < 0.0001$), however the increase was significantly higher in the control catheter compared to the L-NMMA catheter ($p < 0.035$), see Figure 11. Dialysate concentration of insulin was clearly higher in the control catheter compared to the L-NMMA catheter, but the limit for statistical significance was not reached ($p = 0.30$), see Figure 11.

Figure 10. Experimental setup in paper II.

Figure 11. Changes in dialysate concentration of glucose (left) and insulin (right) in the skin during continuous intradermal delivery of L-NMMA (open circles) or control substance (black circles) and during an oral glucose provocation. * indicates significant difference between the L-NMMA catheter and the control catheter. Error bars represent mean $\pm$ SEM.

Urea clearance was significantly higher in the control catheter compared to the L-NMMA catheter ($p < 0.006$), indicating higher local blood flow in the absence of L-NMMA (Figure 12). The level of urea concentration in the L-NMMA catheter in this study corresponded to the level of urea clearance observed in the control subjects not receiving an oral glucose load in paper I. This further emphasize that L-NMMA suppresses the insulin dependent, but not the trauma dependent, increase in tissue blood flow. A clear difference in the microvascular response to glucose provocation between the catheters was observed using LSCI, however not statistically significant ($p = 0.61$) (Figure 12).

Figure 12. Changes in local skin blood flow during continuous intradermal delivery of L-NMMA (open circles) or control substance (black circles) and during an oral glucose provocation, measured using the urea clearance technique (left) and LSCI (right). PU = perfusion units. * indicates significant difference between the L-NMMA catheter and the control catheter. Error bars represent mean $\pm$ SEM.

Conclusions

Local administration of L-NMMA suppresses increase in local blood flow and glucose delivery in the human skin after systemic glucose provocation, presumably by decreased local insulin-mediated vasodilation.

Paper III – Sampling insulin in different tissue compartments using microdialysis: methodological aspects

It is challenging to sample the concentration of insulin in the human skin using microdialysis, both because of low intracutaneous concentrations of insulin and low recovery. The aim of this study was therefore to investigate the methodological aspects of microdialysis sampling of insulin. The study was designed to (1) investigate the variation in insulin concentration in different tissue compartments (intracutaneous, subcutaneous and intravenous) during an oral glucose provocation, and also (2) to study how much insulin is adsorbed to the microdialysis catheter membranes and tubings during a typical microdialysis experiment (*in vivo* and *in vitro*).

The hypothesis was that (1) the concentration of insulin decreases from the intravenous compartment to the intracutaneous and subcutaneous tissue, and that (2) adsorption of insulin to the microdialysis membrane and tubing impairs the recovery of insulin from the tissue.

Experimental design

In vivo

Microdialysis catheters were inserted intracutaneously, subcutaneously and intravenously in 11 healthy subjects ($n = 31$ catheters), see Figure 13. An oral glucose load induced a systemic hyperinsulinemia. Microdialysate concentration of insulin, metabolic markers (glucose, lactate and pyruvate) and urea were analyzed. Microdialysis catheter membranes and tubings were also analyzed for adsorbed insulin.

In vitro

Blood samples from the *in vivo* subjects were collected after intake of the oral glucose load. *In vitro* microdialysis experiments were then done on the serum in order to investigate the temporal resolution of the adsorption of insulin to the microdialysis membranes (Figure 13).

Figure 13. Experimental setup in paper III.

Results and discussion

Dialysate insulin concentration increased significantly more intravenously ($p = 0.03$) after an oral glucose load, compared to the intracutaneous ($p = 0.23$) and subcutaneous ($p = 0.53$) compartments (Figure 14). Dialysate concentration of glucose, lactate and pyruvate concentrations all increased significantly in all compartments after an oral glucose load, and was significantly higher in the intravenous compartment compared to the intracutaneous and subcutaneous compartment ($p = 0.03$) during the whole experiment.

Figure 14. Mean change in dialysate concentration of insulin in the intracutaneous, subcutaneous and intravenous compartment during an oral glucose provocation. * indicates a significant difference between the intravenous catheter compared to the intra- and subcutaneous catheters. Error bars represent mean $\pm$ SEM.

The *in vivo* adsorption of insulin to the microdialysis membrane was highest in the intravenous compartment ($p = 0.01$), compared to the intracutaneous and subcutaneous compartments (Table 2). *In vitro*, the adsorption to the microdialysis membrane was highest one hour after sampling, then the concentration gradually decreased after three and five hours of sampling (Table 3).

Table 2. Adsorption to *in vivo* membranes

Catheter compartment	Mean [insulin] mU/L (SD)
Intracutaneous	0.22 (0.24)
Subcutaneous	0.23 (0.14)
Intravenous	0.99 (0.39)

Table 3. Adsorption to *in vitro* membranes.

Subject	Sampling time (h)	Membrane mU/L	Serum mU/L	Dialysate mU/L	Membrane / Serum	Membrane / Dialysate	Dialysate / Serum
1	1	5.7	46.8	18.4	12.2%	31.0%	39.4%
1	3	1.8	53.4	16.1	3.3%	11.2%	30.2%
1	5	1.1	36.5	17.3	2.9%	6.4%	47.5%
2	1	0.96	11.4	4.6	8.4%	20.9%	40.5%
2	3	0.36	10.4	5.3	3.4%	6.8%	50.8%
2	5	0.28	9.6	4.4	2.9%	6.4%	45.8%

Conclusions

- The concentration of insulin in peripheral tissues is low, probably due to decreasing tissue vascularity.
- Adsorption of insulin to the microdialysis membrane is modest but time-dependent, where most of the adsorption occur during the first hour of microdialysis sampling. This finding highlights the importance of a stabilization time for the microdialysis system before sampling tissue analytes.

Paper IV – Investigation of proteins important for microcirculation using *in vivo* microdialysis after glucose provocation – a proteomic study

This study examined the protein expression in microdialysis samples, in order to identify protein biomarkers that are related to microvascular changes in different tissue compartments during an oral glucose provocation.

Experimental design

In vivo microdialysate from 8 healthy subjects was collected from the intracutaneous, subcutaneous and intravenous tissue compartments (Figure 15). To maintain adequate sample volume, the microdialysate samples were pooled into three phases: recovery after catheter insertion, baseline and glucose provocation, and then analyzed using proteomics.

Results and discussion

A total of 126 proteins were identified in the microdialysate using mass spectrometry (LC-MS/MS). The distribution between the different tissue compartments is presented in Figure 16.

Figure 16. Three-way Venn diagram visualizing the distribution of proteins between the different tissue compartments; intracutaneous, subcutaneous and intravenous.

Figure 15. Experimental setup in paper IV.

Multivariate statistical analysis using SIMCA⁺ found 54 proteins with VIP >1 that contributed most to the separation between all different phases (Table 1 in paper IV), while 53 proteins with VIP >1 were found to be most important for the separation before and after glucose provocation (Table 2 in paper IV). An extraction of the 15 most significant proteins (VIP >1) from Table 1 and 2 from paper IV is presented in the Supplementary materials as Table S1 and S2. The variation in expression of proteins over time (Table S1) and after glucose provocation (Table S2) was most pronounced in the intracutaneous and subcutaneous compartments, compared to the intravenous compartment.

Bioinformatic analysis visualizing the protein-protein interactions for these proteins using STRING identified four proteins involved in microcirculatory physiological functions: angiotensinogen, kininogen-1 and alpha-2-HS-glycoprotein and hemoglobin subunit beta (Figure 17). Angiotensinogen (as part of the renin-angiotensin system) and kininogen-1 (as part of kallikrein-kinin system) is both known to be involved in nitric oxide dependent vasodilation.

Conclusions

It is known that glucose provocation causes insulin induced vasodilation through the nitric oxide pathway. The findings in this study indicates that this is also facilitated through the interactions of the renin-angiotensin system (angiotensinogen) and kallikrein-kinin system (kininogen-1).

Figure 17. Pathway analysis of proteins associated with vascular actions of the proteins most important for the separation between the phases in all compartments (intracutaneous, subcutaneous and intravenous) using the STRING database.

Vascular pathways highlighted: red = vasodilation, blue = regulation of cellular response to insulin stimulus, green = regulation of blood vessel diameter, yellow = positive regulation of nitric oxide biosynthetic process. PPI enrichment was $< 1.0E-16$.

Proteins involved in vascular pathways: AGT = Angiotensinogen, KNG = Kininogen-1, AHSG = Alpha-2-HS-glycoprotein, HBB = Hemoglobin subunit beta.

Discussion

This thesis presents an experimental *in vivo* model for studying the microvascular responses and metabolic actions of insulin in the human skin in healthy subjects.

The skin as a microvascular model

The skin microcirculation has been considered a potentially representative vascular bed to study the mechanisms of microvascular dysfunction. It is also believed to mirror generalized systemic vascular dysfunction in magnitude and underlying mechanisms [92]. Therefore, the skin microcirculation is increasingly used as a model for general vascular function, by observing microvascular responses to different pharmacological and physiological provocations. To date, the skin has been used as a circulation model to investigate vascular mechanisms in several different conditions, including type 2 diabetes mellitus [93], hypertension [94], renal disease [95], peripheral vascular disease [96], obesity [97] and heart failure [98].

There are however conditions where the microcirculation in the skin presents physiological disturbances that cannot be detected in the systemic circulation. This can be seen in patients in severe shock, for instance in patients suffering from severe burns or patients treated at the intensive care unit [99]. An impaired endothelium-dependent vasodilation in the skin has also been described in heart transplant recipients [100, 101]. The same phenomenon has been observed in the cerebral blood flow, where a persistent metabolic crisis in the perihemorrhagic zone was detected using microdialysis, although the cerebral blood flow was normalized [102]. In all of these states of critical illness, it thus seems to be an uncoupling between the local and systemic metabolism and circulation. As early changes in microvascular function in the skin can already occur before clinical manifestation of disease or pathology, it is therefore important to look directly in the skin.

All available methods today for assessing responses to insulin are based on systemic measurements and thereby offer an estimate of the systemic insulin resistance. Previous microdialysis data have observed a persistent deranged glucose metabolism and acidosis locally in the skin of deep partial-thickness burns, although systemic glucose homeostasis was restored [99]. The responses to insulin locally in target tissues may thereby differ from the systemic circulation. A method to measure the local responses of insulin in a specific target tissue is therefore of interest in evaluating how insulin function and how insulin resistance affects specific tissues. Microdialysis of the skin enables assessment of physiological functions of a specific end organ, without taking the whole organism into account. Local effects on metabolism and microvascular blood flow can thus be assessed after both increased systemic or increased local insulin concentrations. For the skin this is likely more relevant for local morbidity compared to, for instance, blood samples.

Why is microdialysis a suiting technique?

To study the microvascular effects of insulin in human skin, it can be delivered through the systemic circulation (either through intravenous administration or endogenous production) or by local delivery. Local delivery of insulin to the skin has previously been done using transdermal iontophoresis, in combination with laser Doppler flowmetry (LDF), for measurement of the microvascular responses [103-107]. This has however been limiting in two different ways. Firstly, the delivery of insulin via iontophoresis has been found challenging because of a substantial nonspecific vasodilation caused by the diluting medium of the drugs that have been used [103, 106, 107]. Secondly, transdermal iontophoresis in combination with optical techniques (such as LDF) only offers assessment of the microvascular effects of insulin and not the metabolic events. Microdialysis enables sampling of all kinds of interstitial molecules, such as metabolites, enzymes and hormones. Intracutaneous microdialysis therefore offers a way to simultaneously deliver substances to the skin and measure both changes in the concentration of metabolites [108] and blood flow [32, 109] in the same tissue volume.

Challenges with microdialysis technique

There are however several challenges using microdialysis technique. This thesis mainly addresses challenges regarding recovery and adsorption, and how these factors might affect the results.

Recovery

The concentration of an analyte of interest in a microdialysate sample does generally not represent the actual concentration in the tissue, but is a product of the recovery of the analyte during the microdialysis sampling. *In vivo*, the recovery is rarely 100%. Numerous different tissue specific factors affect the recovery *in vivo*, where the effect of local blood flow on recovery is of high importance in this thesis. Recovery profiles differs when sampling in highly vascularized organs (brain and skeletal muscle) compared to less vascularized organs (skin and adipose tissue) [68, 110]. This is the origin to the development of different clearance techniques, such as the urea clearance. The effect of blood flow on recovery must therefore be considered when analyzing the results in this thesis, as it comprises sampling in both peripheral tissues (skin and adipose tissue) and intravenously (paper III–IV).

There are other tissue specific factors that affects the recovery, that are somehow difficult to control for. Diffusion in a tissue is typically slower than in an aqueous solution, due to the reduced fluid volume and increased diffusional path (or tortuosity) characteristic of tissue [111, 112]. Tortuosity is a measure of the complexity of a molecules route of diffusion, navigating between cell membranes in the interstitium to the dialysate. The differences in recovery of metabolites and insulin observed in the different tissue compartments (paper III) is likely partly due to differences in blood flow and tortuosity.

The endothelium itself also seems to affect recovery. In a previous study, the subcutaneous concentration of insulin during steady state conditions (by intravenous insulin infusion) was investigated using microdialysis [113]. The subcutaneous dialysate concentration of insulin was 40-50 % lower compared to plasma. The

peak in dialysate subcutaneous interstitial fluid was also found to be delayed by 20 minutes compared to plasma. In a parallel experiment using inulin as a reference compound to insulin, no delay in peak concentration was observed. This suggests that there is an endothelial barrier for insulin in the subcutaneous tissue. It is highly likely that the dialysate insulin concentration peaked earlier in the intravenous compartment in paper III compared to the subcutaneous and intracutaneous compartments, but as the samples were pooled hourly the temporal resolution could not detect this difference.

Recovery does however also depend on several controllable variables, determined by the researcher, where perfusion flow rate and membrane properties (pore size and surface area) are the most important factors [64, 65, 68]. These variables are important to consider when designing a microdialysis experiment. Recovery is proportional to the catheter membrane surface area, due to increasing the area available for diffusion, while recovery is inversely proportional to the perfusion flow rate. High perfusion flow rate gives less time for diffusion over the membrane and thus lower recovery, while a low perfusion flow rate enables higher recovery. However, low perfusion flow rate can make the sampling time required for adequate sampling volume inappropriately long, which is clinically limiting. In addition, longer sampling time also lower the temporal resolution.

Physicochemical properties of the analyte (charge, molecular weight and hydrophobicity) affect recovery. The main application for microdialysis has previously been to sample small, hydrophilic molecules, e.g. glucose, lactate, pyruvate, urea, glutamate and glycerol [114]. As the interest increased to study larger molecules, microdialysis membranes with higher molecular weight cut-off (pore size) were introduced on the market. Higher cut-off (≥ 100 kDa) enables sampling of lipids, cytokines and other proteins [115-122]. A drawback with higher membrane cut-off is however increased fluid loss from the perfusate into the tissue. To compensate, a colloid is often added to the perfusate to increase the osmotic pressure. Microdialysis experiments using a 30 mm membrane and no colloid added to the perfusate have shown a 50% fluid loss at flow rate 0.33 $\mu\text{l}/\text{min}$ and 80% fluid loss at 0.16 $\mu\text{l}/\text{min}$ [123]. The importance of a colloid is thus great. Albumin is a

commonly used colloid, proved to effectively prevent fluid loss [122, 124]. When combining microdialysis with proteomics it is however disadvantageous to use albumin as a colloid, as high concentrations of albumin in the microdialysate sample can interfere with the protein quantification and thereby mask the identification of other proteins in LC-MS/MS. In order to properly investigate the proteome, each sample needs to be depleted of albumin before protein quantification. This is time-consuming but necessary to obtain reliable results. Although, since albumin is a carrier protein, albumin depletion might cause depletion of other proteins as well that can affect the results.

Adsorption

Proteins, in general, adsorb to solid surfaces [125] and the microdialysis membrane and tubings are no exception. Adsorption of proteins to the microdialysis membrane has previously been studied *in vitro* [126-128], and *in vivo* in the skin [129] and brain [130]. Sampling of large molecules (e.g. proteins) using microdialysis technique is particularly challenging. Apart from the necessity to use high molecular weight cut-off membranes, an adequate recovery of large molecules is a challenge. Studies have reported that the recovery may be as low as 1.5-5% for some large peptides *in vitro* [117, 131] and 3-18% for insulin [117, 132]. It has been suggested that this is partly due to adsorption to the microdialysis membrane and tubings. Adsorption can cause a physical blockage of the pores of the microdialysis membrane, hence either completely prevent diffusion or causing analytes to pass over to the microdialysate in an unpredictable manner. Insulin is known to be adsorbed by different materials, such as glass and polyacrylamide [133], but the adsorption to the microdialysis membrane (polyarylethersulphone) and tubings (polyurethane) have previously been unknown. The results in paper III on adsorption is therefore of great interest.

In paper III, adsorption of insulin to the microdialysis membrane during an oral glucose provocation test was investigated. The *in vivo* adsorption of insulin to the microdialysis membrane was highest intravenously, compared to the intracutaneous and subcutaneous compartments. This was expected, and in accordance with the dialysate concentration of insulin that was also highest intravenously.

Figure 18. In a pilot experiment, the *in vivo* adsorption of insulin to the microdialysis catheter membrane was illustrated with chemiluminescence using antibodies directed to insulin. A = insulin catheter membrane. B = control catheter membrane.

The experiments in paper III using *in vitro* microdialysis on human serum showed a time-dependent adsorption of insulin to the membranes, with a rapid initial adsorption that subsided over time. Approximately 10% of the insulin in the surrounding serum adsorbed to the membrane during the first hour, but thereafter gradually detached. In theory, this could of course affect the relative recovery. In these pilot experiments, of only two test subjects (six microdialysis catheters), no statistical calculations could be

done due to the limited number of samples. The data suggests, however, that the amount of insulin that adsorb to the membrane is proportional to the serum concentration, regardless of the actual concentrations. Thence, relative recovery *in vitro* is not affected to a large extent by adsorption to the membrane.

The adsorption to, and subsequent detachment of insulin from the membrane could, however, possibly affect *in vivo* results in an unpredictable manner. It would therefore be very interesting to investigate whether the same time-dependency of insulin adsorption can be observed *in vivo*. This is however challenging, as the concentration of insulin is dynamic *in vivo*. It would also require insertion of numerous catheters in each subject, which ethically would be questionable. In a previous study in which endocannabinoids was studied in human muscle and skin, a similar dynamic adsorption pattern to the membrane was observed, with rapid initial adsorption followed by gradual detachment over time [134]. This indicates that there may be a need to not only let the tissue adapt to the catheter insertion, but also let the microdialysis system have time to stabilize and adapt before dialysate sampling is initiated. Perhaps a longer recovery period after catheter insertion is needed, than the 90-minute period used in this experimental *in*

in vivo model, in order to decrease the risk that the dynamic detachment of adsorbed molecules affects the dialysate concentration.

Different modifications to the microdialysis catheter, for instance siliconizing [135] or coating the membrane, have been explored, with the aim to decrease adsorption of different analytes to the membrane. Dahlin et al. coated the microdialysis membrane with the triblock copolymer Pluronic F-127 [126, 136] and Poloxamer 407 [137] to reduce protein adsorption and increase fluid recovery, the latter with a 33% decrease of protein adsorption [137]. Introducing some form of catheter modifications to prevent adsorption into this study model in the future could be a way to improve the recovery profile of insulin.

Effects of insulin on local blood flow

The effects of insulin on local skin blood flow are the main focus of this thesis. As microdialysis offers a way to simultaneously study the local metabolic effects with a high temporal resolution, the interplay between blood flow and metabolism can be addressed.

Local and systemic delivery of insulin and L-NMMA

In paper I, it was found that local delivery of insulin through the microdialysis catheter immediately increased microvascular blood flow, as measured by both laser speckle contrast imaging (LSCI) and urea clearance (UC). Simultaneously, an increase in interstitial concentrations of pyruvate and lactate was observed, indicating increased glucose uptake. After oral glucose intake, and hence systemically increased insulin concentration, a peak in interstitial insulin was observed approximately 98 minutes after the oral glucose load. The peak in interstitial glucose concentration was however observed at approximately 68 minutes after oral glucose intake, while pyruvate and lactate concentrations peaked at approximately 83 minutes. Thus, the availability of insulin and the resulting metabolic effects in the interstitium of the skin are delayed after a systemic glucose load compared to when insulin is delivered locally. This suggests that the endothelium, i.e. the transendothelial transport, is the limiting factor for the availability of insulin in the interstitium in the skin, which has also been observed in skeletal muscle [138] and subcutaneous adipose tissue [113].

In continuation to paper I, paper II investigated whether these vasodilatory effects observed in the skin after a systemic insulin provocation through oral glucose intake (seen in paper I) was mediated through the nitric oxide pathway. This was investigated using the endothelial nitric oxide synthase (eNOS) inhibitor L-NMMA. The results indicate that local delivery of L-NMMA suppresses the insulin dependent increase in skin blood flow seen after oral glucose intake. This is in agreement with previous studies, demonstrating reduced insulin stimulated microvascular perfusion in skeletal muscle (intravenous administration) [139] and skin (locally delivered through iontophoresis) [45]. In a recently published study,

intravenous administration of L-NMMA also reduced global microvascular perfusion in brain during magnetic resonance imaging (MRI) scanning [140].

It is however known that catheter insertion induces a local trauma and production of inflammatory markers, persistent for at least 24 hours [75]. As the insertion trauma affects the local blood flow, the control subjects not receiving any oral glucose load (in paper I) are of great importance for comparison. Previous studies have observed increased skin blood flow after microdialysis catheter insertion for 60-120 minutes before return to baseline [24, 141]. The control subjects in paper I presented a slight increase in urea clearance during the course of the experiment, indicating a trauma induced increase in local blood flow surrounding the catheter insertion area. However, the increase in urea clearance and local blood flow was significantly higher in the control catheters of the subjects receiving an oral glucose load than in the control subjects (paper I). This suggests that the increase in local blood flow is insulin dependent and not solely the result of a local trauma effect. Furthermore, the degree of urea clearance in the L-NMMA catheter (paper II) corresponded to the urea clearance in the control subjects not receiving an oral glucose load (paper I), indicating that L-NMMA suppresses the insulin dependent, but not the trauma dependent, increase in skin blood flow.

This could however not be confirmed using LSCI, which is somewhat contradictory. LSCI showed increased local blood flow in the skin surrounding both the insulin catheter and the control catheter compared to baseline, but no change in tissue blood flow was observed in the remote skin site of subjects receiving oral glucose load compared to the subjects not receiving any oral glucose load. The absence of blood flow changes in unprovoked skin indicates that a systemic glucose load does not increase total skin blood flow, despite increased insulin levels and an increased glucose metabolism in the skin. A possible explanation might be differences in sensitivity and measurement depth between LSCI and urea clearance. The catheters were carefully placed as superficially as possible, at a depth previously confirmed to be approximately at 0.8 mm using a DermaScan ultrasound device [88]. This represents the border between dermis and subcutis, drained by the deep superficial venous plexus (DSVP). The LSCI is however

sensitive to changes in perfusion in the superficial dermis, possibly to a larger extent drained by the upper superficial venous plexus (USVP) [142]. Another explanation might be that the sample size was too small for the LSCI to detect the difference in perfusion.

Immediately after local delivery of L-NMMA, before the oral glucose load, a tendency towards decreased basal blood flow was observed in the tissue surrounding the L-NMMA catheter. This can be seen in terms of significantly lower glucose concentration and a tendency towards suppressed urea clearance in the L-NMMA catheter compared to the control catheter. This may be caused by an increase in vessel tone (vasoconstriction) due to the inhibition of eNOS, which in turn may restrict the delivery of other vasoactive agents, not necessarily related to the glucose-insulin pathway. An improvement to investigate this further could be to locally deliver L-NMMA to the skin in subjects not receiving an oral glucose load, to see what effects solely local delivery of L-NMMA has on the basal blood flow and metabolism.

Other biomarkers relevant for microcirculation

So far, the microcirculatory effects of a glucose provocation regarding the vascular effects of insulin have been discussed. The interactions between different signaling pathways through hormones, proteins etc. in the human body is however extremely complex. A lot is known, but there is still much to further investigate. For instance, the effects of a glucose provocation on changes in local protein expression are lacking. To understand what mechanisms that orchestrate the microcirculatory effects of a glucose provocation, investigation for other markers important for the microcirculation during a glucose provocation is of interest.

Paper IV used an innovative approach of combining microdialysis and tandem mass spectrometry (LC-MS/MS) to investigate changes in the human proteome in the skin and surrounding tissues (subcutaneous fat and venous blood) before and after an oral glucose provocation. The most relevant findings, from the perspective of local microcirculatory physiological functions, were the strong association of four proteins: angiotensinogen (AGT), kininogen-1 (KNG1), alpha-2-HS-glycoprotein (AHSG) and hemoglobin subunit beta (HBB). These four proteins were upregulated in all three compartments (intracutaneous, subcutaneous and intravenous) after glucose provocation compared to baseline.

Both angiotensinogen (as part of the renin-angiotensin system, RAS) and kininogen-1 (through the kallikrein-kinin system) is involved in nitric oxide dependent vasodilation. Kininogen-1 is cleaved into the vasoactive and proinflammatory bradykinin, that in turn acts on endothelial cell bradykinin B2 receptors to induce nitric oxide production [143-147]. RAS is involved in the regulation of, for instance, arterial blood pressure, vasoconstriction and proinflammatory processes [148-150]. The interaction between nitric oxide and angiotensin II is complex. Angiotensin II is the primary effector of RAS and is formed by enzymatically cleavage of first angiotensinogen and then angiotensin I. Angiotensin II regulate vascular resistance. It mainly induces vasoconstriction through angiotensin 1 receptors (AT1-R). Angiotensin 2 receptor (AT2-R)-mediated signaling pathways and function are not very well understood but in general appear to antagonize the effects of the AT1 receptor, by inducing a compensatory vasodilation through the

bradykinin dependent activation of endothelial nitric oxide synthase [151, 152]. A simplified illustration of how the different pathways by which insulin, angiotensinogen and kininogen-1 affect the nitric oxide production is presented in Figure 19.

Figure 19. Simplified illustration of the different pathways by which insulin, angiotensinogen and kininogen-1 affect the endothelial nitric oxide production, resulting in smooth muscle cells relaxation and thereby vasodilation. Illustration made by Simon Farnebo. IR = insulin receptor, AT1/2-R = angiotensin 1/2 receptors, B2-R = bradykinin 2 receptor, NO = nitric oxide, eNOS = endothelial nitric oxide synthase, P-eNOS = phosphorylated endothelial nitric oxide synthase, cGMP = cyclic guanosine monophosphate, GLUT4 = glucose transporter type 4.

Figure 20. Extended pathway analysis of proteins associated with vascular actions of the proteins most important for the separation before (baseline) and after glucose provocation using the STRING database.

Vascular pathways highlighted: red = vasodilation, blue = regulation of cellular response to insulin stimulus, green = regulation of blood vessel diameter, yellow = positive regulation of nitric oxide biosynthetic process, turquoise = regulation of vasoconstriction, orange = vasoconstriction, purple = regulation of tube diameter, brown = regulation of blood pressure. PPI enrichment was $< 1.0E-16$.

Proteins involved in vascular pathways: AGT = Angiotensinogen, KNG = Kininogen-1, AHSG = Alpha-2-HS-glycoprotein, HBB = Hemoglobin subunit beta, ACE = Angiotensin converting enzyme, REN = Renin, BDKRB2 = B2 bradykinin receptor.

In conclusion, these findings support that the insulin dependent vasodilation is facilitated through insulin itself and the kallikrein-kinin system, whereas the renin-angiotensin system regulates vessel tone through AT₂-R and bradykinin (vasodilation) and AT₁-R (vasoconstriction). The extended pathway analysis (using the STRING database) showed the involvement of the identified proteins in these pathways (Figure 20).

The number of human studies combining microdialysis and proteomics is limited, but the human proteome has been investigated in brain [153], subcutaneous interstitial fluid [154] and skin [155, 156]. The cutaneous proteome has however only been investigated with non-commercial large pore microdialysis catheters, with cut-off of 2000-3000 kDa [155, 156]. The results from these two studies, using microdialysis sampling during trauma phase (caused by catheter insertion) and during vasodilation (by local histamin provocation), obtained identification of approximately 150 proteins. Both angiotensinogen and kininogen-1 were identified in these studies, which is in agreement with the findings in paper IV. Hitherto, the proteome in microdialysate from the intravenous compartment has not previously been investigated. Neither has the human proteome in the skin related to insulin been investigated. This makes the results in paper IV particularly interesting.

It is however important to remember that paper IV is an exploratory study, aiming to identify proteins. The dynamics in protein expression during a glucose provocation has been presented, but conclusions regarding concentration of proteins or interactions on molecular levels cannot be drawn. Further studies are warranted to investigate the differences in protein expression in patients.

Limitations

There is always room for improvements, and it is important to consider possible limiting factors which might restrict the generalizability.

To begin with, all subjects in these papers are young and healthy individuals. As age and comorbidities influence the microvascular response [157], this limits the generalizability. Subjects were furthermore consecutively recruited through advertising on different social platforms, hence depending on who confirmed to the advertisement. Potentially this generates a risk for selection bias. There is also a risk of type II error, as the sample volume (number of subjects) were rather small.

A challenge in all microdialysis experiments is the handling of extremely small sample volumes (μl). As the sample volume is limited, duplicates were not possible to analyze. This makes the data sensitive to missing values, for instance due to technical issues during the analytical process.

It is important to consider that the identified proteins in paper IV are limited by the cut-off of the catheter membrane, so there might be other proteins larger than 100 kDa that are important for microcirculation that could not be identified.

Another limitation in paper I-II is that the absolute dose delivered to the intracutaneous interstitium is unknown. In paper I, the dialysate concentration of insulin could not be determined due to the insensitivity of the ELISA assay for insulin aspart. The added concentration to the perfusate (16.7 mU/L) was however several orders of magnitude higher than the fasting serum insulin concentration (0.007 mU/L). It can therefore surely be assumed that the actual delivered insulin dose was supraphysiological. In paper II, L-NMMA was added to a perfusate concentration of 1.87 mg/mL (equal to 10 mmol/L), based on previous studies using microdialysis [158, 159]. L-NMMA was chosen as eNOS inhibitor because of regulatory issues, as it was commercially available as a sterile compound for *in vivo* use. The analysis of dialysate concentration of L-NMMA was however not

possible, thereby can the actual delivered dose of L-NMMA to the skin not be determined.

In paper I, control subjects not receiving an oral glucose load were used to separate the insulin dependent vasodilation from the vascular effects caused by the catheter insertion trauma. It would have been interesting to use control subjects not receiving an oral glucose load in paper II as well, to solely investigate the effects of local delivery of L-NMMA on the basal blood flow and metabolism. Furthermore, dialysate concentration of insulin was not assessed in neither the control catheter nor the L-NMMA catheter (paper II), which would have been interesting to know for comparison.

Conclusions

This thesis describes an experimental *in vivo* model for assessing the microvascular effects of insulin in the human skin in healthy subjects using microdialysis technique.

Local (through the microdialysis catheter) and systemic (through oral glucose provocation) delivery of insulin to the skin increased the intracutaneous blood flow, measured by both urea clearance and laser speckle contrast imaging (LSCI) (paper I). The physiological mechanisms orchestrating these vascular effects are however complex and multifactorial. Insulin itself affects the endothelial cells and induces vasodilation, and thereby increased blood flow, through the nitric oxide pathway (paper II).

The concentration of insulin was lowest in peripheral tissues (skin and subcutaneous fat) compared to venous blood (paper III), probably due to decreasing tissue vascularity. Paper III also confirmed that adsorption of insulin to the microdialysis catheter membrane occurs both *in vivo* and *in vitro*, however presumably less pronounced than previously thought. The adsorption *in vitro* seems to be time-dependent, with rapid initial adsorption but then gradually detaching.

Proteomic analysis of the microdialysate did also reveal other biomarkers upregulated after a glucose provocation, indicating that the increased skin blood flow after a glucose provocation is facilitated through other pathways as well (paper IV). The pathways identified were the renin-angiotensin system and the kallikrein-kinin system.

Future perspectives

The papers in this thesis have investigated the physiological metabolic and microvascular responses to insulin in the skin. The interaction between glucose metabolism and microvascular dysfunction is complex and the result in this study add to the basic physiological data. The model designed in these studies is further applicable on different patient groups, where it would be interesting to study how metabolic and microvascular responses to insulin in the skin differ, for instance, in obese or insulin resistant patients. A better understanding of the local microvascular effects of insulin in these patients may open for new therapeutic means to decrease tissue morbidity.

Proteomic analysis found upregulation of proteins originating from the renin-angiotensin system and kallikrein-kinin system after glucose provocation compared to baseline. Further investigation of these pathways in relation to glucose provocation and insulin would be interesting. An animal model using insulin resistant or knockout mice, genetically inactivating these pathways, and investigate the differences in microvascular responses to insulin could for instance be of interest.

The adsorption of proteins to the microdialysis membrane is considered a limitation, as it may affect the recovery of the analytes of interest. Another perspective of this phenomenon is to take advantage of the fact that adsorption occur and instead consider the data conserved in the membrane as an addition to the microdialysate data. An interesting approach to a future study would be to investigate all biomarkers adsorbed to the microdialysis membrane during a glucose provocation, to assess biomarkers that for some reason have not been able to diffuse over the membrane to the microdialysate.

Acknowledgements

I would like to express my sincere gratitude and appreciation to all the people who have been by my side in different ways throughout this journey!

My main supervisor **Simon Farnebo**. Thank you for your enthusiasm and limitless energy! You have a fantastic ability to inspire and encourage, and for that I am ever grateful. After every meeting with you I feel that nothing is impossible, and whenever I've been struggling you magically steer me in the right direction. Thank you for your support and belief in me! Without you this would not have been possible!

My supervisor **Bijar Ghafouri**. You are one of the most brilliant women I know! You have not only been a wise research supervisor, but also a great mental support during these years. I don't know how you can achieve so much and still always make time for me. The proteomic part of the dissertation has sometimes been challenging for me, but thanks to your endless patience and explaining I have managed through!

My supervisor **Erik Tesselaar**. Thank you for your valuable support! Your ability to connect data together and draw conclusions have always impressed me. Your knowledge has been extremely valuable and really guided me through these years, and your kindness a great support. I hope that you eventually will find your way back to us in Sweden!

My supervisor **Folke Sjöberg**. Thank you for your wise input. Since my first day as a PhD-student you have asked me when it is time to defend my thesis. Now I can tell you for sure, that the day has come!

Also, a collective thanks to all of my supervisors mentioned above – my research parents! When I first met you, I was a young 21-year-old medical student. You

have all raised me to become the researcher but, in a way, also the medical doctor I am today. Thank you for that! You are all great role models to me. My only regret is that we couldn't drink Aperol Spritz on the beaches in Rio 2020.

My sincerest gratitude to **Fredrik Iredahl**. You introduced me to the practical and experimental work and set an excellent example for me that it was possible to combine medical studies and research. Thanks for your support!

Thanks to all at the Painomics lab, and especially **Karin Wåhlén** and **Anders Carlsson**. Life as a clinical PhD-student can sometimes be a bit lonely, but the moment I sat my foot inside your lab I felt as one in your family. Thank you for “pipettkalibreringsparty”, fika breaks and many happy times.

To all personal at BRIVA that have helped me in different ways during my long and intense experimental days. Especially thanks to **Ingrid Steinwall** and **Eva Lago**, whom always have welcomed me to BRIVA with smiles and nice chats.

As a young medical student without any experience of experimental research and setup, **Joakim Henricson** friendly welcomed me to his lab and helped me with the experiments with patience and calmness. Thank you!

I would also like to acknowledge all my test subjects (and their arms, stomachs and blood), without whom there would never have been any studies at all.

From the bottom of my heart, I would like to thank all my friends and relatives for the support and patience during these years. Especially thanks to my cousins **Elsa Mattsson** and **Saga Bergman** for assisting me and entertaining my test subjects during some of the experimental days at BRIVA. Lucky no one caught you on the scooters in the hospital tunnels underground...

And to my mum **Åse Högstedt**, dad **Bill Högstedt** and sister **Gabriella Högstedt**. You are the best and I love you all so much!

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Supplementary

Protein	Gene name	Protein name	Compartment	VIP
P02766	TTR	Transthyretin	Intracutaneous	1.37
P04217	A1BG	Alpha-1B-glycoprotein	Subcutaneous	1.37
P00450	CP	Ceruloplasmin	Subcutaneous	1.35
P02768	ALB	Albumin	Intracutaneous	1.35
P00738; P00739	HP	Haptoglobin/Haptoglobin-related protein	Subcutaneous	1.32
P02652	APOA2	Apolipoprotein A-II	Intracutaneous	1.31
P02768	ALB	Albumin	Subcutaneous	1.30
P02763	ORM1	Alpha-1-acid glycoprotein 1	Subcutaneous	1.28
P01042	KNG1	Kininogen-1	Subcutaneous	1.28
P43652	AFM	Afamin	Intracutaneous	1.27
P02652	APOA2	Apolipoprotein A-II	Subcutaneous	1.24
P01011	AACT	Alpha-1-antichymotrypsin	Intravenous	1.24
P02763	ORM1	Alpha-1-acid glycoprotein 1	Intracutaneous	1.23
P02765	AHSG	Alpha-2-HS-glycoprotein	Intravenous	1.23
P02750	LRG1	Leucine-rich alpha-2-glycoprotein	Subcutaneous	1.23

Table S1. Extraction of Table 1 in paper IV, showing the 15 most significant proteins (VIP >1) in the orthogonal partial least square discriminant analysis (OPLS-DA) model of all three compartments (intracutaneous, subcutaneous and intravenous) that contributed most to the separation between the different time periods. Proteins were considered significant if VIP (variable influence of projection) value was >1. Protein ID (accession number) and gene name are referred to according to the protein database Uniprot (<http://uniprot.org>).

Protein	Gene name	Protein name	Compartment	VIP	Change from baseline
P02652	APOA2	Apolipoprotein A-II	Intracutaneous	1.54	↑
Q9NZP8	C1RL	Complement C1r subcomponent-like protein	Intracutaneous	1.47	↑
Q96PD5	PGLYRP2	N-acetylmuramoyl-L-alanine amidase	Intracutaneous	1.47	↑
P43652	AFM	Afamin	Intracutaneous	1.46	↑
P04217	A1BG	Alpha-1B-glycoprotein	Intracutaneous	1.44	↑
P02750	LRG1	Leucine-rich alpha-2-glycoprotein	Intracutaneous	1.41	↑
P02765	AHSG	Alpha-2-HS-glycoprotein	Intracutaneous	1.39	↑
P02768	ALB	Albumin	Subcutaneous	1.38	↑
Q96PD5	PGLYRP2	N-acetylmuramoyl-L-alanine amidase	Subcutaneous	1.37	↑
P02768	ALB	Albumin	Intracutaneous	1.36	↑
P00738; P00739	HP	Haptoglobin/Haptoglobin-related protein	Intravenous	1.35	↑
P01019	AGT	Angiotensinogen	Intracutaneous	1.34	↑
P68871	HBB	Hemoglobin subunit beta	Intravenous	1.34	↑
P04217	A1BG	Alpha-1B-glycoprotein	Intravenous	1.34	↑
P04217	A1BG	Alpha-1B-glycoprotein	Subcutaneous	1.33	↑

Table S2. Extraction of Table 2 in paper IV, showing the 15 most significant proteins (VIP >1) in the orthogonal partial least square discriminant analysis (OPLS-DA) model of all three compartments (intracutaneous, subcutaneous and intravenous) that contributed most to the separation in protein expression before (baseline) and after glucose provocation. “Change from baseline” indicates alteration in protein expression level after glucose provocation compared to baseline, where ↑ = Upregulated; ↓ = Downregulated after glucose provocation compared to before (baseline). Proteins were considered significant if VIP (variable influence of projection) value was >1. Protein ID (accession number) and gene name are referred to according to the protein database Uniprot (<http://uniprot.org>).

Papers

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FACULTY OF MEDICINE AND HEALTH SCIENCES

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