

From the Department of Molecular Medicine, the Endocrine and
Diabetes Unit
and
Department of Physiology and Pharmacology
Karolinska Institutet, Stockholm, Sweden

MICRODIALYSIS AS A TOOL FOR THE MANAGEMENT OF DIABETES MELLITUS

Neda Rajamand Ekberg

M.D.



Stockholm 2005

All previously published papers were reproduced with permission from the publisher.

Published and printed by Karolinska University Press

Box 200, SE-171 77 Stockholm, Sweden

© Neda Rajamand Ekberg, 2005

ISBN 91-7140-334-5

To Jonas and our unborn child

1 ABSTRACT

Diabetes complications are the result of impaired metabolic control. A continuous glucose monitoring system could be a tool to improve metabolic control. Microdialysis is a minimal invasive method for investigating tissue metabolism in-vivo and provides a novel opportunity for glucose monitoring in patients with diabetes (DM). The general aim of this thesis was to investigate if microdialysis technique can be used for reliable monitoring of glucose and other metabolites in abdominal subcutaneous (sc) adipose tissue during everyday activity in patients with DM. Moreover, we wanted to investigate if the microdialysis technique can be used for assessment of insulin sensitivity in subcutaneous adipose tissue.

Methodological studies

Comparing the effect of different microdialysis flow rates (0.3, 1, 2 and 5 $\mu\text{L}/\text{min}$) on the interstitial metabolites measured in subcutis we found that at the basal fasting state the dialysate glucose obtained by 0.3 $\mu\text{L}/\text{min}$ was equal to capillary glucose concentration. A decrease in dialysate glucose levels during the basal state was observed for higher flow rates but not for 0.3 $\mu\text{L}/\text{min}$ when no glucose was added to the perfusion fluid. This indicates a depleting effect for higher flow rates ($> 0.3 \mu\text{L}/\text{min}$). The relative increase in glucose after OGTT was similar for capillary glucose and dialysate glucose measured with flow rate 0.3 $\mu\text{L}/\text{min}$ but not for higher flow rates (I).

In long term studies with microdialysis the changes in dialysate glucose and other metabolites over eight days differed significantly in rats and humans. Caution should therefore be used when extrapolating data from subcutaneous tissue in Sprague Dawley rat to human applications. In healthy subjects and in patients with DM significant increases in dialysate glucose levels and relative glucose recovery were observed over five days. Histological signs of local inflammation in sc adipose tissue were observed around the microdialysis catheters. However, when using a flow rate of 0.3 $\mu\text{L}/\text{min}$ over a period of 5 days no changes were observed in dialysate glucose levels or relative glucose recovery. These data suggest that the measurements are not affected by inflammation around the catheter when using a microdialysis flow rate of 0.3 $\mu\text{L}/\text{min}$. The actual glucose concentration can be measured using a flow rate of 0.3 $\mu\text{L}/\text{min}$ without any calibration adjustments that would be needed for 2 $\mu\text{L}/\text{min}$. Using a flow rate of 0.3 $\mu\text{L}/\text{min}$ the dialysate glucose measured by microdialysis catheters which had been rinsed prior to the insertion were significantly higher compared to the levels measured by dry catheters. We recommend that the low flow rate should be used in a microdialysis-based glucose sensor (II, III). In fasting and hyperglycemic state the dialysate and capillary glucose levels correlated in healthy subjects and patients with type 1 DM but not in patients with type 2 DM. The correlation increased over time with the highest correlation being observed at the last measurements in all three groups. This indicates stronger correlation over time between capillary and interstitial glucose in type 2 DM (IV).

Metabolic studies

Using a low flow rate (0.3 $\mu\text{L}/\text{min}$) the true concentrations of interstitial metabolites can be determined. We use the term interstitial (instead of dialysate) metabolite in our metabolic studies. Our studies showed that the microdialysis technique can be used for assessment of insulin sensitivity in sc adipose tissue. Moreover, there were significant correlations between insulin sensitivity in sc adipose tissue, whole body and liver (IV). A mixed meal resulted in lower capillary and interstitial glucose as well as lactate levels compared to OGTT. There were no differences in relative glucose recovery after mixed meal compared to OGTT. The microdialysis technique was reliable also for studies after mixed meal on the insulin effect in sc adipose tissue. (V)

In conclusion microdialysis technique can be used for reliable monitoring of glucose and other metabolites in sc abdominal adipose tissue during everyday activity in patients with DM. Pre-rinsing the microdialysis catheter in combination with a low (0.3 $\mu\text{L}/\text{min}$) perfusion flow rate gave similar interstitial and capillary glucose levels during fasting state, after oral glucose test and standardized mixed meal and two hours after meals in healthy subjects and patients with type 1 and type 2 DM. The low flow rate is also recommended for long term (five days) measurements of sc interstitial glucose, since at this flow rate the measurements were not affected by the inflammation around the catheter. Furthermore, the microdialysis technique can be used for studying the effect of insulin on glucose utilization and lipolysis in abdominal sc adipose tissue.

Keywords: Microdialysis, microdialysis flow rate, adipose tissue, glucose, lactate, pyruvate, glycerol, insulin, IGFBP-1, OGTT.

LIST OF PUBLICATIONS

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

- I. N. Rajamand Ekberg, N. Wisniewski, K. Brismar, U. Ungerstedt.
Measurement of Glucose and Metabolites in Subcutaneous Adipose Tissue during Hyperglycemia with Microdialysis at Various Perfusion Flow Rates.
In press in Clinica Chimica Acta.
- II. N. Wisniewski, N. Rajamand, Ulf Adamson, Per Eric Lins M. Reichert, B. Klitzman, and U. Ungerstedt.
Analyte Flux through Chronically Implanted Subcutaneous Polymeric Membranes Differs in Humans and Rats.
Am J Physiol Endocrinol Metab. 2002 Jun;282(6):E1316-23.
- III. N. Rajamand Ekberg, N. Wisniewski, U. Adamson, M-A Hedblad, U. Ungerstedt and K. Brismar.
Analyte Flux through Chronically Implanted Subcutaneous Microdialysis Catheters in Patients with Diabetes Mellitus.
Submitted for publication.
- IV. N. Rajamand, U. Ungerstedt and K. Brismar.
Subcutaneous Microdialysis before and after an Oral Glucose Tolerance Test: A Method to Determine Insulin Resistance in the Subcutaneous Adipose Tissue in Diabetes Mellitus. In press in Diabetes, Obesity & Metabolism.
- V. N. Rajamand, L Gave and K. Brismar
Comparison between oral glucose tolerance test and a standardized meal with regards to glucose metabolism in subcutaneous adipose tissue in healthy subject.
Manuscript.

CONTENTS

1	Abstract.....	4
2	List of abbreviations.....	7
3	INTRODUCTION	9
	3.1 <i>Basic principles of microdialysis</i>	10
	3.2 <i>Long term sampling</i>	13
	3.3 <i>Microdialysis in subcutaneous adipose tissue</i>	13
	3.4 <i>Microdialysis technique a tool for investigating tissue metabolism</i>	14
4	THE AIMS	17
5	MATERIALS	18
6	METHODS.....	19
	6.1 <i>Study designs</i>	19
	6.2 <i>The microdialysis material used in this thesis</i>	21
	6.3 <i>Analytical methods used in this thesis:</i>	21
	6.4 <i>Statistical analysis</i>	23
7	RESULTS.....	24
	7.1 <i>Methodological studies</i>	24
	7.1.1 <i>Study I</i>	24
	7.1.2 <i>Study II and III</i>	24
	7.1.3 <i>Study IV</i>	27
	7.2 <i>Metabolic studies:</i>	29
	7.2.1 <i>Study IV</i>	30
	7.2.2 <i>Study V</i>	35
8	GENERAL DISCUSSION	37
	8.1 <i>Methodological studies</i>	37
	8.2 <i>Metabolic studies</i>	39
9	THE MAIN FINDINGS	42
10	Acknowledgements	43
11	References	45

2 LIST OF ABBREVIATIONS

AUC-glucose	Area under the curve for C-glucose 0-2 hours after OGTT
AUC-ins	Area under the curve for serum insulin 0-2 hours after OGTT
BMI	Body mass index
C-glucose	Capillary glucose
CV	Coefficients of variance
DM	Diabetes mellitus
Ed	Transport of glucose through the membrane
EM	Electron microscopy
H	Hour
HOMA	Homeostasis model assessment
IGF-I	Insulin-like growth factor-I
IGFBP-1	Insulin-like growth factor binding protein-1
OGTT	Oral glucose tolerance test
OHA	Oral hypoglycemic agents
Min	Minute
mM	mmol/L
MM	Mixed meal
RR	Relative recovery
S	Second
SD	Standard deviation
SE	Standard error of the mean
SEM	Standard error of the mean
Sc	Subcutaneous
v.s.	Versus

3 INTRODUCTION

Diabetes mellitus (DM) is characterized by hyperglycemia and dyslipidemia. Elevated glucose levels are associated with late diabetes complications. Diabetes is a major cause of blindness, end stage renal failure and limb amputations. There is a 2 to 4 times increased risk of cardiovascular disease or stroke. The number of diabetic patients in Sweden is estimated to be 350000. There are two types of DM; type 1 DM is caused by the autoimmune destruction of the insulin producing β -cells in the pancreas. Type 2 DM is caused by the dysfunction in postprandial insulin secretion in combination with insulin resistance. Patients with type 2 DM stand for 80-90 % of the diabetic population. A more sedentary life style and changes in food habit cause an epidemic of type 2 DM around the world.

The Stockholm Diabetes Intervention Study (SDIS), Diabetes Control and Complications Trial (DCCT) and UK Prospective Diabetes Study have shown that improved metabolic control delays or prevents the development of long term complications in diabetic patients (Reichard, et al. 1990, Wang, et al. 1993, Wang, et al. 1993, UKPDS 1998). Current guidelines target a HbA1c lower than 6.5 % as a goal for intensified therapy and recommend that self monitoring of blood glucose should be performed at least three or four times a day (Miller, et al. 2000). Glucose fluctuations occurring postprandially, moreover, appear to increase the risk of micro- and macrovascular complications. However, in order to lower their elevated blood glucose levels patients with DM have to perform tight blood glucose control by frequent finger pricking. The motivation for frequent finger pricking is not easy to sustain, possibly because high blood glucose is often not felt by the patients. Moreover, an intensive therapy gives an increased risk for hypoglycaemic events, which may be dangerous for the patients (Keen 1994). To avoid the risk for hypoglycemia many patients target at higher blood glucose levels. A continuous monitoring technique may give a timely signal of hyper- and hypoglycemia and relieve the patients from the task of finger pricking. Reliable continuous glucose monitoring provides more detailed information which is helpful for improving the therapy in diabetic patients.

Continuous access to a vessel is correlated with increased risk for sepsis; therefore attempts have been made to make a glucose sensor that can be placed in other tissues. Subcutaneous adipose tissue is the tissue of choice because of the accessibility and the neglectable risk of serious infections. Different approaches are used for continuous glucose monitoring. One is the use of an implantable sensor containing a surface, which via an oxidizing process gives rise to a signal directly proportional to the amount of glucose molecules around the sensor (CGMS, Medtronic, MINIMED) (Chase, et al. 2001, Ludvigsson, et al. 2003) . Another promising approach for continuous glucose monitoring is the use of microdialysis technique, which makes continuous monitoring of biochemical events in vivo possible through use of a semi-permeable tubular membrane which is placed in the tissue and perfused with a sterile Ringer solution (Ungerstedt 1991). In this approach analytes are transported outside the body, where the sensor is located and the chemical reaction giving raise to a signal is performed. Recently microdialysis-based glucose sensors have been introduced for continuous measurement of subcutaneous (sc) adipose tissue glucose concentration in diabetic patients (Jungheim, et al. 2001).

The microdialysis sampling technique as it is known today were originally used in the neuroscience field for measurement of transmitter substances in rat brain (Ungerstedt, et al. 1974). Microdialysis has been shown to offer information about substances directly at the site of action while being well tolerable and safe. The technique is used in many different fields as well as in pharmacological studies in animals as in studies of tissue metabolism in for example brain, liver, muscle and adipose tissue of human (Lonnroth 1997, Amer 1999, Henriksson 1999, Rooyackers, et al. 2004, Ungerstedt, et al. 2004, Plock, et al. 2005). Until today 9760 articles using microdialysis technique for studies of unbound tissue concentrations of both endogenous and exogenous substances have been published (Medline search word “microdialysis”).

3.1 BASIC PRINCIPLES OF MICRODIALYSIS

A microdialysis system consists of the microdialysis pump, the microdialysis catheter probe and a microvial in which the sample is collected. During the process of microdialysis the catheter is inserted into the tissue or other materials (e.g. fluid or plants). The microdialysis catheter consists of a thin dialysis tube with an inner diameter of 0.15 – 0.3 mm and a semi-permeable membrane at the proximal end of the catheter (figure 1). As shown in figure 1 a perfusion fluid enters the catheter through the inlet tubing at a constant flow rate (0.1-5 $\mu\text{l}/\text{min}$), passes the membrane and is then transported through the outlet tubings and collected in a microvial. The direction of the diffusion process is dependent on the concentration gradient. While the perfusion fluid passes the membrane, molecules up to a certain molar mass diffuse into (recovery) or out of (delivery) the perfusion fluid. In general the perfusion fluid is an aqueous solution that mimics the composition of the surrounding medium. Usually the perfusion fluid lacks the substances of interest for example glucose or neurotransmitters. The perfusion fluid after passing the semi-permeable membrane contains the substances of interest and is referred to as dialysate. The ratio between the concentration in the dialysate and the concentration in the pericatheter fluid is termed relative recovery (RR) and is discussed later.

Microdialysis catheters: Catheters used for microdialysis experiments differ in their shape and material, depending on where they are going to be used during the experiment. Different catheter types have their own specific molecular weight cut off determined by the pore size of the probe membrane and materials (Jansson, et al. 1994, Hagstrom-Toft, et al. 1997, Rosdahl, et al. 1998). Because the molecular cut off usually ranges between 6 – 100 kDa the catheter is impermeable to large molecules, e.g. proteins. A protein free sample allows direct determination of the sample without the need for time-consuming and elaborating preparations.

The recovery: The concentration of a substance in the dialysate is referred to as the recovery of that substance. The recovery is either an absolute or a relative recovery.

Absolute recovery: Absolute recovery of a substance refers to the amount of molecules transported away from the tissue by the perfusion fluid. The absolute recovery is used commonly in pharmacological studies in animal brain, when neurotransmitter substance release is studied. In these set up a very short membrane (1 mm) is used in combination with a high flow rate ($> 0.3 \mu\text{L}/\text{min}$), which enables to detect small changes in the fluctuation of

transmitter substances. The absolute recovery increases with increased perfusion flow rate in contrast to relative recovery (Hernandez, et al. 1986).

The relative recovery: The equilibration between the interstitial fluid and the perfusion fluid is referred to as relative recovery (RR). This term is describing the concentration of a substance in the dialysate compared to the actual concentration of the substance pericatheter. In an in-vitro set up the relative recovery can easily be calculated by

$$RR = (C_{\text{dialysate}}/C_{\text{outside the membrane}})$$

The relative recovery is usually converted to a percentage. In contrast to in-vitro studies the pericatheter (e.g. interstitial) concentration of substances like for example glucose is not known in in-vivo studies. Usually the RR is calculated as $C_{\text{dialysate}}/C_{\text{blood}}$. The relative recovery is converted to percent.

The relative recovery is dependent on the

I. Velocity of the diffusion process which depends on:

- Temperature
- Weight cut-off and membrane area
- Concentration gradient

II. Composition of perfusate

III. The length of the membrane

IV. Tortuosity of the sample matrix

V. Flow rate

I. Velocity. As given by Stokes-Einstein equation the relative recovery for different molecules can differ according to their diffusion capacity, which is dependent of temperature and the particle radius. Therefore, the entire microdialysis experiment has to be carried out at a constant, preferably body temperature (Benveniste 1989).

II. The composition of perfusate determines if a substance will diffuse into or out of the membrane as described previously.

III. The length of the membrane. According to the Fick's Law of diffusion the rate of perfusion across a membrane is proportional to its area. Therefore increasing the length and thus the area of microdialysis membrane will lead to an increase in relative recovery (Plock, et al. 2005). For studying metabolism in the subcutaneous tissue a membrane length of 30 mm is desirable (Bolinder, et al. 1992).

IV. Tortuosity. Comparing in-vitro - and in-vivo experiments the relative recovery is reduced in the later one because of tortuosity of the sample material. Tortuosity is the sum of increase diffusion length caused by cellular structures and the connectivity of the space (Benveniste, et al. 1989, Nicholson, et al. 1998, Hoistad, et al. 2002). This is an important fact when it comes to determination of relative recovery because it shows that a determination in-vitro will usually differ from the in-vivo one as (so far) the exact simulation of in-vivo conditions is impossible.

V. Flow rate. It is documented that the relative recovery increases non-linearly when perfusion flow rate is decreased (Alexander, et al. 1988, Benveniste 1989). At very low perfusion flow rate, for example flow rate $\leq 0.3 \mu\text{L}/\text{min}$ passing a 30-mm membrane, the relative recovery approaches 100% (Bungay, et al. 1990, Bolinder, et al. 1992, Rosdahl, et al. 1998). Low perfusion flow rate are limited by the small sample volume, which increases the sample collection interval and consequently results in worse time resolution. A higher flow rate provides a better time resolution and changes in concentrations are detected sooner.

However, the relative recovery will never reach 100% when using higher flow rates ($> 0.5 \mu\text{L}/\text{min}$). When using higher non-equilibration flow rates the microdialysis catheter has to be calibrated before drawing any conclusions about the pericatheter fluid concentrations.

Calibration: There are many different methods for calibration and thereby determination of relative recovery. In *method of flow rate variation* introduced in 1985 the calibration is accomplished by varying the perfusion flow rate. Plotting the flow rate against measured concentrations and extrapolating to zero flow will estimate the concentration at zero flow and thus the relative recovery (Jacobson, et al. 1985). The disadvantages of this method are the long procedure time and that the analyst is only able to estimate the actual recovery without being able to determine the actual concentration. Therefore Rosdahl et al determined the actual concentration of analytes by subsequently decreasing the flow rate until further decreased in flow rate did not result in an increased RR (Rosdahl, et al. 1998). The no net flux method developed by Lönnroth et al makes use of different perfusate concentrations (Lönnroth, et al. 1987). In this calibration method the compound of interest is added to the perfusate in different known concentrations. If the concentration in the perfusate ($C_{\text{perfusate}}$) lies below the concentration in the pericatheter fluid (e.g. interstitial fluid) ($C_{\text{interstitial}}$) the analytes diffuses into the perfusate, resulting in higher dialysate concentration ($C_{\text{dialysate}}$) than that of the perfusate. If the $C_{\text{perfusate}} > C_{\text{interstitial}}$ the analytes will diffuse out of the membrane and $C_{\text{dialysate}}$ will be $< C_{\text{perfusate}}$. If the $C_{\text{perfusate}} = C_{\text{interstitial}}$ no changes in $C_{\text{dialysate}}$ is measured. The loss or gain measured in dialysate ($C_{\text{dialysate}} - C_{\text{perfusate}}$) is plotted against the different perfusate concentrations. The intercepts of the abscissa will provide the concentration in the pericatheter fluid. In-vivo catheter recovery is determined by the slope of the regression line. The disadvantage might be that the method is very time consuming and can only be used under steady state conditions.

All the calibration techniques mentioned previously are time consuming and not suitable for clinical studies. Therefore *retrodialysis / internal reference technique* has been developed (Ståhle, et al. 1991, Jansson, et al. 1994, Lönnroth, et al. 1995). It operates by using a perfusate spiked with the analyte in a known concentration. As the diffusion process is assumed to be equal in both directions the substance loss through the membrane is the same as its in vivo recovery. Another variation of the retrodialysis is the use of an internal standard to determine in vivo relative recovery (Larsson 1991). The standard is added to the perfusate during the experiment. Afterwards the concentrations both of the analyte and the standard are determined. The internal standard should match the characteristics of the analyte and therefore its loss through the microdialysis catheter predicts the relative recovery of the analyte, the ratio between measured values gives the recovery of the analyte. Another variation of this method is the *external reference technique* (Jansson, et al. 1993) where authors assume that the ratio for the standard and the analyte recoveries in-vitro equals the ratio in-vivo. Knowing the recovery for the standard in-vivo the recovery for analyte can be calculated. However, one should bear in mind that even if the in-vitro recoveries of the internal standard and the analyte are the same this is not imperative for in-vivo recovery.

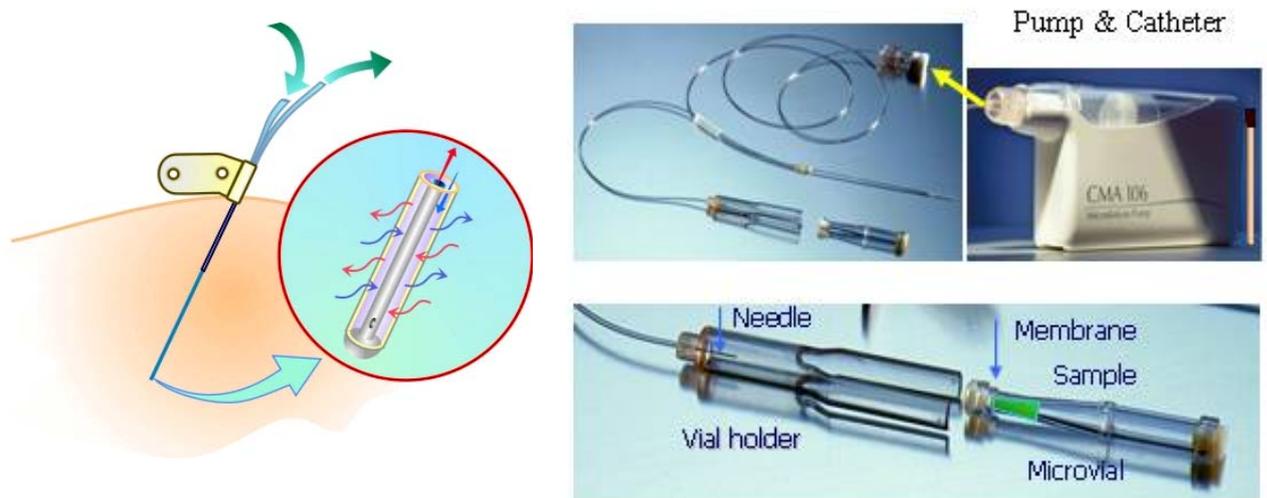


Figure 1. Left. The microdialysis catheter (CMA 60) used in this thesis was placed approximately 1-2 cm subcutaneously in abdomen. Small substances diffuse into or out of the perfusion fluid passing the dialysis membrane. Right. The microperfusion pump, the microdialysis catheter, the vial holder and microvial.

3.2 LONG TERM SAMPLING

Microdialysis has been used to obtain long term (more than 24 hours) profiles of endogenous or exogenous substances in different tissues for example muscle, brain and intestines in human (Edsander-Nord, et al. 2002, Ungerstedt, et al. 2003, Engstrom, et al. 2005). It can be a valuable tool in patients with minimal blood supply, such as neonates (Hildingsson, et al. 1996, Baumeister, et al. 2001).

3.3 MICRODIALYSIS IN SUBCUTANEOUS ADIPOSE TISSUE

Since 1987 microdialysis technique has been used for studies in abdominal subcutaneous adipose tissue (Lönnroth, et al. 1987). Previous studies have shown that that this technique can be used for study of metabolic events in adipose tissue (Lönnroth, et al. 1987). It has been shown the concentrations and the relative kinetic of glucose in interstitial fluid of sc adipose tissue and in blood are similar (Jansson, et al. 1988, Bolinder, et al. 1989). Abdominal subcutaneous adipose tissue is a source of lactate production (Hagstrom, et al. 1990, Jansson, et al. 1990). Being a minimal invasive method which is tolerated well microdialysis technique will in future provide the advantage of combining monitoring of glucose fluctuation with insulin delivery in patients with DM. Recently microdialysis-based glucose sensors have been introduced for continuous measurement of sc adipose tissue glucose concentration in diabetic patients (Jungheim, et al. 2001).

When determining the tissue glucose concentration instead of using blood glucose as basis for diabetes therapy, several issues should be considered. An important question is if the glucose concentrations in these two body compartments are identical / similar and if the same relationship between glucose concentrations is observed during both basal state and during drastic changes. Previous studies on patients with type 1 DM have shown that blood glucose

and interstitial glucose measured by microdialysis are closely parallel (Bolinder, et al. 1993). In a series of experiments Rosdahl et al have shown that changes in blood flow affects the dialysate concentration of glucose, lactate and several amino acids measured with microdialysis at non-equilibration conditions (Rosdahl, et al. 1993). A complete equilibration between interstitial fluid and perfusate is possible if low flow rates ($\leq 0.3 \mu\text{L}/\text{min}$) are used (Rosdahl, et al. 1997, Rosdahl, et al. 1998).

Most of previous studies on the interstitial glucose concentrations and on the glucose kinetic in interstitial fluid compared to blood are performed with non-equilibration flow rate with addition of glucose in the perfusates. Because of the possible increased infection risk it is not recommended to use a glucose containing perfusion fluid in a glucose-sensor which will remain in subcutis in patients for several days. An important issue is which flow rate should be used in a microdialysis-based glucose sensor. Other important issue to be considered is if a microdialysis membrane will function over a period of several days. Jungheim et al have shown that during rapid changes in blood glucose levels, glucose concentrations in alternate sites for example forearm shows a lag time compared to capillary blood samples from the fingertips (Jungheim, et al. 2002, Koschinsky, et al. 2003). There is subsequently a risky delay in detection of hypoglycemia if patients test their glucose concentrations at the alternate sites instead of fingertips. This is referred to as the alternate site testing-like phenomena (Koschinsky, et al. 2001, Jungheim, et al. 2002, Koschinsky, et al. 2003). Different concentrations or a lag time in capillary glucose at the abdomen compared to the levels measured at the fingertips may affect the interstitial glucose levels in subcutis. Therefore capillary glucose from fingertips and abdomen should be compared during drastic changes in glucose.

3.4 MICRODIALYSIS TECHNIQUE A TOOL FOR INVESTIGATING TISSUE METABOLISM

Microdialysis technique is a minimal invasive method suitable for studying tissue metabolism in-vivo (figure 2). Sensors analyzing not only glucose but also other metabolites, if available, would be useful in both research and clinical practice. For example a combined glucose and lactate monitoring technique would, if available, offer the opportunity to investigate tissue glucose utilization or detect a tissue oxygen deficit in time for therapeutic intervention. The analysis of glycerol could be used for studies of lipolysis and the antilipolytic effect of insulin, which may be disturbed in an insulin resistant state.

Insulin resistance is a major predictor and a central feature of type 2 DM and a complication in type 1 DM (Yki-Jarvinen, et al. 1986, Stumvoll, et al. 2001). It is characterized by decreased response to insulin in the insulin sensitive organs, liver, muscle and adipose tissue. Genetic and life-style factors such as low physical activity, obesity, abdominal adiposity, high fat diet, increased levels of free fatty acids and hyperglycemia all contribute to the state of whole body insulin resistance (Yki-Jarvinen 1992, Stumvoll, et al. 2001). Whole body insulin resistance is the sum of tissue specific disruption of insulin action in liver, muscle, adipose tissue, pancreas and brain (DeFronzo, et al. 1982, Stumvoll, et al. 1999, Mauvais-Jarvis, et al. 2000).

Most in vivo studies on insulin sensitivity can be divided into one of two major categories:

I) Whole body insulin sensitivity, mainly reflecting the sensitivity of skeletal muscle, measured by euglycemic hyperinsulinemic clamp (DeFronzo, et al. 1979), by the oral glucose tolerance test (OGTT) (Matsuda, et al. 1999, Stumvoll, et al. 2000) or by homeostasis model assessment ($HOMA = (I * G)/22.5$. Where I is fasting serum insulin and G is fasting blood glucose levels) (Matthews, et al. 1985).

As a measure for post load insulin sensitivity a ratio between the area under the curve for C-glucose 0-2 hours after OGTT (AUC-glucose) and the area under the curve for insulin (AUC-ins) at the same time can be calculated (Albareda, et al. 2000).

II) Hepatic insulin sensitivity diagnosed with isotope techniques or as suggested by determining fasting levels of the hepatic insulin-like growth factor binding protein-1 (IGFBP-1) in relation to insulin levels (Brismar, et al. 1994, Brismar, et al. 1995, Lee, et al. 1997, Rajkumar, et al. 1999, Brismar, et al. 2004). We introduce an index for hepatic insulin resistance (HeIR) using the formula $HeIR = \text{Fasting S-insulin} * \text{fasting S-IGFBP-1}$. This formula is based on the fact that the hepatic protein IGFBP-1 is regulated at transcriptional level by insulin. Fasting IGFBP-1 levels are inversely correlated to insulin and increase in relation to insulin in conditions with hepatic insulin resistance (Brismar, et al. 1995, Hilding, et al. 1995).

Prolonged hyperglycemia, increased free fatty acid concentrations, dyslipidemia, obesity and age are all known factors affecting insulin sensitivity in the body (Stumvoll, et al. 2001). Question may be raised if insulin sensitivity is a stable status or is changing over time in the same patients. To our knowledge there are no studies on the natural course of whole body or tissue insulin sensitivity in patients with DM. Easy methods for investigation of whole body and tissue insulin sensitivity are desirable.

Adipose tissue is an active endocrine organ producing different cytokines and hormones such as leptin and adiponectin. Local production of interleukin -6 in sc tissue has been studied with microdialysis previously (Sopasakis, et al. 2004). Correlations between increasing levels of S-leptin and or low adiponectin concentrations and the insulin resistant state have been shown in previous studies (Trayhurn, et al. 2001, Trujillo, et al. 2005). Recent studies of transgenic animal models have provided evidence that adipose tissue contributes not only to the insulin resistance state but also to the development of hyperglycemia and diabetes (Mauvais-Jarvis, et al. 2000). Adipose tissue is envisioned as an organ with a central role in glucose homeostasis (Stumvoll, et al. 1999). Both glucose utilization and lipolysis in sc adipose tissue are influenced by insulin and therefore glycolysis as well as interstitial glycerol levels may be related to the insulin sensitivity in the tissue (figure 2). Thus tools for studying insulin sensitivity in adipose tissue are of great interest. Such a tool can be of great help when evaluating the effect of new drugs on insulin sensitivity. Furthermore the effect of insulin therapy depends on the insulin sensitivity. A glucose-sensor determining tissue insulin sensitivity if available would be of great help during insulin therapy. Interstitial glycerol concentrations measured using microdialysis technique have been used as an index of lipolysis (Arner, et al. 1988). It has been shown that following glucose ingestion the lactate generation is impaired in obese subjects (Lovejoy, et al. 1990). We suggest that a ratio between interstitial glucose and lactate in abdominal sc adipose tissue in healthy subjects and patients with DM can be used as a measure of glucose utilization.

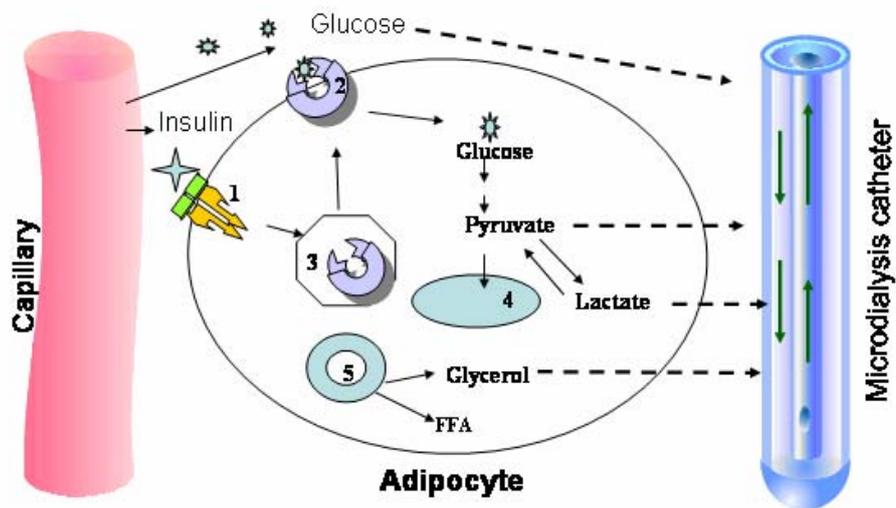


Figure 2. Insulin stimulates GLUT-4 associated glucose transport into the cell and inhibits the lipolysis. Glucose enters the glycolysis and is converted to pyruvate and lactate. 1= Insulin receptor, 2= glucose transporters (GLUT-4), 3= Vesicle containing GLUT-4, 4=mitochondria, 5=Fat drop.

The hyperinsulinemic normoglycemic glucose clamps are suggested as the golden standard method for investigating the insulin sensitivity in whole body, the sum of insulin sensitivity in liver, adipose tissue and muscle. The effect of insulin in liver, adipose tissue and muscle is observed at different S-insulin concentrations. When using hyperinsulinemic clamp one may affect the insulin sensitivity in adipose tissue and liver because in these organs the insulin effect is observed at lower levels compared to muscle. Therefore a two-step insulin clamp is to be preferred. This is on the other hand very time consuming. Recently several studies have shown that a glucose load can be used for studying insulin sensitivity in the body (Stumvoll, et al. 2000).

4 THE AIMS

The general aim of this thesis was to investigate if microdialysis technique can be used for reliable monitoring of glucose and other metabolites (lactate, pyruvate and glycerol) during everyday activity in patients with DM. In view of its simplicity and similarity to an everyday situation we decided to use oral glucose tolerance tests (OGTT) (containing 75 g of glucose dissolved in 3 dl of water) and standardized mixed meal (MM) rather than glucose clamps in our studies.

The specific aims

- To validate the microdialysis flow rate suitable for use in a glucose sensor.
- To investigate if long term applications of a microdialysis catheter in subcutaneous adipose tissue may affect the function of the membrane measured as the relative glucose recovery.
- To investigate the relation between capillary and interstitial glucose in patients with type 1 and type 2 DM and healthy subjects, and to study the feasibility of using microdialysis to assess local insulin sensitivity in adipose tissue.
- To compare OGTT and mixed meal (MM) with regard to insulin induced changes in glycerol, glucose and other metabolites in sc adipose tissue.

5 MATERIALS

A total of 46 healthy subjects, 13 patients with type 1 and 13 patients with type 2 diabetes mellitus and 6 animals (rats) were included in the studies. The animals were male Sprague – Dawley rats with a weight range of 300-400 g (II).

Table 1. The age and BMI range of subjects included in this thesis.

Paper	Subjects	Ratio female/male	Age range (year)	BMI range (kg/m²)	N
I	Healthy subjects	10/2	21 – 25	16 - 23	12
II	Healthy subjects	3/3	24 – 25	19.6 – 26.3	6
III	Type 1 DM,	4/1	40-62	23-31	5
	Type 2 DM	2/3	59-75	26-40	5
	Healthy subjects	2/1	24 – 37	20 – 24	3
IV	Type 1 DM	5/2	37 - 56	20 - 25	7
	Type 2 DM	4/3	46 - 70	21 - 43	7
	Healthy subjects	3/4	38 – 64	23 - 30	7
V	Healthy subjects	4/4	22 - 26	20 - 25	8
Wet vs dry catheters	Healthy subjects wet catheter	6/2	22 -30	19 – 27	8
	Healthy subjects dry catheter	6/3	24 - 32	20 - 30	9
Gender differences	Healthy subjects	7/0	21 -30	19 -25	7
	Healthy subjects	0/7	20 - 27	19 - 26	7
Capillary glucose finger vs. abdomen	Healthy subjects	4/4	20 - 24	19 - 24	8
Glucose clamp	Type 1 DM	1/1	60 and 62	29 and 23	2
	Type 2 DM	2/1	61 - 71	31 - 46	3
Plasma vs. interstitial lactate and glycerol	Type 1 DM	0/3	40 – 60	24 – 28	3
	Type 2 DM	2/1	57 – 70	21 – 44	3
	Healthy subjects	2/1	44 - 57	20 - 27	3

6 METHODS

6.1 STUDY DESIGNS

Paper I.

The subjects arrived at the hospital at 7:30 in the morning after an overnight fast. Four wetted microdialysis catheters were inserted in the sc adipose tissue. Each catheter was connected to a microperfusion pump set at one of four different flow rates (0.3, 1, 2 or 5 $\mu\text{L}/\text{min}$). Samples of dialysate (perfusion fluid after passing through the catheter) were collected every 20 minutes for 260 minutes (4 hours and 20 minutes). At the same time, capillary blood samples were collected from the fingertips for analysis of glucose. After the insertion of microdialysis catheters and before the start of the experiment a 30 minute equilibration period was allowed, consisting of a 6 minute flush at 15 $\mu\text{L}/\text{min}$ and 24 minute perfusion at the four different flow rates. After a baseline period of 60 minutes subjects received an OGTT. No other food or drink was allowed during the test period. To study the direct effect of different flow rates on the detection of changes in dialysate metabolite levels during different glucose concentrations, excluding the effect of tissue diffusion coefficient. Four microdialysis catheters were connected with four micro-perfusion pumps with adjustable flow rates (CMA 107, CMA Microdialysis, Sweden); each pump was pre-set at one of the four different flow rates (0.3, 1, 2 or 5 $\mu\text{L}/\text{min}$). The catheters were placed in a jar containing 100 mL of a control solution with basal concentration of glucose 4.7 – 5.7 mmol/L, lactate 2.9 – 3.5 mmol/L, pyruvate 198-242 $\mu\text{mol}/\text{L}$ and glycerol 198 -242 $\mu\text{mol}/\text{L}$. After a flush period of 30 minutes 20-minute dialysate fractions were collected for 60 minutes. Thereafter the catheters were moved to a second jar containing 100 mL of a solution with higher concentration of metabolites (glucose 14.0 – 17.2 mmol/L, lactate 8.6 – 10.6 mmol/L, pyruvate 594-726 $\mu\text{mol}/\text{L}$ and glycerol 594 – 726 $\mu\text{mol}/\text{L}$). 20-minute dialysate fractions were collected for 80 minutes before the catheters were moved to a third jar containing 100 mL of a solution with low concentration of metabolites (glucose 1.1 – 1.5 mmol/L, lactate 0.7 – 0.9 mmol/L, pyruvate 47 – 63 mmol/L and glycerol 55-75 $\mu\text{mol}/\text{L}$) and 20-minute dialysate fraction were collected for 80 minutes.

Paper II.

Each rat or human received 2 microdialysis catheters that remained implanted for one week. Steady state conditions were confirmed, as there was no net increase or decrease in the concentrations of the four subsequently collected dialysate samples. On day 0 and each subsequent day, four 10-minute dialysate fractions were collected immediately after the equilibration period. Corresponding blood glucose values were collected between dialysate samples 1 and 2 and between samples 3 and 4. Human blood was obtained through standard finger pricks, and rat blood was obtained from the tail. On the eighth day catheters were withdrawn. Samples were taken for sterility verification, histology or scanning electron microscopy (EM). Before catheter insertion on day 0 and each day before collection periods, rats were anesthetized with sodium pentobarbital. On the eighth day after the final dialysate collection period, but before excising probes, rats were killed with an overdose of sodium pentobarbital. No anesthetics were administered to the human subjects at any time.

Paper III.

In patients with type 1 and 2 DM the same protocol as previously described for healthy subjects in study II were used. In addition in three healthy volunteers microdialysis catheters were inserted at both sides of the umbilicus. A microdialysis flow rate of 0.3 $\mu\text{L}/\text{min}$ was used. Since our previous study showed changes during the first four to five days after catheter insertion, the catheters remained in subcutis for five days. Each day before sample collections, a 30-minute equilibration period was allowed consisting of a 6 minute flush at 15 $\mu\text{L}/\text{min}$ and a 24 minute perfusion at 0.3 $\mu\text{L}/\text{min}$. Steady state conditions were confirmed, as there was no net increase or decrease in the concentrations of the three subsequently collected dialysate samples. On insertion day and each subsequent day, three 30-minute dialysate fractions were collected immediately after the equilibration period. Corresponding blood glucose values were collected at the same time. On the fifth day catheters were withdrawn.

In one healthy volunteer (age 59 year, BMI 25 kg/m^2) four microdialysis catheters were inserted on both sides of umbilicus. The same protocol as in study II was followed. The flow rate through two of the catheters on each side of umbilicus was 2 $\mu\text{L}/\text{min}$ and through the other two catheters was 0.3 $\mu\text{L}/\text{min}$. After the last measurement on the seventh day in the healthy volunteer three out of four catheters were explanted in situ in the sc adipose tissue through dissection. One sample of subcutaneous adipose tissue 2 cm away from any microdialysis catheter was also dissected. The fourth catheter was withdrawn. Tissue samples were fixed in 4% buffered formaldehyde, routinely prepared and paraffin embedded. Histopathological examination of sections stained with haematoxylin and eosin was performed.

Paper IV.

Healthy subjects and patients with type 1 and type 2 DM were studied after an overnight fast. No insulin was taken after 21:00 hours the evening before the study. A polyethylene catheter was placed in a forearm vein for blood sampling. Pre-wetted microdialysis catheters were inserted in the peri-umbilical subcutaneous tissue. Microdialysis flow rate was set at 0.3 $\mu\text{L}/\text{min}$. Dialysate samples were collected every 30 minutes during five hours for analysis of glucose, lactate, pyruvate and glycerol. After one hour of base time sampling the subjects received an OGTT. Patients with type 1 DM concomitantly received a rapid acting insulin analogue (8 IU Humalog^R, Lilly) subcutaneously. Capillary blood was sampled every 30 minutes for determination of C-glucose. Venous blood was collected concomitantly for analysis of serum (S) insulin, insulin like growth factor-I (IGF-I) and IGFBP-1.

Two step hyperinsulinemia euglycemic glucose clamp.

After an overnight fast patients with type 1 (n=2) and type 2 DM (n=3) arrived to the hospital at 08:00 in the morning. The subjects were studied in supine position. In each subject two polyethylene catheters were placed in forearm veins one for insulin and glucose infusion (200 mg/ml) and one for blood sampling. The insulin dosage was 0.25 mU/kg body weight and minute during the first 180 minutes of the experiment. Thereafter the insulin dose was increased to 1 mU/kg body weight and minute. Blood glucose concentrations were determined every 10 minutes. The glucose infusion rate was adjusted so the blood glucose levels were constant, 5 mmol/L. Two pre-rinsed microdialysis catheter were inserted in the periumbilical subcutaneous tissue in each subject. The catheters were connected to pumps. A sterile solution was perfused at a rate of 0,3 $\mu\text{L}/\text{min}$. Fractions of dialysate were collected

every 30 minutes. The first fractions were discarded thereafter the fractions were collected for six hours for analysis for the concentrations of glucose, lactate, pyruvate and glycerol. At the same time capillary blood were taken from the fingertips for analysis of capillary blood glucose (B-glucose). Venous blood samples were taken every 30 minutes for analysis of S-insulin, IGFBP-1. For each insulin dose the M-value was calculated during 30 minutes of blood glucose steady state according to the formula ($[\text{Sum of glucose infusion during glucose steady state (mL/h)} * 10/60 \text{ (h)} * 200 \text{ (mg/mL)} / \text{Kg body weight}] / 30 \text{ min}$).

Paper V.

The same protocol as described previously for study IV was used twice in each healthy subject with the exception that after one hour of baseline sampling the subjects received as a drink an OGTT at one occasion and a standardized breakfast (mixed meal) including two cheese sandwiches, a bowl of 200 g yoghurt with 25 g musli, one apple and 2.5 dl of lemonade sweetened with aspartan, at the second occasion. The interval between the two occasions was 3 to 60 days. The standardized breakfast was composed of 17% proteins, 25 % fat and 58 % carbohydrates. The total amount of calories in the MM was 489 kCal.

6.2 THE MICRODIALYSIS MATERIAL USED IN THIS THESIS

In all studies the microdialysis catheter consisted of a 30-mm-long dialysis membrane (polyamide with a 20,000 molecular weight cut-off) with an outer diameter of 0.60-mm (CMA/60; CMA/Microdialysis AB, Solna, Sweden). The microdialysis catheters were connected to sterile syringes, which were placed in a microinfusion pump (CMA/107; CMA/Microdialysis AB, Solna, Sweden). Sterile Ringer's solution (Perfusion fluid, CMA Microdialysis AB, Sweden) was perfused through the microdialysis catheters. The dialysate (the perfusion fluid after passing through the dialysis membrane) was collected in microvials (small sealed holders).

6.3 ANALYTICAL METHODS USED IN THIS THESIS:

- Capillary glucose concentrations were determined using blood glucose monitors (Glucotrend, Roche Diagnostic, Mannheim, Germany or Freestyle, Abbott Laboratories, Illinois, U.S.A).

- The concentrations of interstitial glucose, lactate, pyruvate and glycerol were measured using a CMA 600 analyzer (CMA Microdialysis AB, Stockholm, Sweden). CMA 600 Microdialysis Analyzer is an instrument designed for rapid analysis of the small volumes as in microdialysis samples, using a kinetic measurement, by enzymatic photometric assay using peroxidase methodology modified after Lloyd et al (Lloyd, et al. 1978). The instrument uses a high precision pipeting device for handling the small sample and reagent volumes and a filter photometer equipped with a mercury lamp for measuring the absorbance of the test solution in a capillary flow cell. During operation, the instrument begins with aspiration of the sample, typically 0.5 μl . The reagent (typically 15 μl) is then aspirated in the same tubing. Thereafter the sample and reagent are moved to the flow cell. During the passage to the flow cell the sample is mixed with the reagent and the reaction starts. When the test solution has been placed in the flow cell the photometer reads the absorbance during 30 seconds, extracting the maximal absorbance change per second from the generated absorbance versus time curve. After the measurement is complete the test solution is dispensed to waste and the

flow system is rinsed. The complete measurement cycle takes between 60 and 120 seconds, depending on analyte. Glucose, lactate and glycerol are assayed using Trinder type reagents using glucose oxidase, L-lactate oxidase and glycerolphosphate oxidase, respectively. Glycerol is first converted to glycerolphosphate in a reaction catalysed by glycerol kinase. The formation of the quinoneimin reaction product is measured at 546 nm (Barham, et al. 1972, Foster, et al. 1978, Shimojo, et al. 1989) and (H.F. Kühnle et al., J. Chem. Clin. Biochem. 15(1977)171). Urea is hydrolysed using urease. The liberated ammonium ions react with 2-oxoglutarate and NADH to produce glutamate and NAD⁺ in a reaction catalysed by glutamate dehydrogenase. The decrease of NADH is monitored at 365 nm (Kassirer 1971, Young, et al. 1975) and (N. W. Teitz, Fundamentals of Clinical Chemistry W. B. Saunders Company, 1970 Philadelphia).

- Plasma glycerol concentrations were determined using an automatic luminometric kinetic assay.
- Plasma lactate levels were measured by an enzymatic method.
- Serum IGFBP-1 concentrations were determined by RIA as described by Povia et al (Povia G 1984). The intra- and inter-assay coefficients of variation (CV) were 3 and 11%, respectively, and the detection limit was 3.0 µg/l. Cross-reactivity with IGFBP-2 and IGFBP-3 was less than 0.5 and 0.05%, respectively.
- Serum concentration of IGF-I was determined using RIA after separation of IGFs from IGFBPs by acid-ethanol extraction and cryoprecipitation. To minimize interference of remaining IGFBPs, des 1-3 IGF-I was used as the radioligand (Bang Peter 1991). The recovery of unlabelled IGF-I was 95% and the intra- and inter-assay CV were 5 and 11%, respectively. The detection limit was 8 µg/L. The standard deviation was calculated according to IGF-I SD score = $[(^{10} \log \text{IGF-I}_{\text{observed}} + 0.00693 * \text{age}) - 2.581] / 0.120$ (Hilding, et al. 1995).
- Serum insulin was measured using RIA (Pharmacia & Upjohn Diagnostic AB, Uppsala, Sweden). The detection limit was < 2 mU/L and within assay CV was 5.8 for a mean insulin value of 11.6 mU/l and 5.7 for mean value 65.2 mU/L. In paper V and in the two-step insulin clamp study insulin was determined with ELISA method (K6219, DakoCytomation, Diagnostika, Falkenberg). The intra and inter assay CVs are shown in table 2.

Value (µU/mL)	Intra assay CV (%)	Value (µU/mL)	Inter assay CV (%)
6.5	7.5	6.7	9.3
44.8	7.5	47.8	8.9
206	5.1	206	4.2

Table 2. The intra and inter assay variations for ELISA analysis. The values are based on 20 assays.

- Serum adiponectin was analyzed using RIA (HADP-61-HK, Linco, Diagnostika, Falkenberg). The intra and inter assay variations were 3.59% and 9.25% for a value of 1.5 mg/L, 6.21% and 6.9% for a value of 3 mg/L and 1.78% and 9.25% for a value of 7.5 mg/L.
- Serum leptin was analyzed using RIA (Human leptin RIA kit. HL/81K, Linco, Diagnostika, Falkenberg). The intra and inter assay CVs are shown in table 3.

Value (µg/L)	Intra assay CV (%)	Inter assay CV (%)
4.9	8.3	6.2
7.2	4.6	5.0
10.4	3.9	4.7
15.7	4.7	3.0
25.6	3.4	3.4

Table 3. The intra and inter assay CV for leptin RIA analysis. The values are based on five duplicates in five assays.

6.4 STATISTICAL ANALYSIS

Data are expressed as mean \pm SEM or SD. Significance for multiple samples were tested using repeated measure ANOVA. Mann-Whitney U test was used for testing the difference between the groups at specific time points. Wilcoxon matched pair test was used for testing the difference between values in the same group. To study the correlation between two variables, regression analysis was performed. Correlations between capillary and interstitial glucose were tested using paired sample correlations. When multiple correlations were tested the Bonferroni corrected p-value was used. A p-value of <0.05 was considered significant. In paper II the differences between rat and human metabolite levels were tested for significance using t-tests comparing data from each day. $P < 0.05$ was considered significant. Significance of each metabolite trend over time in both individual species was tested using repeated measures analysis of variance (ANOVA) with a Student-Newman-Keuls Multiple Comparisons Post Test with significance attributed to $p < 0.05$.

7 RESULTS

7.1 METHODOLOGICAL STUDIES

7.1.1 Study I

At the fasting state in healthy subjects despite a steady state situation in blood glucose levels we observed decreasing dialysate glucose concentrations measured using higher flow rates ($> 0.3 \mu\text{L}/\text{min}$). After the glucose load the relative increase in glucose levels measured by higher flow rates ($> 1 \mu\text{L}/\text{min}$) were significantly higher compared to capillary blood glucose and dialysate glucose measured by $0.3 \mu\text{L}/\text{min}$ (figure 3). This difference was not observed in the in-vitro study.

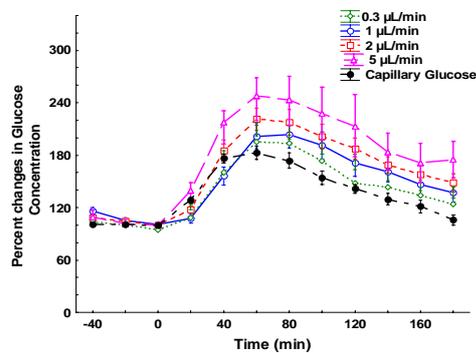


Figure 3. The variations in concentrations of capillary and dialysate glucose after OGTT, measured by microdialysis using flow rate 0.3 (diamonds), 1 (circles), 2 (squares) and 5 $\mu\text{L}/\text{min}$ (triangles), expressed as percentage of basal fasting levels at time 0. The dialysate glucose curve for 0.3 $\mu\text{L}/\text{min}$ is shifted 20 minutes to the left.

7.1.2 Study II and III

Using a microdialysis flow rate of 2 $\mu\text{L}/\text{min}$ in abdominal sc tissue of healthy subjects ($n=6$) and patients with type 1 ($n=5$) and type 2 DM ($n=5$) we observed that the dialysate glucose levels, the relative glucose recovery and dialysate urea concentrations increased during five days after catheter insertion and were stable thereafter (Figure 4). The ratio dialysate lactate / pyruvate was stable over the study period. In contrast to humans in rats the dialysate glucose concentrations and the relative glucose recovery decreased and the ratio lactate / pyruvate increased over the study period. Rat catheter histology sample showed an increased cellular density and fewer blood vessels around the microdialysis catheter. Human catheter histology sample showed increased amount of inflammatory cells and dilated small capillaries close to the microdialysis membrane in the sc adipose tissue. Using a microdialysis flow rate of 0.3 $\mu\text{L}/\text{min}$ during five days we observed stable dialysate glucose concentrations and relative glucose recovery in subcutis in healthy subjects (Figure 5).

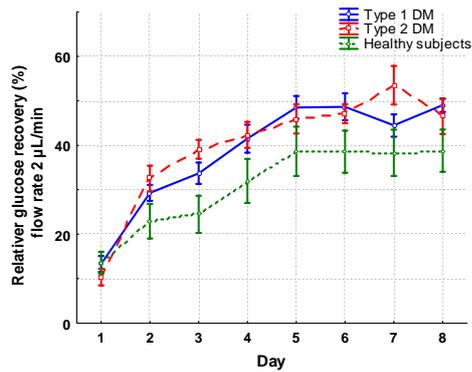


Figure 4. Mean \pm SEM for relative glucose recovery, calculated as (dialysate glucose / capillary glucose) *100, for flow rate 2 μ L/min in patients with type 1 (diamonds, n = 5) and type 2 DM (squares, n = 5) and healthy subjects (triangles, n = 6) over one week. The value for each day in each patient is the mean of four subsequent samples during one hour.

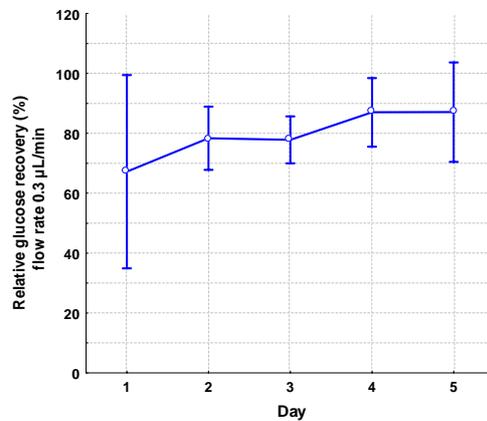


Figure 5. The mean \pm SD for relative glucose recovery measured by flow rate 0.3 μ L/min in healthy subjects (n = 3). The value for each day in each subject is the mean of three subsequent samples during 90 minutes.

As shown in figure 4 the relative glucose recovery increased significantly 24 hours after the catheter insertion. We hypothesize that part of this increase may be due to air being trapped in the membrane of catheter affecting the diffusion of water soluble substances through the membrane. Consequently rinsing the membrane before the insertion could improve the relative glucose recovery. Thus we have compared the concentration of metabolites measured by microdialysis using dry catheters (n=9 catheters in 9 subjects) with the corresponding concentrations measured using pre-wetted catheters (n = 8 in 8 healthy subjects). The same protocol as described previously for study IV was used.

There were no differences between the groups with regards to age, BMI or capillary glucose (figure 6a), see table 1 for BMI and age. The dialysate glucose and pyruvate in the group using pre-rinsed catheters were significantly higher ($p=0.042$ and $p < 0.001$ for dialysate glucose and pyruvate, respectively) compared to the group using dry catheters (figures 6b and c). The dialysate lactate and relative glucose recovery were higher in the group using pre-rinsed catheter but the differences ($p = 0.050$ for lactate and $p = 0.056$ for glucose RR) did not reach statistical significance (figure 6d and e). There were no significant differences between the dialysate glycerol levels between the two groups.

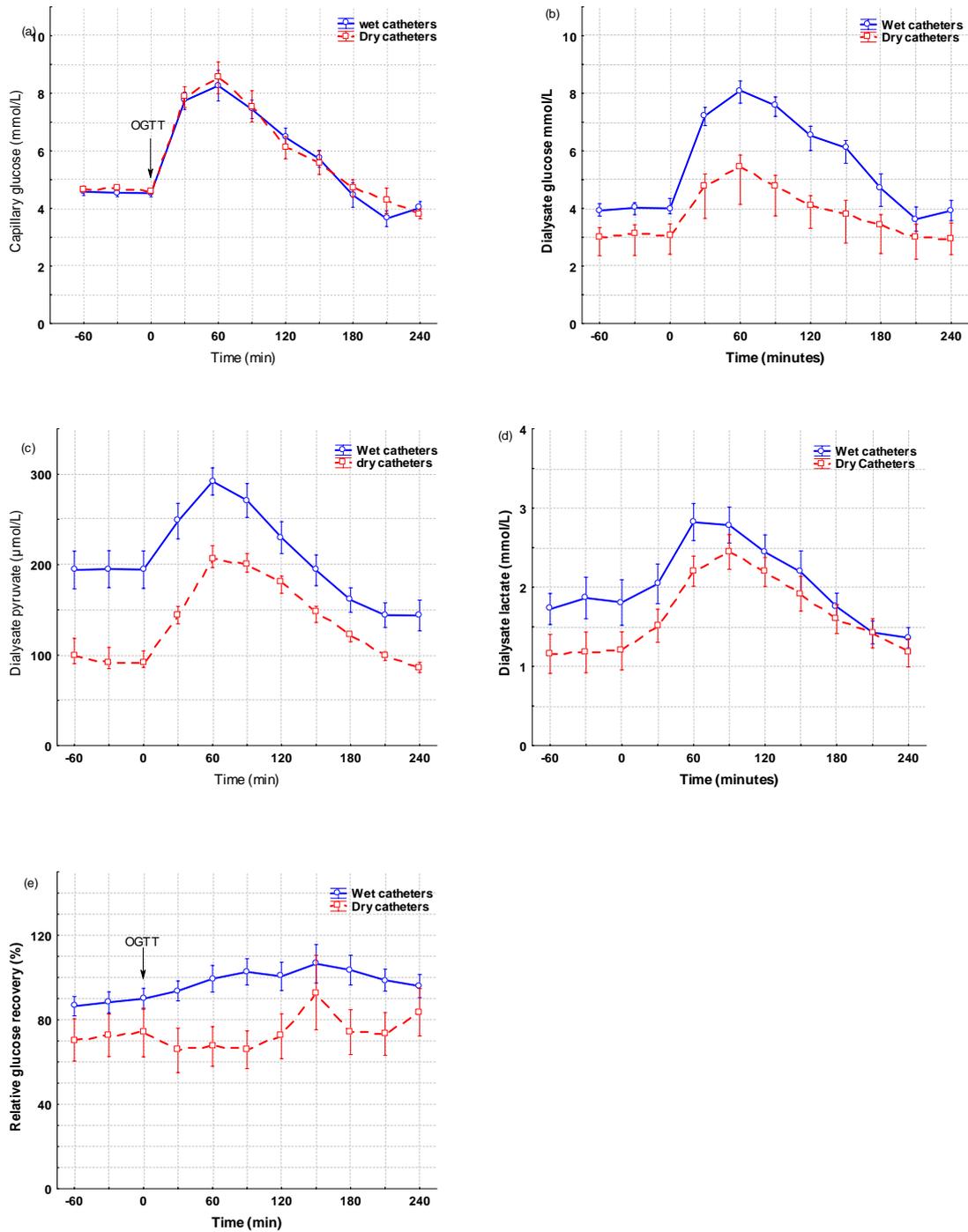


Figure 6. The mean \pm SEM of capillary glucose (a), dialysate glucose (b), pyruvate (c), lactate (d) and relative glucose recovery (e) in sc adipose tissue in healthy subjects measured using dry or pre-rinsed microdialysis catheters.

7.1.3 Study IV

Before and during the OGTT the capillary and interstitial glucose changed in parallel in healthy subject and in patients with type 1 and type 2 DM (figure 7 a-c). The correlation coefficient between capillary and interstitial glucose ranged from 0.94 ($p= 0.002$) at the fasting state to 0.99 ($p< 0.001$) post OGTT in type 1 DM, from 0.35 ($p= 0.437$) at the fasting state to 0.77 ($p= 0.071$) after OGTT in type 2 DM and from 0.78($p= 0.040$) at the fasting state to 0.97 ($p < 0.001$) post OGTT in healthy subjects. In all three groups the correlation coefficient increased over the study time with the highest correlation coefficient being observed at the last measurements. The relative glucose recovery at baseline was 98 ± 4 , 87 ± 8 and 92 ± 8 % for type 1 DM, type 2 DM and healthy subjects, respectively. Similar results for relative glucose recovery were observed in young healthy subjects (V).

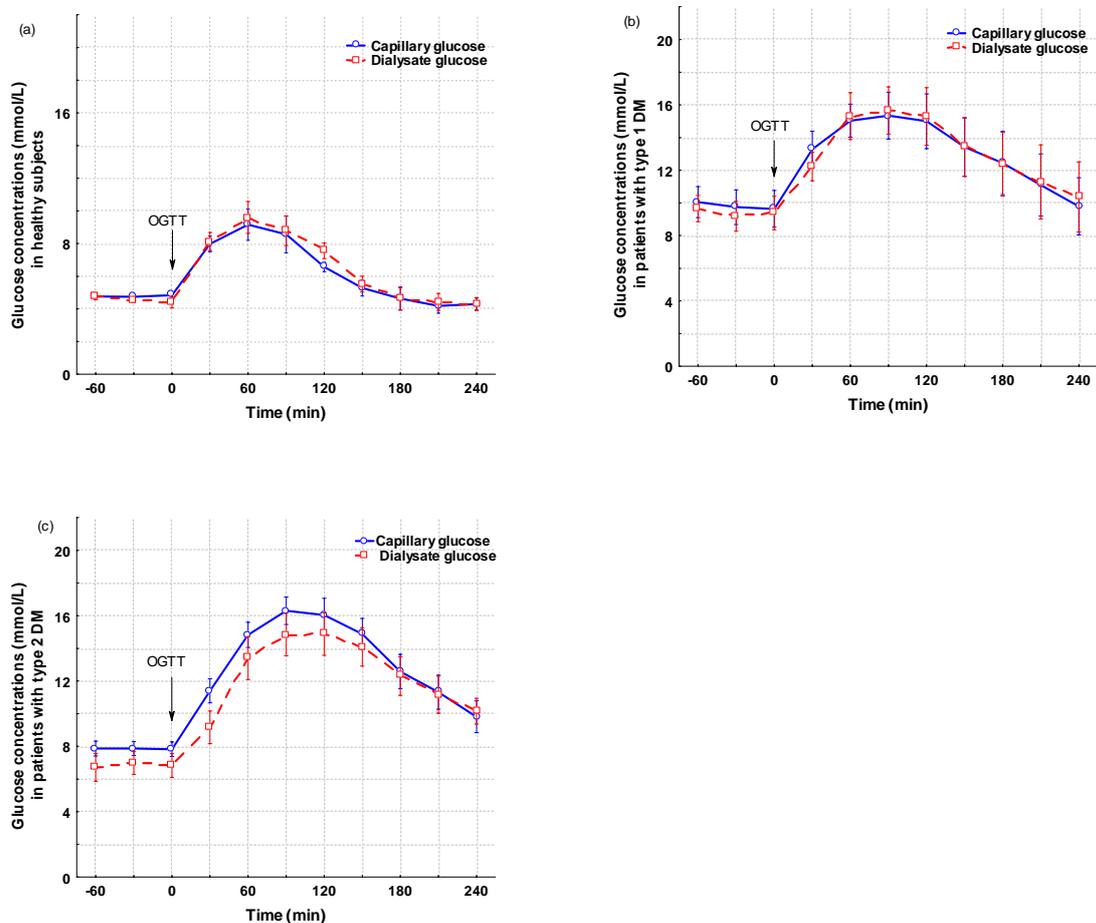


Figure 7 a-c. The mean \pm SEM of capillary (circles) and dialysate (squares) glucose concentrations measured using flow rate $0.3 \mu\text{L}/\text{min}$ in abdominal subcutaneous adipose tissue before and after an OGTT in healthy subjects (a), patients with type 1 (b) and type 2 DM (c).

We compared glucose concentrations in capillary blood samples from the fingertips with capillary blood from abdomen in eight healthy young subjects (4 female and 4 male subjects) before and during an OGTT containing 75 g glucose. The subjects were studied after an overnight fast in supine position. As shown in figure 8 no differences between capillary glucose concentrations from abdomen and finger were observed.

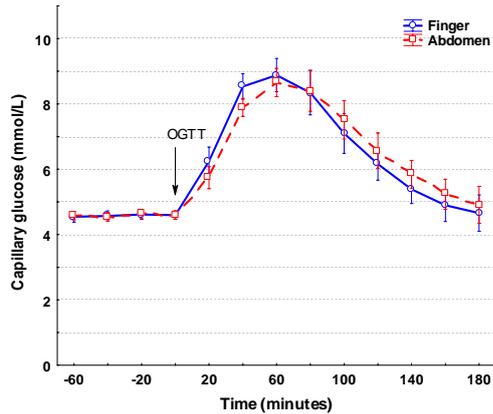


Figure 8. The mean \pm SEM of capillary glucose concentrations tested at fingertips (circles) and at abdomen (squares) in healthy subjects

When measuring interstitial glycerol and lactate concentrations it is important to be certain that the majority of the metabolites are produced locally and not brought to the tissue by blood. We, therefore compared the plasma glycerol and lactate levels with those measured locally in interstitial fluid of subcutis with microdialysis technique in healthy subjects (n = 3), patients with type 1 (n = 3) and type 2 DM (n = 3).

In healthy subjects and patients with type 2 DM the interstitial glycerol levels were significantly higher compared to plasma levels ($p = 0.021$, healthy subjects and $p = 0.001$ type 2 DM, respectively) (Figure 9a and c). In type 1 DM there was a difference in glycerol levels over time ($p = 0.020$) (Figure 9b). But the plasma and interstitial glycerol levels were not significantly different during 180 minutes (-60 to 120 min) ($p = 0.076$). An interaction between plasma/interstitial levels and time was however observed indicating differences during part of the experiment.

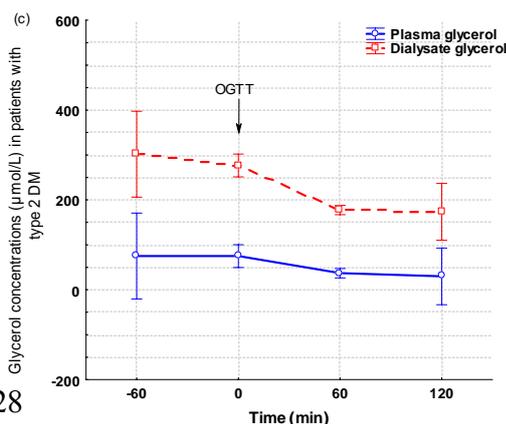
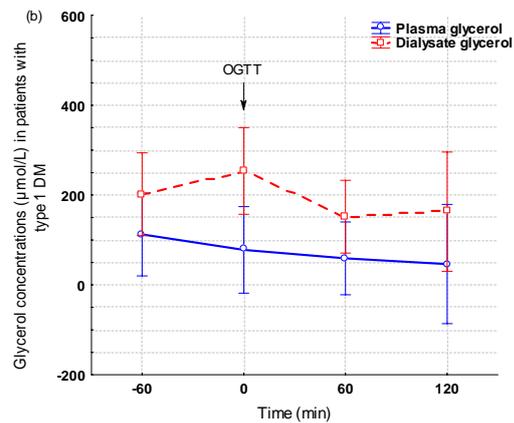
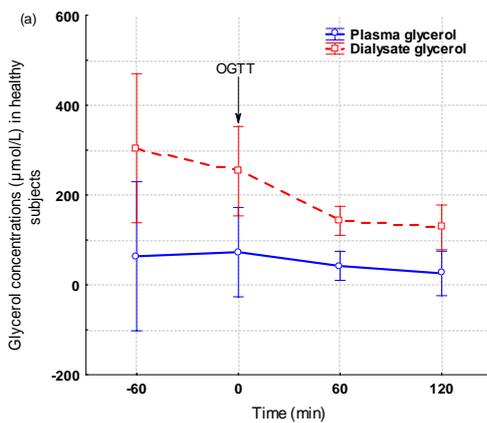


Figure 9 a-c. Mean \pm SD of plasma (circles) and dialysate (squares) glycerol concentrations measured with flow rate 0.3 μ L/min in abdominal sc tissue before and during an OGTT in healthy subjects (a), patients with type 1 (b) and type 2 DM (c).

In healthy subjects there was a difference in lactate concentrations over time ($p < 0.001$). But no differences in plasma and interstitial lactate concentrations during the whole experiment (-60 to 210 min) ($p = 0.099$). An interaction between plasma/interstitial levels and time was however observed indicating difference during part of the experiment (figure 10a). Interstitial lactate levels were significantly higher compared to the concentrations measured in plasma in patients with type 2 DM ($p = 0.029$) (figure 10c). There was no significant difference between interstitial and plasma lactate levels in patients with type 1 DM (figure 10b).

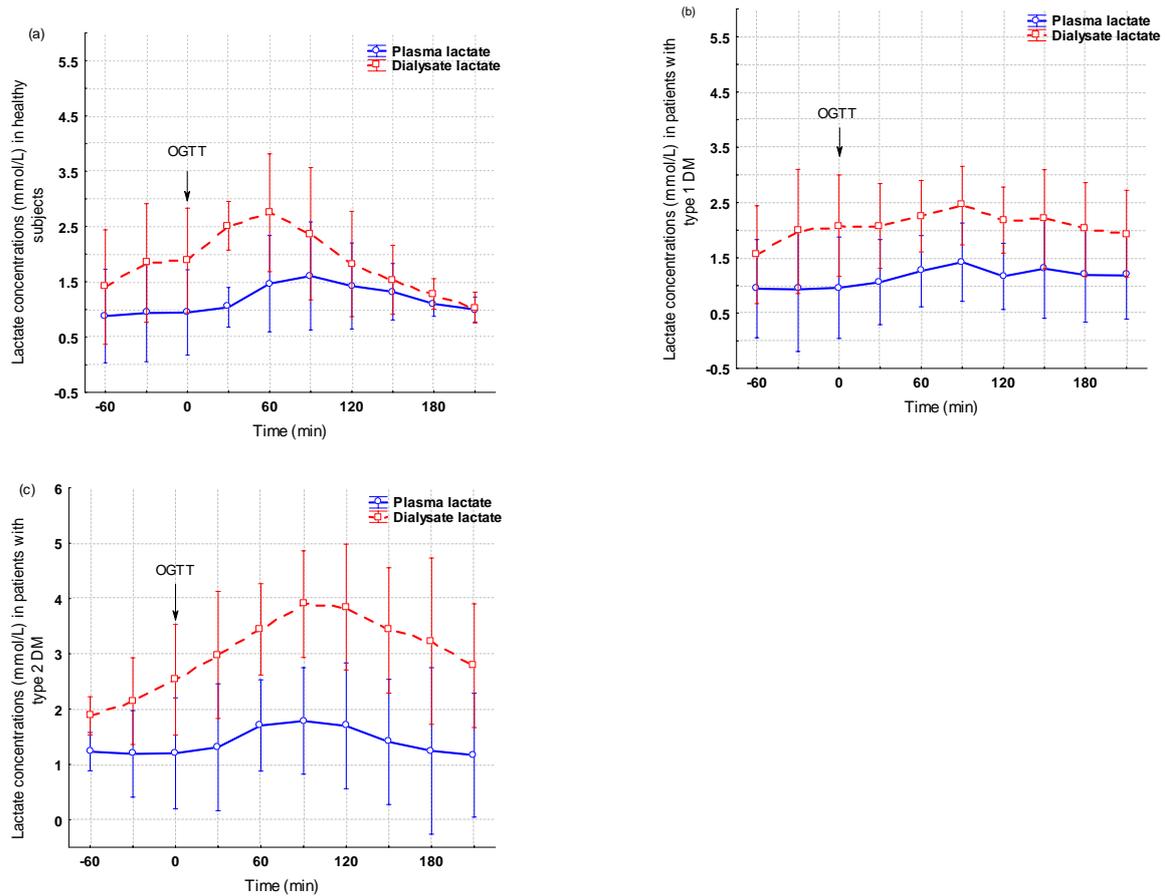


Fig 10 a-c. Mean \pm SD of plasma (Circles) and dialysate (squares) lactate concentrations measured with flow rate 0.3 μ L/min in abdominal sc tissue before and during an OGTT in healthy subjects (a), patients with type 1 (b) and type 2 DM (c).

7.2 METABOLIC STUDIES:

In the metabolic studies the microdialysis flow rate 0.3 μ L/min was used. As we assume that the concentrations of substances in dialysate are the same as in the interstitial fluid we use the term interstitial (instead of dialysate) glucose, lactate, pyruvate and glycerol. At a low flow rate, such as 0.3 μ L/min, there is a delay before any changes can be detected in the interstitial fluid since the dialysate needs time to reach the vials. In the figures showing interstitial concentrations of glucose, lactate, pyruvate and glycerol we have therefore shifted the curves 30 minutes to the left to compensate for the known delay.

7.2.1 Study IV

In 21 participants (7 healthy subjects, 7 type 1 and 7 type 2 DM) we compared insulin sensitivity in whole body, in sc adipose tissue and in the liver.

Measures of whole body insulin sensitivity: Fasting whole body insulin sensitivity was determined according to HOMA. The mean HOMA value for type 2 DM, 10 ± 4 , was higher ($p=0.002$) compared to healthy subjects (1 ± 0.1). Whole body insulin sensitivity during OGTT was determined as the ratio AUC-glucose/AUC-ins. AUC-glucose/AUC-ins for healthy subjects was 0.3 ± 0.03 . The ratio for type 1 DM was 0.5 ± 0.09 ($p=0.025$ compared to healthy controls) and that for type 2 DM was 0.4 ± 0.1 ($p=0.110$ compared to healthy controls).

Subcutaneous adipose tissue insulin sensitivity: Despite significantly higher insulin levels in both type 1 DM ($p=0.007$) and type 2 DM ($p=0.002$) the interstitial glycerol levels were higher in both groups ($p=0.035$ and $p=0.018$; respectively) compared to healthy subjects in the fasting state (figure 11). The higher insulin and interstitial glycerol levels remained for two hours after OGTT for patients with type 2 DM. Patients with type 1 DM had similar insulin levels as healthy controls but the concentrations of interstitial glycerol were significantly higher ($p=0.018$) compared to healthy subjects two hours after OGTT (figure 11). The glucose/lactate ratio was higher in patients with type 1 DM both at baseline ($p=0.013$) and after OGTT ($p=0.013$) and in patients with type 2 DM it was higher post-OGTT ($p=0.024$) compared to controls (figure 12).

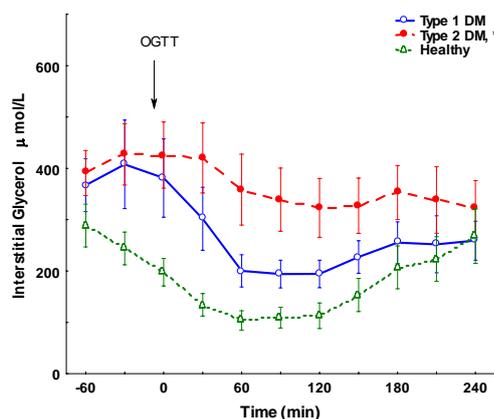


Figure 11. Mean \pm SEM of interstitial glycerol concentrations in abdominal subcutaneous tissue in type 1 DM (empty circles), type 2 DM (filled circles) and healthy subjects (triangles) before and after oral glucose tolerance test (OGTT). N= 7 for each data point. *=significant difference for repeated measures ($p<0.005$) in type 2 DM compared to healthy subjects.

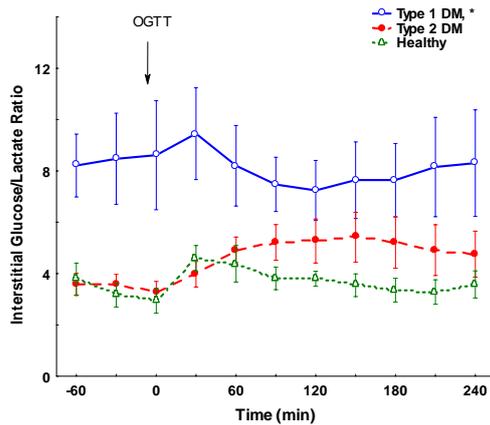


Figure 12. Mean \pm SEM of interstitial subcutaneous glucose/lactate ratio in type 1 DM (empty circles), type 2 DM (filled circles) and healthy subjects (triangles) before and after oral glucose tolerance test (OGTT). N= 7 for each data point. *=significant difference for repeated measures ($p < 0.005$) in diabetic patients compared to healthy subjects.

Other groups have reported differences in whole body lipid-metabolism between men and women (Williams 2004, Lima, et al. 2005). We compared interstitial concentrations of glucose, lactate, pyruvate and glycerol in subcutis in a group of healthy young females (n=7) and a group of healthy young male (n=7). There were no significant differences between the two groups with regards to age and BMI, see table 1 for age and BMI data. The same protocol as described in paper IV was used. Capillary glucose concentrations and interstitial glucose and glycerol for the two groups are shown in figure 13 a-c. We found no significant gender differences between capillary glucose, interstitial glucose, lactate, pyruvate or glycerol (data for interstitial lactate and pyruvate not shown).

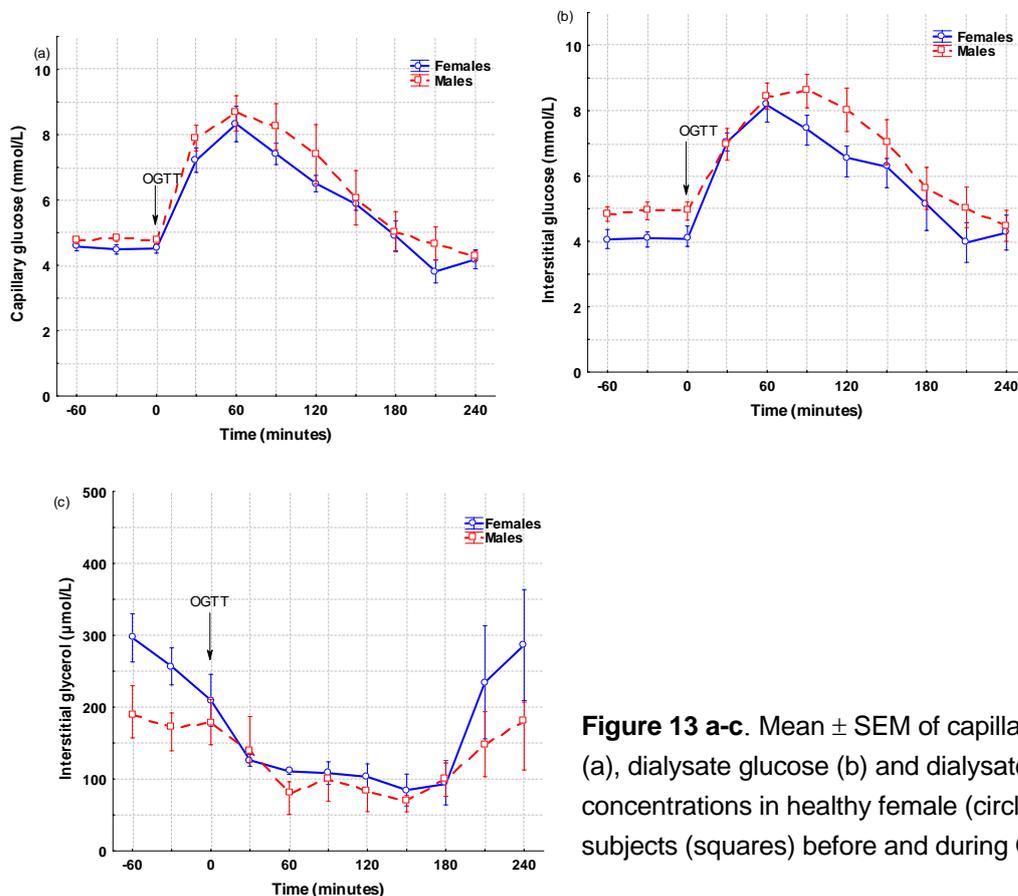


Figure 13 a-c. Mean \pm SEM of capillary glucose (a), dialysate glucose (b) and dialysate glycerol (c) concentrations in healthy female (circles) and male subjects (squares) before and during OGTT.

We analyzed S-leptin and S-adiponectin concentrations for all the participants in study IV. No effect of OGTT on the levels of S-adiponectin or S-leptin was observed 120 and 240 minutes after OGTT neither in healthy subjects nor in diabetic patients (figure 14 and 15). Compared to healthy subjects the S-leptin levels were significantly higher in patients with type 2 DM ($p= 0.004$) but not in patients with type 1 DM. In type 1 DM we observed higher S-adiponectin levels, but the difference was not significant compared to type 2 DM and healthy subjects. There was no significant difference between the adiponectin concentrations before and after OGTT in patients with type 2 DM compared to healthy subjects.

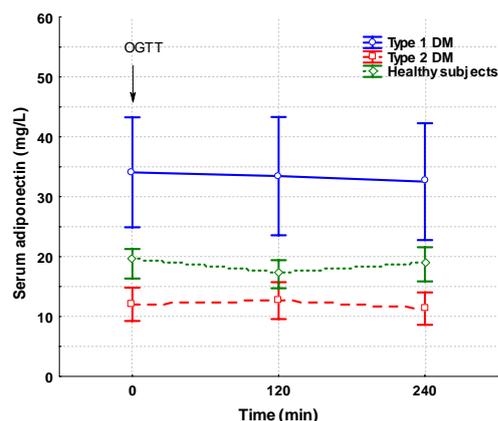


Figure 14. Mean \pm SEM of serum adiponectin concentrations at basal fasting state and 120 and 240 minutes after the OGTT in patients with type 1 (circles) and type 2 DM (squares) and healthy subjects (diamonds).

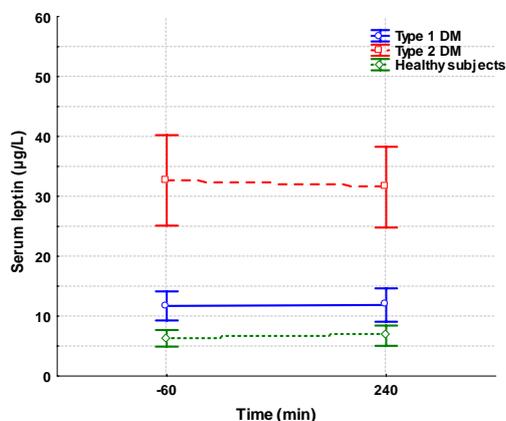


Figure 15. Mean \pm SEM of serum leptin concentrations 60 minutes before and 240 minutes after the OGTT in patients with type 1 (circles) and type 2 DM (squares) and healthy subjects (diamonds).

Hepatic insulin sensitivity. Despite higher (at basal and four hours after OGTT) or similar (at two and three hours post-OGTT) insulin levels, patients with type 1 DM had higher IGFBP-1 levels (64 ± 12 and 37 ± 10 $\mu\text{g/L}$, fasting and 240 minutes post-OGTT, respectively) compared to healthy subjects (25 ± 3 and 12 ± 3 $\mu\text{g/L}$, fasting and 240 minutes post-OGTT levels) during the whole study. Patients with type 2 DM had higher insulin levels during the whole study (23 ± 10 and 30 ± 8 mU/L, fasting and 240 minutes post-OGTT

levels) compared to healthy subjects (4 ± 0 and 8 ± 3 mU/L, fasting and 240 minutes post-OGTT levels) but their IGFBP-1 levels were not decreased (20 ± 4 and 16 ± 5 μ g/L, fasting and 240 minutes post-OGTT levels) compared to healthy subjects. The mean HeIR values were significantly higher in patients with type 1 (HeIR= 612 ± 99) ($p=0.002$) and type 2 DM (HeIR= 558 ± 214) ($p=0.034$) compared to healthy subjects (HeIR= 106 ± 27).

Correlations. We correlated the measures of insulin sensitivity in the adipose tissue (basal interstitial glycerol concentration and interstitial glucose/lactate ratio 120 minutes post-OGTT) and that in the liver (HeIR and percent decrease in IGFBP-1 after OGTT) to whole body insulin sensitivity measured as HOMA in patients with type 2 DM (table 4a).

In patients with type 1 DM + controls as a group we found correlations between interstitial basal glycerol and AUC-glucose/AUC-ins ($p=0.001$, $r=0.76$, p Bonferroni corrected = 0.002) and between interstitial glucose/lactate ratio 120 minutes post-OGTT and AUC-glucose/AUC-ins ($p=0.014$, $r=0.64$, p Bonferroni corrected = 0.028).

The percent decrease in IGFBP-1 120 minutes post-OGTT correlated to HeIR in patients with type 1 ($p=0.030$, $r=-0.58$, p Bonferroni corrected = 0.060) and type 2 DM ($p=0.006$, $r=-0.70$ and p Bonferroni corrected = 0.0012).

We found no correlations between interstitial glucose/lactate ratio post-OGTT and fasting serum levels of leptin and adiponectin in patients with type 1 and type 2 DM. The correlations between fasting glycerol and S-leptin and S-adiponectin are shown in table 4b

Insulin sensitivity in adipose tissue	Whole body insulin sensitivity (HOMA)	Hepatic insulin sensitivity (% BP-1 decrease 120 min post-OGTT)
Basal interstitial glycerol	$p < 0.001$ $r=0.81$ $p 2 < 0.001$	ns
Interstitial glucose/lactate 120 min post-OGTT	$p=0.051$ $r=0.52$ $p 2=0.102$	$p=0.006$ $r = -0.70$ $p 2=0.012$

Table 4a. Correlations in type 2 DM + healthy controls (N=14) between measures of peripheral basal insulin sensitivity (HOMA) and hepatic insulin sensitivity determined as percent decrease in IGFBP-1 120 min post-OGTT (% BP-1 decrease) and measures of insulin sensitivity in adipose tissue (basal interstitial glycerol and interstitial glucose/lactate ratio 120 minutes post-OGTT). $p 2$ = Bonferroni corrected p-value.

Basal dialysate glycerol ($\mu\text{mol/L}$) in group	Basal S-leptin $\mu\text{g/L}$	Basal S-adiponectin mg/L
Type 1 DM + healthy	ns	P=0.002 r = 0.66
Type 2 DM + healthy	p = 0.006 r = 0.7	ns

Table 4b. Correlations between basal interstitial glycerol and basal S-leptin and S-adiponectin in patients with type 1 DM + healthy (n=14) and patients with type 2 DM + healthy (n=14). Ns= not significant.

Comparing the measures of insulin sensitivity in adipose tissue (basal interstitial glycerol concentration and interstitial glucose/lactate ratio 120 minutes post-OGTT) and S-leptin and S-adiponectin levels in patients with type 2 DM + healthy subjects as a group (n = 14) we observed correlations between fasting basal interstitial glycerol and fasting S-leptin (p= 0.006, r= 0.7), but not with basal adiponectin levels. No correlations were found between glucose/lactate ratio and S-leptin or S-adiponectin levels. In patients with type 1 DM + healthy subjects as a group (n=14) a correlation was observed between basal interstitial glycerol and basal S-adiponectin (p= 0.002, r= 0.66) but not with S-leptin. No correlations were found between glucose/lactate ratio and S-leptin (and p = 0.239 r = 0.28 and p= 0.556 r=0.17 in type 1 and 2 DM, respectively) or S-adiponectin levels (p = 0.010 r = 0.38 and p = 0.373 r = 0.21 in type 1 and 2 DM, respectively) in any group.

In 5 patients with DM (2 with type 1 DM and 3 with type 2 DM) a two-step hyperinsulinemic euglycemic glucose clamp was preformed. Insulin doses of 0.25 mU/kg body weight and minutes and 1 mU/kg body weight and minute were used. During the clamp the blood glucose levels were kept constant at 5 mmol/L. The interstitial glucose and lactate did not change during the study period. The glycerol levels decreased when the insulin dose was increased. In contrast to type 2 DM the IGFBP-1 concentrations decreased during the whole study period in type 1 DM. The results are shown in table 5.

The interstitial glucose/lactate ratio can not be used as a measure of local insulin sensitivity since the blood glucose concentrations are kept at a constant level. We hypothesize that interstitial glycerol levels may be used as a measure of adipose tissue insulin sensitivity during insulin clamp studies. Therefore basal interstitial glycerol measured 60 minutes after the start of the experiment was correlated to M-values. There were no significant correlations between basal glycerol and M1 (p=0.651 r = 0.28) or M2 (p = 0.541 r= 0.37)

	Patients with type 1 DM N=2	Patients with type 2 DM N=3
M-value (mg/kg.min)		
❖ Low insulin dose	1.2 ± 1	1.5 ± 0.7
❖ High insulin dose	4.7 ± 1	4.5 ± 0.6
Insulin (mU/L)		
❖ Low insulin dose	27 ± 16	24 ± 5
❖ High insulin dose	91 ± 34	101 ± 1
Interstitial glycerol (µmol/L)		
❖ Low insulin dose	292 ± 3	276 ± 13
❖ High insulin dose	142 ± 48	176 ± 25
Interstitial lactate (mmol/L)		
❖ Low insulin dose	2.5 ± 1.0	1.5 ± 0.3
❖ High insulin dose	1.9 ± 0.2	1.7 ± 0.2
Interstitial Glucose / Lactate ratio		
❖ Low insulin dose	2.4 ± 0.1	2.6 ± 0.5
❖ High insulin dose	3.3 ± 0.7	2.3 ± 0.5
IGFBP-1 µg/L		
❖ Low insulin dose	23 ± 1	28 ± 36
❖ High insulin dose	15 ± 1	25 ± 26

Table 5. The mean ± SD. The concentrations determined at 150 (at the end of low dose insulin) and 300 minutes (at the end of high dose insulin) after the start of the experiment. Low (0.25 mU/kg body weight min) and high-dose insulin clamp (1 mU/kg body weight min).

7.2.2 Study V

Comparing an oral glucose tolerance test with a standardized mixed meal (MM) we observed lower capillary glucose ($p=0.012$) as well as lower interstitial glucose ($p=0.003$) and lactate ($P=0.046$) levels after MM compared to OGTT despite similar insulin levels. The relative glucose recovery was similar to that during OGTT (Figure 16a-c)

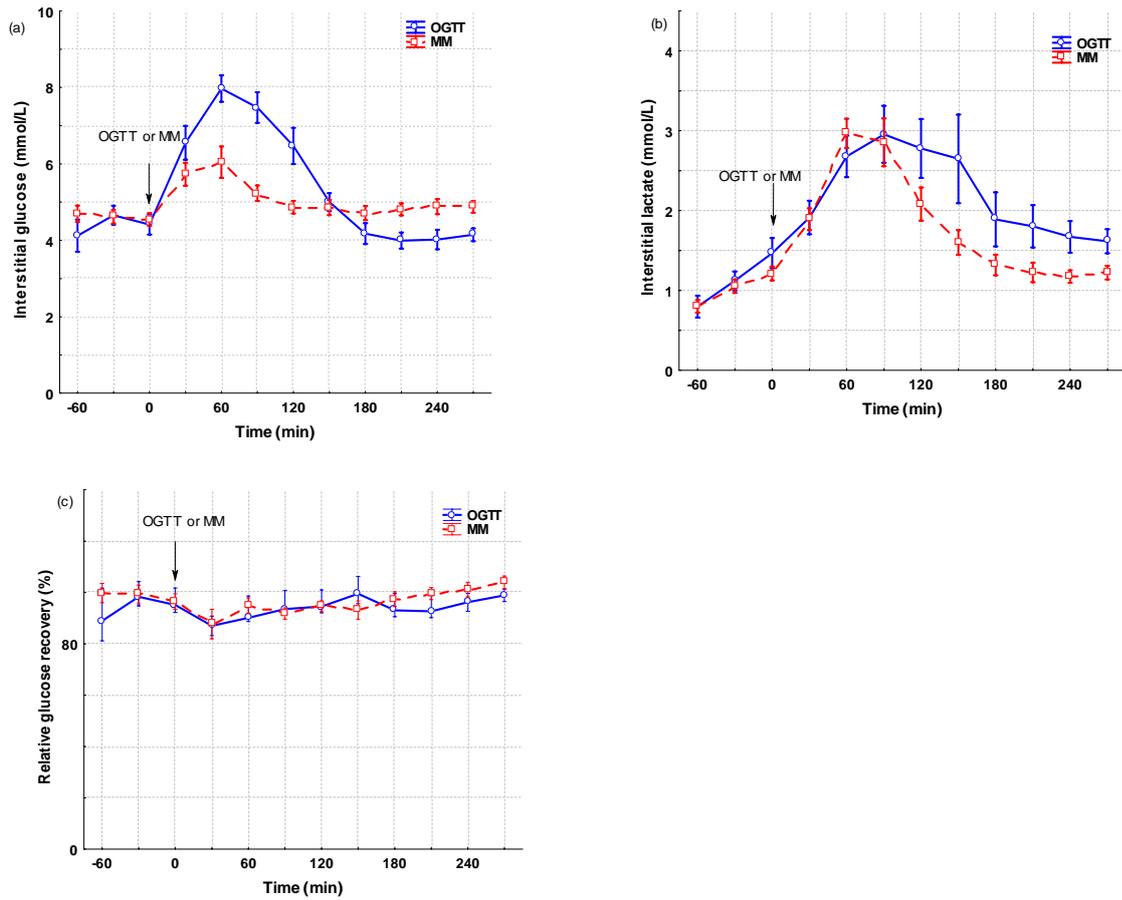


Figure 16a-c. Mean \pm SEM for interstitial glucose (a), interstitial lactate (b) and relative glucose recovery (c) levels in subcutaneous adipose tissue in healthy subjects before and after an OGTT (circles) and a mixed meal (squares).

8 GENERAL DISCUSSION

8.1 METHODOLOGICAL STUDIES

We investigated the differences between high vs. low microdialysis flow rates for measuring small molecules in the subcutaneous tissue during an OGTT.

The baseline was stable for capillary glucose and flow rate 0.3 $\mu\text{L}/\text{min}$, however for 1, 2 and 5 $\mu\text{L}/\text{min}$ the dialysate glucose levels decreased significantly over the same period. The amount of fluid passing through the catheter during a certain time period is larger for higher flow rates and a greater amount of substances around the catheter will be removed from the tissue as compared to when very low flows are used (e.g. 0.3 $\mu\text{L}/\text{min}$). Therefore there is a concern about possible alterations of small molecules concentrations in the tissue adjacent to the catheter, also referred to as a depletion effect of microdialysis on the subcutaneous adipose tissue (Lonnroth, et al. 1987). The decreasing basal glucose levels before OGTT observed during higher flow rates ($> 0.3 \mu\text{L}/\text{min}$) could be due to such a depletion effect. In most studies using higher flow rates (2.5 $\mu\text{L}/\text{min}$) glucose has been added to the perfusion fluid, in an attempt to compensate for this depletion (Lonnroth, et al. 1987).

During glucose load the percent increase in glucose compared to baseline level at time zero was the same for 0.3 $\mu\text{L}/\text{min}$ as for capillary blood. However, the percent increase was substantially greater for the higher flow rates. From a clinical perspective this effect would be a problem. A glucose-sensor is calibrated at time of insertion. This means that the patient performs a capillary glucose measurement and the value is programmed in the sensor so the sensor signal (based on the interstitial glucose levels) will be equal to the capillary glucose concentration. A 200% increase in the interstitial glucose levels means a 200 % increase in sensor signal and subsequently a 200% increase in the values displayed for the patients. This would be a problem if the patient will compensate a high increase in glucose levels by higher doses of insulin than needed. It highlights the interpretation problem encountered when using higher microdialysis flow rates.

On the contrary to the in-vivo results there was no difference between the percent increases in glucose levels in our in-vitro bench study after the change from basal solution to the solutions with high or low glucose concentration measured by different flow rates, speaking in favor of a biological event in the tissue affecting the higher flow rates ($> 0.3 \mu\text{L}/\text{min}$). As shown by Jansson et al and Van der Merwe et al the adipose tissue blood flow increases during a glucose load (Jansson, et al. 1994, van der Merwe, et al. 2001). In addition to above mentioned studies Hickner et al (Hickner, et al. 1992) have shown that a change in blood flow around a microdialysis catheter increases the dialysate glucose levels measured with a flow rate of 1 $\mu\text{L}/\text{min}$ which is explained by more available glucose molecules close to the microdialysis catheter when capillary flow increases. Previous studies have show that a low flow rate (0.3 $\mu\text{L}/\text{min}$) and a sufficiently long dialysis membrane (30 mm) provide enough time for complete equilibration of small molecules, as for glucose between the dialysate and interstitial fluid (Bolinder, et al. 1992, Rosdahl, et al. 1998). This means that during these conditions the concentration of glucose in the dialysate is not dependent upon capillary delivery. Instead, it reflects the true concentration regardless of any increase in supply i.e. capillary flow. However, at higher microdialysis flow rates and increase in

capillary flow (caused by the glucose load) will increase supply per time unit and cause a higher percent increase over baseline as compared to what happens in capillary blood and dialysate at 0.3 $\mu\text{L}/\text{min}$ flow. Low perfusion flow rate are limited by the small sample volume, which increases the sample collection interval and consequently results in worse time resolution. This problem maybe compensate for by shortening the outlet tubings and by performing a trend analysis (I).

Rinsing the microdialysis catheter prior to the insertion in sc adipose tissue improved the measurement of dialysate glucose and pyruvate. The microdialysis membrane may contain different amount of air, which affects the measurement of interstitial metabolite levels. Pre-rinsing the microdialysis catheters minimizes the differences between different microdialysis catheters. We recommend that the microdialysis membrane should be rinsed prior to insertion of the catheters in order to avoid differences in results due to the air trapped in the dialysis membrane.

When using a flow rate of 2 $\mu\text{L}/\text{min}$ in sc adipose tissue in healthy subjects and rats, we observed that in the rat subcutis the changes in dialysate glucose and other metabolites over eight days differ from that in the humans. The rat model is a poor model of the human subcutaneous tissue reaction to a foreign body. With a flow rate of 2 $\mu\text{L}/\text{min}$ in abdominal sc tissue in healthy subjects and patients with DM we observed increasing mean levels of both dialysate glucose and urea and relative glucose recovery over five days. Supported by the findings in the biopsies of sc adipose tissue, we suggest that these changes are due to an inflammatory response. On the contrary using a flow rate of 0.3 $\mu\text{L}/\text{min}$ in sc adipose tissue in healthy volunteers, metabolite levels did not change over five days, indicating that the metabolite flux into the catheter at a low flow rate is not affected by the inflammation (II and III).

We observed higher dialysate compared to plasma glycerol levels in healthy subjects and patients with type 2 DM. Our data is in accordance with previous study (Jansson, et al. 1990, Hagstrom-Toft, et al. 1997, Kerckhoffs, et al. 1998, van der Merwe, et al. 2001). In patients with type 1 DM the interstitial glycerol levels were higher compared to plasma levels over time. Higher interstitial than plasma glycerol levels in patients with type 1 DM have been shown by other groups (van der Merwe, et al. 1994). The plasma lactate levels were significantly higher in patients with type 2 DM. This is in accordance with previous findings in patients with diabetes and in obese subjects (van der Merwe, et al. 2001). In healthy subjects we found higher dialysate lactate levels over time. Previous studies with microdialysis have shown that lactate is produced in subcutaneous adipose tissue (Hagstrom, et al. 1990, Jansson, et al. 1990, Jansson, et al. 1994). The plasma and interstitial lactate levels did not differ significantly in our patients with type 1 DM. Others have shown higher interstitial lactate levels compared to plasma in patients with type 1 DM (van der Merwe, et al. 1994). The reason for this discrepancy are not known but could partly been explained by the fact that we studied only three patients with type 1 DM. In summary subcutaneous interstitial glycerol and lactate were higher compared to plasma levels in our healthy subjects and type 1 and 2 DM. Based on our results and previous studies we suggest that the majority of studied sc interstitial glycerol and lactate are produced locally.

Dialysate and capillary glucose levels correlated in healthy subjects and patients with type 1 DM but not in patients with type 2 DM in the fasting and hyperglycemic state. This

supports the idea of using microdialysis for monitoring glucose levels in normal weight type 1 DM. Patients with type 2 DM were more obese, i.e. a more subcutaneous adipose tissue and larger adipocytes with decreased capillary density as shown by Jansson et al (Jansson, et al. 1994). This means that the distance between capillaries and the microdialysis catheter is greater and glucose molecules have to diffuse over a longer distances, which may affect the correlation between C-glucose and interstitial glucose. A similar observation were made by Rosdahl et al (Rosdahl, et al. 1998) who found a negative correlation between skin fold thickness and interstitial glucose levels measured by microdialysis technique. The correlation, however, increased over time with the highest correlation being observed at the last measurements in all three groups. This may indicate a stronger correlation between capillary and interstitial glucose exists in patients with type 2 DM if measurements are taken for a longer period of time (IV).

In summery based on these findings we suggest that the microdialysis membrane preferably should be rinsed before the insertion. The actual glucose concentration can be measured directly using flow rates of 0.3 μ l/min without any calibration adjustments that would be needed at 2 μ L/min. Thus the low flow rate is preferred in a microdialysis-based glucose sensor and in studies of local tissue metabolism.

Other groups have shown higher basal plasma glycerol levels as well as higher total lipolysis rate in female compared to male subjects (Lima, et al. 2005). In that study the S-insulin concentrations were lower in females compared to males. We found that for similar S-insulin levels in healthy male and female subjects, there were no significant differences between capillary glucose, interstitial glucose, lactate and pyruvate or glycerol levels. Therefore, we concluded that there are no differences between glucose utilization in sc adipose tissue, measured as levels of interstitial glucose, lactate and pyruvate, in male and female subjects. Neither is there any difference between male and female subjects in subcutaneous lipolysis measured as glycerol concentrations. Thus both men and women were included in the study groups.

8.2 METABOLIC STUDIES

To evaluate the ability of microdialysis to assess insulin sensitivity in sc adipose tissue we compared microdialysis data with fasting whole body insulin sensitivity measured as HOMA in patients with type 2 DM and healthy subjects and whole body insulin sensitivity post OGTT assessed as AUC-glucose/AUC-ins in type 1 DM and healthy subjects as well as hepatic insulin sensitivity measured as insulin-like growth factor binding protein-1 (IGFBP-1). We observed insulin resistance in sc adipose tissue both in the fasting state and after OGTT in patients with type 1 and type 2 DM. In two of seven patients with type 1 DM and three of seven patients with type 2 DM we preformed a two-step hyperinsulinemic euglycemic clamp. The M-values for our patients were in accordance with the values calculated in patients with type 2 DM in a previous study with similar study design (Bavenholm, et al. 2003). The M-values in patients with type 1 DM as well as type 2 DM were lower compared to those in the healthy subjects previously reported (Bavenholm, et al. 2003). These findings further strengthen our assumption that whole body insulin resistance was present in our patients. There was no significant correlation between fasting interstitial glycerol and M-values measured in the five patients. Because of the limited number of

patients included in insulin clamp study there is difficult to draw any conclusion from this result.

In patients with type 1 DM the higher basal interstitial glycerol levels compared to healthy subjects despite higher S-insulin levels indicates a less effective inhibition of lipolysis. After the glucose load and addition of exogenous insulin the insulin levels similar to those in healthy subjects were sufficient to suppress lipolysis but not to normalize glucose metabolism. During OGTT the ratio AUC-glucose/AUC-ins was higher in patients with type 1 DM compared to controls, suggesting whole body insulin resistance which could be due to the harmful effect of chronic hyperglycemia, referred to as "glucose toxicity" (Yki-Jarvinen 1992). We found that both basal interstitial glycerol levels and glucose/lactate ratio post OGTT correlated to AUC-glucose/AUC-ins in patients with type 1 DM and healthy subjects. These results suggest a relation between insulin sensitivity in sc adipose tissue and in whole body.

In patients with type 2 DM we observed insulin resistance in whole body indicated by the higher HOMA and insulin resistance in adipose tissue indicated by higher fasting and post-OGTT glycerol levels in relation to insulin. Nurjahn et al have described increased lipolysis in patients with type 2 DM (Nurjahn, et al. 1992). Our results support their findings. Further increase in insulin concentrations may normalize the interstitial glycerol concentrations in type 2 DM as shown in the insulin clamp study (table 5). Post-OGTT the delayed glycolytic response in patients with type 2 DM compared to healthy subjects could be due to delayed absorption of glucose from the gastrointestinal tract or it could be a feature of insulin resistance at endothelial level as suggested by Jansson et al (Jansson, et al. 1993). Post-OGTT we observed insulin resistance in sc adipose tissue as indicated by significantly higher glycerol levels and higher interstitial glucose/lactate ratio compared to healthy subjects. Leptin is a measure of the amount of adipose tissue and insulin sensitivity in the body (Trayhurn, et al. 2001). We observed for the first time a correlation between fasting interstitial glycerol (index of lipolysis) and fasting S-leptin concentrations in type 2 DM + controls. This finding suggests that fasting S-leptin may be a measure of insulin sensitivity in sc adipose tissue. A correlation was observed between fasting S-adiponectin and fasting interstitial glycerol in type 1 DM + controls. Thus increase adiponectin maybe a response to overcome the insulin resistance induced by the lipotoxic effect by increased lipolysis in patients with type 1 DM. In summary we found correlations between insulin sensitivity in adipose tissue and in whole body in both type 1 DM and type 2 DM and between insulin sensitivity in sc adipose tissue and liver in type 2 DM. We conclude that the microdialysis technique can be used to study in vivo insulin sensitivity in adipose tissue over time and that it may be useful for evaluating the effects of new drugs on insulin sensitivity.

The general aim of this thesis was to investigate if microdialysis technique can be used for reliable monitoring of glucose and other metabolites (lactate, pyruvate and glycerol) in everyday life in patients with DM. Our knowledge on fluctuations in interstitial glucose and other metabolites in subcutis is mostly based on studies performing oral glucose tolerance test (OGTT) or hyperinsulinemic euglycemic or hyperglycemic clamps (Jansson, et al. 1990, Hagstrom-Toft, et al. 1997, Kerckhoffs, et al. 1998, Rosdahl, et al. 1998). However, none of

these study designs are comparable with a normal meal, which contains not only glucose but also fat and protein. The insulin response to glucose differs from the response to fat and amino acids (Del Guerra, et al. 2005, McCarty, et al. 2005). Patients with type 2 DM have an intact insulin response to amino acids despite impaired insulin response to glucose. A fat rich meal may affect not only the insulin response but also the insulin sensitivity in diabetes mellitus (Daly 2003). There are obvious benefits for patients with DM if the effect of different therapies would be evaluated after a mixed meal (MM). Therefore in paper V we compared the insulin induced changes in glycerol, glucose and other metabolites in sc adipose tissue after an OGTT and a MM. We found lower capillary glucose and interstitial glucose and lactate after MM compared to OGTT despite similar insulin levels. There were no differences in relative glucose recovery after MM and OGTT. A mixed meal gives a more clinically relevant metabolic status. However, further studies with MM in patients with DM are needed to evaluate the microdialysis technique for continuous glucose monitoring during everyday activities in patients with DM

9 THE MAIN FINDINGS

- Comparing the effect of different microdialysis flow rates (0.3, 1, 2 and 5 $\mu\text{L}/\text{min}$) on the interstitial metabolites measured in subcutis we found that at the basal fasting state the dialysate glucose obtained by 0.3 $\mu\text{L}/\text{min}$ was equal to capillary glucose concentration. A decrease in dialysate glucose levels during the basal state was observed during 60 minutes for higher flow rates but not for 0.3 $\mu\text{L}/\text{min}$ when no glucose is added to the perfusion fluid. This indicates a depleting effect for higher flow rates ($> 0.3 \mu\text{L}/\text{min}$). The relative increase in glucose after OGTT was similar for capillary glucose and dialysate glucose measured by flow rate 0.3 $\mu\text{L}/\text{min}$ but not for higher flow rates (I).
- The dialysate glucose and other metabolite trends over eight days at a microdialysis catheter interface differ significantly in rat and human subcutis. Caution should be used when extrapolating Sprague Dawley rat biomaterial data to human applications. In healthy subjects and in patients with DM significant increase in dialysate glucose levels and glucose recovery was observed over five days after catheter insertion in subcutis. Histological signs of local inflammation in sc adipose tissue were observed around the microdialysis catheters. However, using a flow rate of 0.3 $\mu\text{L}/\text{min}$ over a period of 5 days no changes were observed in dialysate glucose levels or glucose recovery. These data suggest that the measurements are not affected by inflammation around the catheter when using a microdialysis flow rate of 0.3 $\mu\text{L}/\text{min}$. The actual glucose concentration can be measured directly using a flow rate of 0.3 $\mu\text{L}/\text{min}$ without any calibration adjustments that would be needed at 2 $\mu\text{L}/\text{min}$. We recommend that this flow rate should be used in a microdialysis-based glucose sensor (II, III).
- In fasting and hyperglycemic states the interstitial and capillary glucose levels correlate in healthy subjects and patients with type 1 DM but not in patients with type 2 DM. The poor correlation in patients with type 2 DM may due to the obesity and increased waist circumference observed with possibly decreased capillary density. The correlation increased over time with the highest correlation coefficient being observed at the last measurements in all three groups. This may indicate that a stronger correlation between capillary and interstitial glucose exists in patients with type 2 DM if measurements are taken for a longer period of time (IV).
- The microdialysis technique can be used for assessment of insulin sensitivity in abdominal subcutaneous adipose tissue. We observed correlations between insulin sensitivity in abdominal sc adipose tissue (measured with microdialysis) and whole body and hepatic insulin sensitivity. A correlation between fasting interstitial glycerol and fasting serum leptin was observed, suggesting that fasting serum leptin may be a measure of insulin sensitivity in sc adipose tissue (IV).
- A mixed meal results in lower capillary glucose and interstitial glucose and lactate levels compared to an oral glucose tolerance test. A mixed meal is a more physiological situation and provides a more clinically relevant metabolic status. The microdialysis technique is reliable for studies on the insulin effect after oral glucose load and mixed meal in abdominal sc adipose tissue (V).

10 ACKNOWLEDGEMENTS

My warmest thanks are reserved for my tutor and main supervisor **Kerstin Brismar**, for her generous sharing of scientific knowledge, for always being encouraging and never failing me down. I am very grateful to have her as a friend and a mentor for my professional development as a young doctor.

My sincere gratitude to:

Urban Ungerstedt, my co-supervisor, for giving me the opportunity to start my work at his lab and for his generous support.

Natalie Wisniewski, my friend and co-worker, for being such a good collaborator and always being there across the Atlantic when I needed someone to discuss my papers with.

My co-authors Prof **Ulf Adamson**, Prof **Per-Erik Lins**, Docent **Mari-Anne Hedblad**, Prof **Monty Reichert** and dr. **Bruce Klitzman** for their scientific contributions to my papers.

Prof **Suad Efendic** for generously sharing his knowledge of science with me.

Åse Elfving, Monica Pace-Sjöberg, Elham Rosstami, Michel Goiny, Silvia Di Maio, Maricanata Bondi, Marjan Ponten and Johan Ungerstedt at the Department of Physiology and Pharmacology for valuable help with analyzing microdialysis samples, assistance with administration, inspiring discussions and most of all for creating a joyful and friendly atmosphere.

Agneta Reinholdsson, Alice Skogholm, Eva Lena Forsberg, Kajsa Sundqvist, Carolynn Lidbeck, Lovisa Gave and Emilie Sunqvist, medical technologists, research nurses and students, for valuable help taking care of participants in my studies and helping me perform the studies.

Ella Wallerman, Elvi Sandberg, Inga-Lena Wivall and Gloria Ursu, for excellent laboratory work with hormone analysis and for excellent work with histology preparations.

Dr Jonas Malmström for valuable help with surgically removing samples of subcutaneous adipose tissue.

All the colleagues at the Department of Molecular Medicine **Sergiu-Bogdan Catrina, Jacob Grunler, Kerstin Hall** and **Moira Lewitt** and the colleagues at Department of Physiology and Pharmacology **Göran Engberg, Sophie Erhardt, Lilly Schwieler** and **Margaretha Westling** for creating friendly and stimulating atmospheres at the departments, during journal clubs and during the Pharmacology education.

Janet Holmen for your help with revision of my papers.

Renee Andersson, Department of Physiology and Pharmacology, and **Lennart Helleday**, Department of Molecular Medicine, for technical support.

Prof. **Peter Thoren**, Prof. **Bertil Fredholm** and Prof. **Stefan Eriksson**, former and present heads of the Department of Physiology and Pharmacology, for giving me the opportunity to start my work in the Department.

Prof. **John Wahren** and docent **Pierre Lafolie**, the former and present heads of the Karolinska Clinical Research Trial and Support Center (KCR) giving me the support and access to KCR.

All volunteers, without whom none of the studies had been possible.

My family; to my mother **Mehry**, for endless love and support which makes me strong, to my father **Ali**, for interesting me in science early in life by giving me riddles to solve and to my brother **Poyan**, for just being the best brother in the world.

My parents in law **Margaretha** and **Ingvar** for your love and support and for letting me be a part of your family.

My friends from medical school, **Mari Mahlman**, **Åsa Ryberg**, **Rusana Simonoska** and **Johanna Ungerstedt** for the nice time we have together.

All my other friends –no one mentioned and no one forgotten.

And finally:

Jonas, my husband and friend. Not many men could manage to have a full time job, take care of the housework, settle a new home and be a patient and loving husband to a pregnant woman. Without you this work would not be possible.

11 REFERENCES

EN.REFLIST