Novel Approaches to Treatment and Prevention of Diabetic Nephropathy

LINA NORDQUIST
Dissertation presented at Uppsala University to be publicly examined in Auditorium Minus, Gustavianum, Akademigatan 3, Uppsala, Friday, December 7, 2007 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Several studies have reported beneficial effects of C-peptide supplementation in diabetic patients and animal models of insulinopenic diabetes. However, it is also established that good glycemic control is essential to minimize the risk of diabetes-induced complications. This thesis investigates potential mechanisms for the beneficial effect of C-peptide on glomerular hyperfiltration, and a novel, painless route of insulin administration.

The results demonstrate that both C-peptide and its C-terminal penta-peptide sequence reduce the diabetes-induced glomerular hyperfiltration within an hour. The results also indicate that C-peptide possibly reduces diabetes-induced hyperfiltration via three different mechanisms: 1. Constriction of the afferent arteriole was demonstrated on isolated vessels from diabetic mice. 2. A net dilation of the efferent arteriole was evident in vivo. 3. Inhibition of the Na⁺/K⁺-ATPase was demonstrated in vivo in diabetic rats as well as in vitro on isolated proximal tubular cells from diabetic rats. All these mechanisms are known regulators of the net glomerular filtration pressure.

The last part of this thesis demonstrates that intradermal administration with a newly developed patch-like microneedle device results in similar insulin concentration compared to standard subcutaneous delivery.

These findings provide an insight for the beneficial effects of C-peptide on diabetic kidney function, and shows that this effect can be achieved by infusion of the C-terminal penta-peptide sequence alone. This thesis also presents a novel, painless alternative to insulin injections that is controllable, requires minimal training, and therefore presents several advantages compared to current standard therapy.

Keywords: Diabetes, Nephropathy, C-peptide, Microneedle, Renal, GFR, Oxygen Consumption, Reabsorption

Lina Nordquist, Department of Medical Cell Biology, Box 571, Uppsala University, SE-75123 Uppsala, Sweden

© Lina Nordquist 2007

ISSN 1651-6206
ISBN 978-91-554-7021-0
urn:nbn:se:uu:diva-8308 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-8308)
Till mormor och morfar

Facilius est multa facere quam diu
List of original papers

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

I. The C-peptide fragment EVARQ reduces glomerular hyperfiltration in streptozotocin-induced diabetic rats.  

II. Proinsulin C-peptide constricts glomerular afferent arterioles in diabetic mice. A potential reno-protective mechanism.  

III. Proinsulin C-peptide reduces diabetes-induced glomerular hyperfiltration via efferent arteriole dilation and inhibition of proximal tubular reabsorption in streptozotocin-induced diabetic rats.  

IV. Novel microneedle patches for active insulin delivery are efficient in maintaining glycaemic control: an initial comparison with subcutaneous administration.  

Reprints of papers and figures were made by permission of the publishers.
Contents

Introduction ................................................................................................... 11

Diabetes mellitus .......................................................................................... 11
Renal anatomy and physiology ................................................................. 11
Development of diabetic nephropathy ....................................................... 13
  Alterations in glomerular filtration rate ............................................... 13
Prevention and treatment of diabetic nephropathy .................................... 14
Prevention by proinsulin C-peptide ............................................................ 15
  Mechanisms for C-peptide in diabetic nephropathy ............................ 16
  Reducing diabetic glomerular hyperfiltration ....................................... 17
The importance of glycemic control .......................................................... 19
  Intradermal delivery of insulin ............................................................. 19

Aims of the investigation .......................................................................... 23

Methods ...................................................................................................... 24

Animals and induction of diabetes ............................................................ 24
  Rats (Studies I, III, and IV) .............................................................. 24
  Mice (Study II) ............................................................................. 25
Experimental protocols, treatment regimens, drugs and preparations.... 25
Preparations and measurements ............................................................... 26
  Anesthesia (Study I, III, and IV) and euthanasia ................................. 26
  Surgical procedure (Study I, III and IV) .......................................... 26
  Blood pressure (Studies I, III and IV) ............................................... 27
  Tissue isolation, dissection and perfusion (Study II) .......................... 27
  Measurements of oxygen consumption (Study III) ............................ 28
  Blood flow estimations (Study III) ................................................... 29
  Measurements of tubular pressures (Study III) .................................. 29
  Intradermal infusion of insulin (Study IV) ......................................... 30

Analyses and calculations ........................................................................ 30
  Estimation of glomerular filtration rate (Study I and III) ................. 30
  Electrolyte analysis (Study I and III) ............................................... 31
  Measurements of arteriole contraction (Study II) .............................. 31
  Calculations in Study III .................................................................. 31
  Insulin analysis (Study IV) .............................................................. 31

Statistical method and presentation of data ............................................. 32
Results...........................................................................................................33
Study I ......................................................................................................33
  Glomerular filtration rate ...............................................................33
  Blood glucose concentrations ........................................................43
  Urinary sodium excretion ...............................................................34
Study II .....................................................................................................35
  Afferent arteriole diameter ...............................................................35
  Rho-kinase inhibitor Y-27632 .............................................................37
Study III ...................................................................................................38
  Blood pressure and renal parameters .............................................38
  Fractional Na\(^+\) excretion and transported Na\(^+\) .........................39
  In vivo oxygen consumption ............................................................40
  Micropuncture parameters: filtration pressures ..............................40
  In vitro oxygen consumption ............................................................41
Study IV ...................................................................................................42
  Plasma insulin concentrations ........................................................42
  Blood glucose concentrations ........................................................43

Discussion.....................................................................................................45
  Penta-peptide effects .........................................................................45
  C-peptide and glomerular hyperfiltration .........................................45
    Glomerular filtration rate and tubuloglomerular feedback .............46
    The tubular hypothesis of glomerular filtration ..............................47
    Separate effects on capillary and proximal pressure ......................48
    Renal glomerular arteriolar vascular tone and blood flow .............51
  Cellular C-peptide signaling ..............................................................51
    C-peptide and Rho-kinase .............................................................51
    C-peptide and oxygen consumption ..............................................54
    Potential tissue or state-specificity of C-peptide .........................54
    Lack of effect on normoglycemic animals ......................................55
    C-peptide-induced reduction of blood glucose ..............................55
  Intradermal insulin treatment ............................................................56

Summary and conclusions ..........................................................................57
Future perspectives .....................................................................................58

Acknowledgements ...................................................................................63

References..................................................................................................66
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>bw</td>
<td>body weight</td>
</tr>
<tr>
<td>C-peptide</td>
<td>proinsulin connecting peptide</td>
</tr>
<tr>
<td>ΔΠ onc</td>
<td>onatotic pressure</td>
</tr>
<tr>
<td>FF</td>
<td>filtration fraction</td>
</tr>
<tr>
<td>GFR</td>
<td>glomerular filtration rate</td>
</tr>
<tr>
<td>IU</td>
<td>international unit</td>
</tr>
<tr>
<td>IV</td>
<td>intravenous</td>
</tr>
<tr>
<td>MAP</td>
<td>mean arterial blood pressure</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Na⁺/K⁺-ATPase</td>
<td>sodium/potassium ATPase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>P&lt;sub&gt;BS&lt;/sub&gt;</td>
<td>hydrostatic pressure in Bowman’s space</td>
</tr>
<tr>
<td>P&lt;sub&gt;cap&lt;/sub&gt;</td>
<td>hydrostatic pressure in the glomerular capillary</td>
</tr>
<tr>
<td>P&lt;sub&gt;ff&lt;/sub&gt;</td>
<td>free-flow tubular pressure</td>
</tr>
<tr>
<td>P&lt;sub&gt;net&lt;/sub&gt;</td>
<td>glomerular net filtration pressure</td>
</tr>
<tr>
<td>P&lt;sub&gt;strf&lt;/sub&gt;</td>
<td>stop-flow tubular pressure</td>
</tr>
<tr>
<td>P&lt;sub&gt;tub&lt;/sub&gt;</td>
<td>hydrostatic pressure in early proximal tubulus</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>Q&lt;sub&gt;O₂&lt;/sub&gt;</td>
<td>oxygen consumption</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>RBF</td>
<td>renal blood flow</td>
</tr>
<tr>
<td>RVR</td>
<td>renal vascular resistance</td>
</tr>
<tr>
<td>SC</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>TGF</td>
<td>tubuloglomerular feedback</td>
</tr>
<tr>
<td>T&lt;sub&gt;Na&lt;/sub&gt;</td>
<td>transported sodium</td>
</tr>
<tr>
<td>T&lt;sub&gt;Na&lt;/sub&gt;/Q&lt;sub&gt;O₂&lt;/sub&gt;</td>
<td>transported sodium per consumed oxygen</td>
</tr>
</tbody>
</table>

*Homo prudens non contra ventum mingit*
Introduction

Diabetes mellitus

Diabetes mellitus is a chronic disease where blood glucose concentrations increase as a consequence of insufficient secretion of insulin from the pancreatic beta cells. Insulinopenic diabetes mellitus involves autoimmune destruction of the insulin-secreting cells. The diagnosis therefore mostly means being subject to a life with multiple daily insulin injections, and also being exposed to considerable risks for complications. There are several long-term complications associated with diabetes mellitus. Among the complications are retinopathy, circulatory problems, neuropathy, and nephropathy. The incidence of insulinopenic diabetes mellitus is constantly increasing in Scandinavia, and in addition approximately 30% of all hyperinsulinemic diabetic patients develop insulinopenia.

Renal anatomy and physiology

Our kidneys consist of approximately 1,000,000 structural and functional units known as nephrons (Fig. 1). Blood arrives at the nephron through the afferent arteriole of the glomerulus. In the glomerulus, low molecular weight constituents of the plasma are filtered across the glomerular membrane and into Bowman’s space, creating a filtrate known as primary urine. In the human kidneys, approximately 180 l of primary urine and 1.5 kg of NaCl are formed daily, but the normal urinary excretion equals only 1-2 l and 10-15 g of NaCl. This means most of the constituents of the primary urine must be reabsorbed by the tubular structures following Bowman’s space, before the urine exits the kidneys and enters the urethra leading to the urinary bladder. To achieve this, the different nephron segments contain specialized cells, which via interconnected segments regulate kidney function in order to maintain electrolyte homeostasis, blood pressure, blood volume and acid-base balance, to name a few. Approximately 80% of the total oxygen consumed by the kidneys is related to electrolyte transport, primarily by proximal tubular cells that are responsible for about 2/3 of all tubular electrolyte transport.
To maintain proper kidney function, it is essential to regulate the rate by which the blood is filtered across the glomerular membrane. This rate is referred to as the glomerular filtration rate (GFR). There are several factors influencing the GFR: the filtration area, the membrane permeability, and the hydrostatic and oncotic pressures. The resistance of the afferent and efferent arteriole determines the blood flow, and the relative constriction between the two regulates the hydrostatic pressure within the glomerulus (P$_{cap}$), and thus the driving force for the GFR. A factor opposing filtration is the hydrostatic pressure in Bowman’s space (P$_{BS}$). This pressure is influenced by the rate by which the filtered primary urine is reabsorbed from the early proximal tubule back to the peritubular capillaries. In the glomerular capillaries, the oncotic
pressure ($\Pi_{\text{one}}$) is made up by proteins in the blood. Since proteins are not filtered across to Bowman’s space, the $\Pi_{\text{one}}$ in the primary urine is generally assumed to be close to zero.

**Development of diabetic nephropathy**

Diabetes is the most common cause for chronic and end stage renal failure. More than 30% of all diabetic patients will eventually develop diabetic nephropathy. The first quantifiable change in this development is thickening of the glomerular basement membrane. Later, mesangial expansion, known as diffuse glomerulosclerosis, occurs, inducing glomerular enlargement and clinical manifestations such as glomerular hyperfiltration. Through positive feedback, the decrease in GFR will increase the capillary pressure, inducing glomerular damage.

Podocyte integrity and numbers are related to diabetes-induced alterations in glomerular membrane permeability, although it is not elucidated what is cause and what is effect. However, hypertrophy of the glomerular capillaries might cause the podocytes to stretch. If so, when the hypertrophy of the glomerular capillaries would exceed the stretching ability of the podocytes, spaces would form between the podocytes and result in unwanted leakage.

After the initial phase with renal hyperfiltration and hypertrophic alterations, the diabetic patients experience a quiet, non symptomatic phase, with microscopic renal alterations. Further on in the development of diabetic nephropathy follows a phase of incipient nephropathy with microalbuminuria. The diabetic nephropathy is considered manifest when this phase aggravates, albuminuria is persistent, blood pressure increases, and GFR declines. For some patients, this stage results in renal failure and the need for dialysis or transplantation. It has been demonstrated that diabetic patients requiring hemodialysis have a decreased survival rate compared to other hemodialysed patients.

**Alterations in glomerular filtration rate**

There are several hypotheses for the mechanisms leading to the onset and progression of diabetes-induced, progressive renal dysfunction. Early in the development of diabetic nephropathy, glomerular pressure increases and the glomerular filtration rate is augmented. The glomerular hypertension and hyperfiltration may play a crucial role in the development of diabetic nephropathy, and thus, prevention or inhibition of these increases may have beneficial effects on renal function in diabetes.
In healthy kidneys, the glomerular capillary pressure is maintained by autoregulation\(^5\). However, in the development of nephropathy, it is possible that autoregulated hyperfiltration helps to maintain overall GFR short-term, but concomitantly augments glomerular hydrostatic pressure, which accelerates the renal damage\(^5\). This is thought to be mediated mainly by afferent vasodilation\(^5\). It has been suggested that autoregulation is defect due to hyperglycemia in the diabetic state, but data is unclear\(^5\).

An increased filtration surface area is also associated with the development of diabetic hyperfiltration\(^8\). It seems that patients with glomerular enlargement secondary to mesangial expansion, are more susceptible to diabetic nephropathy if they are unable to compensatory preserve their glomerular filtration surface area\(^5\).

Diabetes is associated with an increase in renal oxygen metabolism\(^9\). The increased oxygen consumption (\(Q_{O2}\)) could be an essential component of renal hypertrophy, hyperperfusion, and hyperfiltration commonly observed in the diabetic kidney. Sodium/potassium adenosine triphosphatase (Na\(^+\)/K\(^+\)-ATPase) is ubiquitously expressed in the basolateral membrane of tubular cells, where it creates the driving force for Na\(^+\) reabsorption\(^10\). Since the Na\(^+\)/K\(^+\)-ATPase is responsible for a majority of the cortical oxygen consumption, it has been proposed that the increased oxygen utilization occurs secondary to increased Na\(^+\)/K\(^+\)-ATPase protein expression. Numerous studies have shown an increased renal Na\(^+\)/K\(^+\)-ATPase activity in the kidneys of streptozotocin (STZ)-induced diabetic animals\(^9, 11-13\), and in other diabetes models\(^14-16\).

It is possible that the increased Na\(^+\)/K\(^+\)-ATPase activity could partially be responsible for diabetes-induced glomerular hyperfiltration, since an increased Na\(^+\)/K\(^+\)-ATPase could affect \(P_{BS}\) by decreasing the hydrostatic early proximal tubular pressure (\(P_{tub}\)), thus opposing filtration across the glomerular membrane.

**Prevention and treatment of diabetic nephropathy**

Relationships between diabetic duration and renal pathology are imprecise, due to variability in susceptibility as well as treatment\(^5\). However, the degree of metabolic control is clearly associated with the risk of developing diabetic nephropathy. The Diabetes Control and Complication Trial concluded that the degree of hyperglycemia predicts long-term diabetic renal complications\(^17\), making a proper glycemic control of utmost importance to prevent renal damage. It seems clear that strict glycemic control is an effective way to reduce hyperfiltration\(^5\). Hence, combinations of slow- and fast-acting in-
sulin, as well as improved delivery via continuous, subcutaneous insulin pumps have been developed to achieve optimal glycemic control. Recently, transplantation of pancreatic beta cells in order to restore islet mass has become a reality for diabetic patients, but mainly for those already on the list for a kidney transplant. In the future, human embryonic stem cells may become a novel source for generation of pancreatic beta cells for the treatment of diabetes mellitus.

Since $P_{cap}$ appears to be implicated in the development of diabetic nephropathy, reducing glomerular hypertension protects against the development of diabetic nephropathy. It has been shown that dilators of the efferent arteriole, such as angiotensin-converting enzyme inhibitors, as well as afferent constrictors, such as low protein diets, improve long-term function in similar manner.

Even patients with good glycemic control may develop complications. Despite strict metabolic control, more than 15% of the patients in The Diabetes Control and Complication Trial developed microalbuminuria during the 9 year duration of the study. Thus, it seems that a factor other than insulinopenia is at least partially, involved in the development of renal complications.

**Prevention by proinsulin C-peptide**

C-peptide is the 31-residue cleavage product of insulin synthesis, and is secreted from the islets of Langerhans, along with insulin in equimolar amounts. In the early 1980s, it was suggested that proinsulin C-peptide may exert biological functions, and during the past few years, studies have reported reno-protective effects from C-peptide. When, as in insulinopenic diabetes, insulin synthesis is impaired, C-peptide will be affected to the same extent. In STZ-treated rats, administration of physiological doses of C-peptide has been shown to reduce diabetes-induced glomerular hyperfiltration, decrease albuminuria and renal hypertrophy, and normalize glomerular volume. Recently, the normalizing effect of C-peptide on diabetic hyperfiltration was confirmed in conscious, unrestrained rats (Fig. 2). In that study, conscious GFR was measured as plasma clearance of a single bolus injection of fluorescein isothiocyanate inulin. It has been shown that pancreatic transplantation reverses glomerulopathy in insulinopenic diabetic patients, an observation that fits in well with the beneficial and reno-protective effects of C-peptide on renal function.
It has also been reported that C-peptide decreases hyperfiltration in patients with insulinopenic diabetes, and that C-peptide supplementation to insulinopenic patients during a period of 1-3 months is accompanied by improved renal function. Notably, peptides with the same amino acid composition as C-peptide, but in randomized sequences, have no such effects. Hence, the lack of C-peptide has successively emerged as a possible mechanism for the development of the disproportionate burden of complications affecting insulinopenic diabetes patients.

Mechanisms for C-peptide in diabetic nephropathy
Although it seems clear that C-peptide reduces most diabetes-induced alterations, the exact mechanisms mediating these beneficial effects of C-peptide remain unclear. Experimental data supports that C-peptide binds to and exerts its effects via a G-protein-coupled receptor, there is still no conclusive evidence. Also, the fact that hyperinsulinemic, diabetic patients develop similar complications and at a similar rate as insulinopenic diabetes patients, may speak in favor of effects dependent on the insulin receptor. Another
factor suggesting insulin-like behavior are the metabolic effects of C-peptide, such as increased glucose utilization and lowering of blood glucose concentrations\textsuperscript{24, 36, 37}.

There are reports of C-peptide-induced intracellular Ca\textsuperscript{2+} increases, activation of phospholipase C, RhoA, and Akt, as well as stimulation of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase via protein kinase C (PKC)-induced activation of mitogen-activated protein kinase (MAPK)\textsuperscript{33, 38, 39}. It is possible that downstream C-peptide effects induce transcriptions factors, but although several intracellular effects of C-peptide have been described \textit{in vitro}, no fully explanatory model has been presented so far\textsuperscript{34}.

Reducing diabetic glomerular hyperfiltration

GFR is determined by the glomerular membrane permeability, filtration surface area, differences in hydrostatic pressures, and the difference in $\Delta\Pi_{\text{ onc}}$. Thus, to lower the diabetic hyperfiltration, C-peptide must alter at least one of the parameters determining GFR.

Although an increased filtration area is associated with diabetic hyperfiltration\textsuperscript{5}, there are few reports of acute alterations in filtration surface area\textsuperscript{40}. One possibility is that C-peptide constricts afferent arterioles and/or dilates efferent arterioles (Fig. 3). By constricting the afferent arteriole or dilating the efferent, C-peptide would reduce the capillary pressure. This would lower the net filtration pressure ($P_{\text{ net}}$) and reduce GFR. However, altered vascular tone of the afferent or efferent arterioles should induce alterations in renal blood flow, which has not been reported for C-peptide\textsuperscript{26}.
Figure 3 Constriction of the afferent or dilation of the efferent arteriole will reduce the capillary pressure. Thus, filtration pressure is lowered, inducing a reduction in glomerular filtration rate.

Another parameter that may affect filtration is early $P_{\text{tub}}^{41-43}$. Diabetes is associated with increased proximal tubular Na\(^+\) reabsorption\(^{44}\). Changes in the proximal fluid reabsorption rate may induce changes in GFR through an effect on $P_{\text{tub}}^{45}$. If increased Na\(^+\) excretion is due to a decreased proximal Na\(^+\) reabsorption that could increase the hydrostatic pressure in the proximal tubule, and thereby affect $P_{\text{BS}}$. An increase in $P_{\text{BS}}$ would decrease the net driving force for filtration across the membrane, leading to a reduction in GFR. Thus, any factor reducing fluid reabsorption in the early proximal tubule could reduce GFR. C-peptide-treated diabetic animals have been reported to display increased urinary Na\(^+\) excretion\(^{27}\), implying an inhibitory effect of C-peptide on tubular Na\(^+\) reabsorption.

In addition, a C-peptide-mediated effect on Na\(^+\) reabsorption could have further beneficial consequences. When glomerular hyperfiltration increases
the tubular Na\(^+\) load, this increases the oxygen demand and thereby tissue Q\(_{O2}\). This is amplified by the enhanced Na\(^+\) reabsorption due to increased glucose co-transport in diabetes. Numerous studies have reported increased renal Na\(^+\)/K\(^+\)-ATPase activity and increased Q\(_{O2}\) during the early hyperfiltration phase in STZ-induced diabetes\(^{46, 47, 9, 11, 12, 15}\), and in other models of experimental diabetes\(^{48}\). Since Na\(^+\)/K\(^+\)-ATPase consumes most of the oxygen in the renal cortex\(^{49}\), an influence of C-peptide on cortical Na\(^+\)/K\(^+\)-ATPase activity could influence not only GFR, but also Q\(_{O2}\) and oxygen availability.

The importance of glycemic control

Optimal glycemic control is essential to minimize the risk for diabetes-induced complications, but the majority of diabetic patients fail to achieve proper long-term glucose levels even in clinical trials, and even more so in clinical practice\(^{17}\). The majority of diabetic patients are reported not to be confident to manage their disease themselves\(^{50}\), and one out of five diabetic children place their injections inappropriately\(^{51}\). By achieving good glycemic control, a subcutaneous insulin pump markedly reduces the risk of complications in diabetic patients\(^{17}\).

A major benefit of the insulin pump is that it reduces the need for multiple daily injections. Injections are painful and cause skin trauma, which causes complications for patients to comply with their treatment. Every fourth diabetes patient taking insulin reports anxiety regarding self-injection\(^{50, 52}\). This reluctance and suboptimal compliance in patients using multiple daily injection regimens, together with the perceived risk of hypoglycemia, are factors which reduce the glycemic control\(^{53}\). Finally, patients express a preference for discrete, easy to use treatments\(^{54}\).

Compliance to a treatment regimen is likely to be higher if the procedure is simple, painless, and discrete. Insulin has so far been suggested for nasal, gastrointestinal, as well as inhalation therapy\(^{52, 55}\). Although gastrointestinal and nasal administrations have been unsuccessful so far, an insulin inhaler was recently developed and approved\(^{56}\). However, since insulin is also a potent growth factor, there is concern that intra-alveolar deposition of insulin could adversely affect pulmonary function\(^{57, 58}\). Other routes are therefore to prefer, which do not compromise the comfort and future health of diabetic patients.

Intradermal delivery of insulin

For drugs that easily diffuse across the skin barrier, patch-based intradermal drug delivery provides convenient drug administration without the draw-
backs of subcutaneous injections. Recently, a device with miniaturized needles was developed that combines the simplicity and discretion of patch-based treatments, but with the potential of peptide and protein administration\textsuperscript{59-62}.

Our largest organ, the skin, has two main functions: it is an excellent physical barrier for protection, and a sensitive receiver of external sensory stimuli. The skin can be divided into three sections; the epidermis, dermis and hypodermis, or subcutaneous tissue (Fig. 4). The epidermis is approximately 100 $\mu$m thick and consists of an outer barrier part, stratum corneum, where stacked, dead cells are continuously replaced by outward moving, new cells produced in the more basal layer. The underlying dermis is more than 1000 $\mu$m thick and constitutes a thick supportive layer, containing nerve endings, blood vessels, sweat glands, and hair follicles. The subcutaneous tissue, finally, is a layer of adipose tissue serving as an energy reservoir and thermal insulation\textsuperscript{63}.

![Figure 4 Schematic illustration depicting a cross section of the skin, with epidermis, dermis, and subcutaneous tissue.](image)

The standard insulin treatment involves subcutaneous injections, which includes that the syringe is inserted through the nociceptor-rich dermis into the subcutaneous tissue (Fig. 4). Microneedles, on the other hand, are shorter
than 1 mm\textsuperscript{62}, and are also considerably thinner (Fig. 5), making microneedle delivery of any substance a minimally-invasive injection technique. Since the microneedles only penetrate part of the dermis, they do not reach most of the nociceptors. Thus, microneedle insertions are perceived as painless\textsuperscript{62}.

\textbf{Figure 5} Scanning Electron Microscopy picture showing a complete microneedle device displayed next to two standard, 20G and 27G syringes\textsuperscript{65}.

If sufficient bioavailability can be obtained using this newly developed technique for intradermal administration, one could achieve the advantages of subcutaneous drug delivery, but in a discrete and minimally invasive manner (Fig. 6). Since studies report needle size and fear of pain as two major reasons for injection anxiety\textsuperscript{52}, such a device could improve patient acceptance, and the development of a “controlled release”-design could further prevent long-term complications. Due to the small size and user comfort, the microneedle approach is a potentially very good candidate for future insulin delivery.
Figure 6  Photomicrograph showing a pattern in the skin of a human subject after dye injection through the patch-like microneedle device\textsuperscript{62}.
Aims of the investigation

Study I investigates and compares the effects of a small C-peptide fragment, EVARQ, with that of the intact C-peptide on diabetes-induced glomerular hyperfiltration in rats.

Study II investigates vascular reactivity of isolated afferent arterioles from diabetic C57/Bl mice to endogenous administration of C-peptide, in order to understand the mechanisms of C-peptide-induced vasoconstriction.

Study III investigates if altered renal afferent-efferent arteriole tonus mediates the reduction of diabetes-induced glomerular hyperfiltration, and how this relates to blood flow alterations. Furthermore, this study investigates the effect of C-peptide on renal sodium handling and oxygen usage in diabetic rats.

Study IV evaluated an integrated patch-like microneedle system in vivo for delivery of insulin.
Methods

Animals and induction of diabetes

The animals were housed in a facility at the Laboratory Animal Resources of the BioMedical Centre, Uppsala University in Uppsala. All animals were fed standard chow (R3, Ewos, Södertälje, Sweden) and water ad libitum. All experiments were approved by the Uppsala Ethical Committee for Animal Experiments, and were performed in accordance with national guidelines for the care and use of laboratory animals.

For all diabetic animals, body weight (bw) and blood glucose concentrations were monitored every other day throughout the entire experimental period, in order to evaluate the degree of hyperglycaemia and severity of diabetes. Blood samples were obtained from the cut tips of the tails and analysed by a glucose oxidase method (15-20 μl, Precision QID, MediSense, Bedford, MA, USA). Animals were excluded if they developed a weight loss of more than 10%, or if the blood glucose concentrations exceeded 30 mM (560 mg/dl) on more than two consecutive measurements. Age-matched normoglycemic animals served as controls.

All chemicals used in these experiments were purchased from Sigma-Aldrich, St Louis, MO, USA and were of the highest grade available unless otherwise stated.

Rats (Studies I, III, and IV)

In Study I, III and IV, 160 male Sprague-Dawley rats were used. Two weeks prior to the acute experiment, the rats were made diabetic by means of a single intravenous injection of STZ (50-55 mg/kg bw) dissolved in 0.2 ml saline. This resulted in blood glucose concentrations above 20 mM. STZ is derived from Streptomyces achromogenes. It was developed in the 1960s as an antibiotic, but was soon found to induce beta cell destruction. STZ consists of a glucose moiety attached to nitrosourea, and the toxicity has been shown to be due to the nitrosourea decomposing to methyl ions after being transported by specific glucose transporters into the beta cells. Today, STZ is widely used to induce diabetes in experimental models of diabetes mellitus.
In Study III and IV, the rats were treated daily with a subcutaneous injection of long-acting insulin (Insulatard, Novo Nordisk, Hjørring, Denmark; 5 IU/kg bw/day).

Mice (Study II)

In Study II, 73 male C57BL/6 mice were used. The mice were made diabetic by means of a single intravenous injection of alloxan (75 mg/kg) two weeks prior to the experiment, increasing blood glucose concentrations to above 18 mM. Diabetes was induced with alloxan. Alloxan, originally used as raw material for a purple dye, is one of the oldest organic compounds named\textsuperscript{73, 74}, and has been used for induction of diabetes for more than 60 years\textsuperscript{75}. Although not toxic to human beta-cells\textsuperscript{76}, it destructs rodent beta cells by interactions with the oxidative metabolism by the formation of superoxide radicals\textsuperscript{77, 78}.

Experimental protocols, treatment regimens, drugs and preparations

Study I

Five groups of animals were studied for two consecutive 20 minute-periods before and after 60 minutes of drug administration: control rats given vehicle only (n=10), control rats given rat C-peptide (50 pmol/kg bw/min; n=9), diabetic animals given vehicle (n=8), diabetic rats given rat C-peptide (50 pmol/kg bw/min; n=9), and diabetic rats given the C-peptide fragment EVARQ (500 pmol/kg bw/min; n=9).

Study II

Seven groups of mouse afferent arterioles were studied before and after drug administration: a normoglycemic group administered vehicle only (n=4), a normoglycemic group receiving scrambled C-peptide (5 nM, n=5), a normoglycemic group receiving C-peptide (5 nM, n=9), a hyperglycemic group receiving vehicle (n=4), a hyperglycemic group receiving scrambled C-peptide (5 nM, n=5), a hyperglycemic group receiving C-peptide (0.5 nM, n=7), and a hyperglycemic group receiving C-peptide (5 nM, n=7).

The involvement of Rho-kinase in C-peptide-induced vasoconstriction was investigated by administering the Rho-kinase inhibitor Y27632 (1 μM, n=7)\textsuperscript{79}.
Study III

In vivo, hyperglycemic (n=9) and normoglycemic (n=8) animals were measured at baseline and after 40 minutes of C-peptide or vehicle infusion. For measurements of tubular pressures, 6 hyperglycemic animals were measured at baseline and after 40 minutes of C-peptide infusion.

In vitro, four groups were studied. The effect of C-peptide on QO2 was measured in isolated proximal tubular cells from normoglycemic (n=9) and hyperglycemic (n=12) animals during baseline and after incubation with ouabain (1 mM) or No-nitro-L-arginine-metyl-ester (L-NAME; 37 μM).

Study IV

Blood glucose concentrations were measured at baseline, and after 60, 120, 180, and 240 minutes of insulin infusion. Plasma concentrations of insulin lispro were measured after 30, 90, 150, and 210 minutes of infusion.

Seven groups of diabetic animals were studied before and after drug administration: intravenous saline solution only (n=9), intravenous insulin infusion (0.14 IU/h, 70 IU/ml, n=7), subcutaneous insulin infusion, (0.20 IU/h, 100 IU/ml, n=9), microneedle-aided intradermal low-rate insulin infusion (0.20 IU/h, 100 IU/ml, n=9), microneedle-aided intradermal high-rate insulin infusion (0.40 IU/h, 100 IU/ml, n=9), microneedle-aided passive intradermal insulin diffusion (100 IU/ml, n=9), and insulin applied onto microneedle-penetrated skin (100 IU/ml, n=9).

Preparations and measurements

Anesthesia (Study I, III, and IV) and euthanasia

13-15 days after the induction of diabetes, the animals were anaesthetized with an intraperitoneal injection of sodium-5-sec-butyl-5-ethyl-2-thiobarbiturate (Inactin).

All mice were sacrificed by cervical dislocation. All rats were sacrificed under anesthesia by an intravenous injection of saturated potassium chloride.

Surgical procedure (Study I, III and IV)

In studies I, III and IV, the anaesthetized rats were placed on a servo-controlled heating pad in order to maintain temperature at 37.5°C, and tracheotomized to facilitate spontaneous breathing. The right femoral artery was catheterized for monitoring of mean arterial blood pressure (MAP) and for blood sampling. The right femoral vein was catheterized for substance
administration and infusion of isotonic saline at a rate of 5 ml/kg bw/h for non-diabetic rats and 10 ml/kg bw/h for diabetic rats. The urinary bladder was catheterized for the collection of urine.

In Study III, the left kidney was exposed by a left subcostal flank incision, immobilized in a plastic cup, embedded in pieces of saline-soaked cotton wool, and the surface covered with paraffin oil (Apoteksbolaget, Gothenburg, Sweden). Kidney weights were determined at the end of the experiment.

Blood pressure (Studies I, III and IV)

Blood pressure was continuously measured by connecting the arterial catheter to a pressure transducer (Statham P23dB; Statham Laboratories, Los Angeles, CA, USA), and recorded on a polygraph (Model 7D Polygraph, Grass Instrument Co., Quincy, MA, USA) or a MacLab instrument (AD Instruments, Hastings, UK).

Tissue isolation, dissection and perfusion (Study II)

After cervical dislocation, the kidneys were removed and sliced along the corticomedullary axis. The afferent arterioles were dissected using sharpened forceps under a stereoscopic microscope at +4°C in Dulbecco’s Modified Eagle Medium enriched with 1% albumin. With intact glomeruli, the afferent arterioles were transferred into a thermoregulated chamber. The use of a flexible perfusion system allows for adjustments of holding and perfusion pipettes (Fig. 7).
To achieve physiological pressure and flow, the perfusion pressure was set to 100 mmHg at the pressure head of the perfusion pipette. The diameter of the perfusion pipette was 26 μm at the tip, and the holding pipette, which was put into the lumen of the afferent arteriole, had a diameter of 5 μm. The experiments were recorded by a video system with a Nikon water immersion lens and a digital camera.

Measurements of oxygen consumption (Study III)

In vitro
Proximal tubular cells were isolated as previously described\(^9\, 46\). In brief, renal cortical tissue was minced through a metallic mesh strainer and immediately placed in an ice-cooled buffer solution and incubated in collagenase (0.05% wt/vol) at 37°C for 60 minutes while equilibrated with 95% O\(_2\)/5% CO\(_2\). Thereafter, the cell suspension was cooled to +4°C and filtrated through graded filters with pore sizes of 180, 75, 53 and 38 μm, in order to separate isolated tubular cells from glomerulus. The isolated cells were then pelleted by slow centrifugation (100 g, 4 minutes) and re-suspended in a collagenase-free buffer, a procedure that was repeated three times. The sus-
pension was kept on ice until \( Q_{O2} \) was measured according to a previously described procedure\(^4\). A custom-made thermostatically-controlled (+37°C) gas-tight plexiglas chamber was filled with buffer solution (in mM: 113.0 NaCl, 4.0 KCl, 27.2 NaHCO\(_3\), 1.0 KH\(_2\)PO\(_4\), 1.2 MgCl\(_2\), 1.0 CaCl\(_2\), 10.0 HEPES, 0.5 Ca lactate, 2.0 glutamine, osmolality 298±2 mOsm), and continuously stirred with an air-driven magnetic stirrer. The glucose concentration in the medium was 5.8 mM (100 mg/dl) for cells from normoglycemic controls and 23.2 mM (400 mg/dl) for cells from diabetic animals. A modified Unisense 500 oxygen-sensing electrode (Unisense, Aarhus, Denmark), calibrated with air-equilibrated or Na\(_2\)S\(_2\)O\(_5\) saturated buffer solution was used. 100 μl of cell suspension was then injected into the chamber, and the rate of oxygen disappearance was measured.

At the end of each experiment, a sample was taken to determine protein concentration. In all groups, the rate of oxygen disappearance was adjusted for protein concentration. The effect of C-peptide on \( Q_{O2} \) was estimated with and without preincubation with ouabain or L-NAME.

**In vivo**
The \( Q_{O2} \) of the whole kidney *in vivo* was measured as the artero-venous difference in blood oxygen content, multiplied by the renal blood flow. Blood gas parameters were analyzed in samples drawn from the left renal vein and femoral artery (ISTAT, Abbott, Solna, Sweden).

**Blood flow estimations (Study III)**
Total renal blood flow (RBF) was measured using an ultrasound probe (Transonic Systems, Ithaca, NY, USA) placed around the left renal artery, and cortical RBF was measured with laser-Doppler flowmetry (PF 4001–2; Perimed, Stockholm, Sweden). All parameters were continuously recorded with a MacLab instrument (AD Instruments) connected to a Macintosh Power-PC 6100.

**Measurements of tubular pressures (Study III)**
\( P_{\text{net}} \) was estimated by stop-flow technique at the baseline and 40 minutes after the infusion of C-peptide was commenced (bolus 5 nmol/kg bw + continuous infusion 50 pmol/kg bw/min). Early proximal tubular segments (5/rat) were randomly chosen on the kidney surface for each measurement. A pipette, filled with 1 M NaCl and Lissamine green, connected to a servonull pressure system (World Precision Instruments, New Haven, CT, USA) was used to determine proximal tubular free-flow (\( P_{\text{ff}} \)) and stop flow pressure (\( P_{\text{str}} \)), the latter after tubular flow was interrupted with a wax blockade distal to the pressure pipette. \( P_{\text{net}} \) was calculated according to \( P_{\text{str}} - P_{\text{ff}} \).
Intradermal infusion of insulin (Study IV)

A patch-like microneedle device was attached on depilated skin (potassium thioglycolate, Veet Creme, Reckitt Benckiser, Slough, UK) using moderate hand force. The device was then fixated onto the skin with surgical tape.

The microneedles were fabricated in monocrystalline silicon by Deep Reactive Ion Etching. To allow active delivery, the needles are hollow with the needle bore-opening located on the side of the needle, and are organized on a chip in arrays (Fig. 8). A sharp and well-defined needle tip allows microneedle insertion into the skin by hand. The system also includes a small, active dispenser mechanism based on a novel thermal actuator consisting of highly expandable microspheres. When actuated, these microspheres expand into a liquid reservoir, dispensing of stored liquid through the microneedles.

![Figure 8 Drawing showing an exploded view of the setup of the used drug delivery micro system. When voltage is passed through the heater, the expandable composite heats up and expands into the drug reservoir, consequently ejecting the liquid through the hollow needles on the chip.](image)

Analyses and calculations

Estimation of glomerular filtration rate (Study I and III)

$^3$H-inulin (3-5 μCi/ml; American Radiolabeled Chemicals, St. Louis, MO, USA) was infused at a rate of 5 ml/kg bw/h for non-diabetic rats and 10
ml/kg bw/h for diabetic rats. GFR was estimated by urinary clearance of $^3$H-inulin before and after the infusion of vehicle, C-peptide, or EVARQ. The $^3$H activities in urine and plasma were measured using a standard liquid scintillation technique. The GFR was calculated according to $\text{GFR} = U \cdot V / P$, where $U$ and $P$ denote the activity of $^3$H-inulin in urine and plasma respectively, and $V$ denotes the urine flow rate (ml/min).

Electrolyte analysis (Study I and III)

Urine volumes were measured gravimetrically. Urinary sodium and potassium concentrations were measured by flame photometry (Model IL 543, Instrumental Laboratory, Milan, Italy). Osmolality was measured by freezing point depression (Model 3MO, Advanced Instruments, MA, USA). Urine excretion rates were calculated by multiplying urinary concentrations with urinary flow rates.

Measurements of arteriole contraction (Study II)

The video sequences were digitalized, and the vessel luminal diameters estimated using customized software. At the end of a recovery period, baseline control values were obtained for each experiment. Hereafter, luminal diameter was measured every 5 minutes for a total of 30 minutes.

Calculations in Study III

The filtration fraction (FF) was estimated as $\text{FF} = \text{GFR/RBF} \cdot (1 - \text{Hct})$. Renal vascular resistance (RVR) was calculated as $\text{MAP} / \text{RBF}$. In vivo renal $\text{Q_O2} (\mu\text{mol/min})$ was estimated from the arterio-venous difference in $\text{O}_2$ content ($\text{O}_{2\text{ct}} = [\text{Hb}] \cdot s\text{O}_2 \cdot 1.34 + \text{PO}_2 \cdot 0.22$) multiplied by total $\text{RBF}^{80}$. Tubular $\text{Na}^+$ transport ($T_{\text{Na}}; \mu\text{mol/min}$) was calculated using $\text{T_{Na}} = \text{[P_{Na}] / GFR}$. $\text{T_{Na}}$ per $\text{Q_O2}$ was calculated according to $\text{T_{Na}} / \text{Q_O2}$. Estimates of the fractional $\text{Na}^+$ excretion were obtained from the calculation $\text{[UNa]} / \text{[Pinulin]} / \text{[P_{Na}] / [U_{inulin}]}$.

Insulin analysis (Study IV)

Plasma insulin lispro concentration was determined using a $^{125}$I-monoiiodinated, competitive-binding radioimmunoassay analysis (Lispro insulin RIA kit, Linco Research, MO, USA).
Statistical method and presentation of data

In all studies, when analyzing two data sets with normal distribution, unpaired or paired Student’s t-tests were applied for comparisons between or within the same group respectively.

In Study I, multiple data sets between groups were analyzed using analyses of variance (ANOVA) followed by Tukey’s post hoc test when appropriate.

In Study II, multiple data sets between groups were analysed with non parametric Kruskal Wallis followed by Dunn’s post hoc test, or ANOVA followed by Fisher’s post hoc test when appropriate. Multiple data sets within groups were analysed with repeated measurements ANOVA followed by Dunnett’s post hoc test for paired comparisons.

In Study III, multiple comparisons between different groups were performed using ANOVA followed by Fisher’s protected least significant difference test. Multiple comparisons within the same group were performed using repeated measures ANOVA followed by Dunnett’s post-hoc test for paired comparisons.

In Study IV, multiple data sets between groups were analyzed with ANOVA followed by Tukey’s post hoc test, when appropriate. Multiple data sets within groups were analyzed with ANOVA followed by Dunnett’s post hoc test, when appropriate.

All statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). Descriptive statistics are presented as mean values ± standard error of the mean (SEM). For all comparisons, P <0.05 was considered to be statistically significant.
Results

Study I

Glomerular filtration rate

All diabetic groups displayed a pronounced glomerular hyperfiltration compared to the non-diabetic rats (Fig. 9). The hyperfiltration persisted during the infusion of vehicle, whereas C-peptide and EVARQ reduced GFR to baseline levels. Infusion of C-peptide in control rats had no effect.

Figure 9  C-peptide and EVARQ decrease glomerular hyperfiltration in diabetic rats. Modified from\textsuperscript{28}.
Blood glucose concentrations
All diabetic animals displayed elevation of blood glucose concentrations (Fig. 10). When compared to baseline values, C-peptide, as well as EVARQ, reduced the blood glucose in diabetic animals. Control animals were unaffected by C-peptide, and vehicle treatment did not affect blood glucose in any group.

![Blood Glucose Concentrations Graph](image.png)

*Figure 10  C-peptide and EVARQ decrease blood glucose in diabetic rats.* denotes $P<0.05$ vs. vehicle-treated control group. Modified from\textsuperscript{28}.

Urinary sodium excretion
Baseline urinary excretion of Na\textsuperscript{+} was similar in all groups (Fig. 11). During the experiments, urinary Na\textsuperscript{+} excretion increased in all groups, except for the vehicle-treated control group.
Study II

Afferent arteriole diameter
C-peptide administered to afferent arterioles from hyperglycemic mice reduced afferent arteriolar diameter within 10 minutes. After 30 minutes, C-peptide had decreased afferent arteriolar diameter by -27±8% (Fig. 12 and 13). Normoglycemic animals given C-peptide showed a minor, but significant decrease in arteriolar diameter after 30 minutes only (4±1%; Fig. 14). Scrambled C-peptide or vehicle had no effect.
Figure 12  C-peptide-induced constriction of an isolated renal afferent arteriole from a diabetic mouse. A. the arteriole at baseline, B. the arteriole after 15 minutes of C-peptide perfusion, and C. the arteriole after 30 minutes of C-peptide perfusion. Photos from Study II.
Figure 13  The effect of C-peptide, scrambled peptide, and vehicle on afferent glomerular arterioles from hyperglycemic C57-Bl mice. * denotes p<0.05 vs. all other groups. Modified from Study II.

Figure 14  The effect of C-peptide, scrambled peptide, and vehicle on afferent glomerular arterioles from normoglycemic C57-Bl mice. Modified from Study II.

Rho-kinase inhibitor Y-27632

The effect of C-peptide was prevented by the Rho-kinase inhibitor Y-27632. The inhibitor did not affect vessel diameter per se (data not shown).
Study III

Blood pressure and renal parameters
Diabetic rats had increased GFR and FF compared to controls (Fig. 15). C-peptide selectively reduced these parameters in the diabetic animals. MAP and RVR (Fig. 16) were similar during baseline, and C-peptide decreased both parameters in both groups.

![Figure 15: Glomerular filtration rate (left panel) and filtration fraction (right panel) in normoglycemic controls and diabetic rats before and after the infusion of C-peptide. Figures from Study III.](image)
Fractional Na\(^+\) excretion and transported Na\(^+\)

Diabetic rats had similar baseline fractional Na\(^+\) excretion to control animals, but C-peptide increased the fractional Na\(^+\) excretion selectively in diabetic animals (Fig. 17). Baseline T\(_{Na}\) was higher in diabetic animals compared to controls, and was only reduced by C-peptide in the diabetic group (Fig. 17).

There was no difference in either total or cortical RBF in the diabetic animals compared to the normoglycemic controls. C-peptide did not alter RBF in any of the investigated groups. The infusion of vehicle did not alter any of the investigated parameters (GFR, FF, MAP, RVR, fractional Na\(^+\) excretion, or RBF).

Figure 16  Renal vascular resistance in normoglycemic controls and diabetic rats before and after the infusion of C-peptide. Figure from Study III.
In vivo oxygen consumption

Diabetic animals displayed increased total in vivo $Q_O2$, although $T_{Na}/Q_O2$ did not differ between diabetic and normoglycemic control rats. C-peptide or vehicle did not affect either total $Q_O2$ or $T_{Na}/Q_O2$ in any of the two groups.

Micropuncture parameters: filtration pressures

C-peptide reduced the $P_{f}$ by 5.2% (11.6±0.2 vs. 11.0±0.2 mmHg), $P_{stf}$ by 8.4% (41.9±0.3 vs. 38.4±0.3 mmHg), and the calculated $P_{net}$ by 9.6% (30.3±0.4 vs. 27.4±0.3 mmHg) in diabetic rats (Fig. 18).
Figure 18 Tubular free-flow pressure (A.), stop-flow pressure (B.), and calculated glomerular net filtration pressure (C.) in diabetic rats before and after C-peptide. Figures from Study III.

In vitro oxygen consumption

Isolated proximal tubular cells from diabetic animals displayed increased baseline $Q_{O_2}$ compared to controls (39±3, vs. 28±2 nmol/mg protein/min) (Fig. 19). Treatment with C-peptide reduced $Q_{O_2}$ in cells from STZ-treated rats (28±3 nmol/mg protein/min), but had no effect on cells from normoglycemic controls (26±5 nmol/mg protein/min). The transport-independent $Q_{O_2}$
was higher in diabetic cells compared to controls (18±2 vs. 13±3 nmol/mg protein/min). C-peptide had no effect on ouabain-pretreated cells (17±6 vs. 18±2 nmol/mg protein/min). Pretreatment with L-NAME had no effect on any of the investigated groups, and did not alter the effect of C-peptide on the diabetic cells.

![Oxygen consumption by isolated proximal tubular cells from normoglycemic controls and diabetic rats during baseline and after incubation with C-peptide, ouabain, L-NAME or a combination of C-peptide and either ouabain or L-NAME. * denotes P<0.05 when compared to baseline in the control group. Figure from Study III.](image)

**Figure 19** Oxygen consumption by isolated proximal tubular cells from normoglycemic controls and diabetic rats during baseline and after incubation with C-peptide, ouabain, L-NAME or a combination of C-peptide and either ouabain or L-NAME. * denotes P<0.05 when compared to baseline in the control group. Figure from Study III.

**Study IV**

**Plasma insulin concentrations**

The subcutaneous and microneedle infusions displayed similar increases in plasma insulin concentrations (Fig. 20). Animals receiving the intravenous infusion did not show any changes in insulin concentrations between the first and last measurement.
Blood glucose concentrations

All animals displayed a pronounced elevation of blood glucose after the induction of diabetes (Fig. 21). Sixty minutes after the start of the insulin infusion, blood glucose was lowered in the intravenously-infused and the microneedle-infused groups. Intravenous, subcutaneous, and intradermal insulin infusion all lowered blood glucose concentration after 180 minutes.
Figure 21 Effect of different administration routes of insulin lispro on blood glucose concentration. IV = intravenous, SC = subcutaneous, microneedle = patch-like microneedle device. * denotes P<0.05 vs. baseline in corresponding group. Modified from65.
Discussion

It has now been established that diabetes-induced glomerular hyperfiltration is reduced by C-peptide in patients\textsuperscript{23, 32} as well as in animal models of insulinopenic diabetes\textsuperscript{20, 22}, with concomitant reduction of diabetes-induced renal damage\textsuperscript{22, 23, 25, 31}. The main new findings in this thesis are that acute administration of C-peptide reduces GFR in diabetic rats via more than one mechanism, and that this effect can be achieved by infusion of only the C-terminal penta-peptide sequence. The results also suggest that the vascular effects of C-peptide involve Rho-kinase. The final part of this thesis demonstrates a new administration route for insulin which may have several benefits compared to today’s standard therapy.

Penta-peptide effects

In Study I, the infusion of the intact C-peptide, as well as the carboxy-terminal penta-fragment EVARQ, abolished diabetes-induced glomerular hyperfiltration. The effect of EVARQ on diabetic hyperfiltration was a reduction of the GFR within an hour from the start of administration, while vehicle-infused diabetic rats maintained a substantial hyperfiltration throughout the experiments. Hence, it is possible that the five amino acid sequence of the carboxy-terminal of C-peptide is the sequence, or one of the sequences, mediating the normalizing effect on GFR in these diabetic rats.

It is common that proteins have defined functional sites. A fragment of gonadotropin-releasing peptide, displays increased activity compared to the intact peptide\textsuperscript{81}. Gastrin, CCK and osteogenic growth peptide, have active sites in their C-terminal penta-peptides\textsuperscript{82-85}.

C-peptide and glomerular hyperfiltration

Evidence is increasing of the beneficial effects of C-peptide on the diabetic kidney, and it seems clear that C-peptide can reduce diabetic hyperfiltration. But what causes this effect of C-peptide on hyperfiltration? A possible mechanism is a direct effect of C-peptide on the renal afferent arteriole. It was concluded in Study II that C-peptide constricts the afferent arteriole.
from diabetic mice, but not from normoglycemic animals. A constriction of the afferent arteriole will lower glomerular filtration pressure and thus decrease GFR.

In study III, two seemingly different mechanisms are reported for the C-peptide-induced decrease of diabetic glomerular hyperfiltration. First, C-peptide dilated the efferent arteriole in vivo, as evident from the decreased RVR, FF, and $P_{stf}$ in the absence of altered RBF in the diabetic rats. Secondly, C-peptide inhibited the tubular Na$^+$ reabsorption as evident from the increased fractional Na$^+$ excretion in diabetic rats and reduction in transport-dependent $Q_{O2}$ by the isolated proximal tubular cells from diabetic rats.

Glomerular filtration rate and tubuloglomerular feedback

It has previously been postulated that diabetic hyperfiltration occurs due to alterations in tubuloglomerular feedback (TGF)$^{86}$. TGF is an intrarenal mechanism that stabilizes GFR, and thus the tubular Na$^+$-load to match the tubular Na$^+$ handling capacity (Fig. 22). The anatomical prerequisite for TGF is the return of the tubule to its own glomerulus. These, together with the macula densa (MD) make up the juxtaglomerular apparatus. The MD consists of specialized epithelial cells localized where the returning tubule passes the glomerulus, and constitutes a sensor mechanism for Na$^+$, but preferentially Cl$. Increased tubular flow rate will increase the tubular NaCl load, which is sensed by the MD, and results in a constriction of the afferent arteriole. The results from Study III show that C-peptide inhibits Na$^+/K^+$-ATPase, and this inhibition will increase the NaCl load to the MD, activating the TGF to constrict the afferent arteriole.
Even though the data may seem to fit into the hypothesis that C-peptide decreases hyperfiltration via a TGF-altering mechanism, this conclusion is not entirely self-evident. Although it is likely that Na⁺/K⁺-ATPase is inhibited, the afferent constriction from Study II occurs in isolated arterioles, where the arteriole is not set up with an intact tubulus. In addition, it was recently shown by Sällström et al. that TGF does not mediate diabetes-induced hyperfiltration⁴¹, since diabetes-induced glomerular hyperfiltration occurs in adenosine A1-receptor-deficient mice known to lack a functional TGF mechanism⁴¹, ⁴², ⁸⁷. If TGF is not the mediator of diabetic hyperfiltration, it is unlikely that C-peptide would exert the effect on filtration via a TGF-dependent mechanism. That would render it unlikely that the two effects seen on the nephron in this thesis are related or interlinked. Therefore, a possible alternative conclusion might include that C-peptide exerts its effect on glomerular filtration via two separate mechanisms.

The tubular hypothesis of glomerular filtration

As an alternative to TGF-mediated regulation of filtration, “The Tubular Hypothesis of Glomerular Filtration” has been stipulated as a possible model for the regulation of GFR⁴³. This hypothesis postulates that proximal tubular
reabsorption will determine GFR through alterations in $P_{\text{tub}}$ that directly influence $P_{BS}$. It has been shown that sustained hyperglycemia in patients as well as experimental animal models, results in increased proximal tubular $Na^+$ reabsorption secondarily to increased $Na^+$-linked glucose reabsorption. Furthermore, it has also been shown that early distal tubular $Na^+$ concentrations decrease during hyperglycemia, together with increased cortical $Na^+/K^+$-ATPase expression. Conversely, decreased $Na^+$ reabsorption in the early proximal tubule will increase $P_{\text{tub}}$ and thereby augment $P_{BS}$, which in turn lowers $P_{\text{net}}$ and therefore reduces GFR. This hypothesis is consistent with reports of a linear correlation between GFR and $Na^+/K^+$-ATPase activity in the kidney cortex in several models of experimental diabetes. Therefore, a reduced or normalized $Na^+$ reabsorption in the early proximal tubule is likely to reduce GFR in the diabetic kidney.

Separate effects on capillary and proximal pressure

If the effects of C-peptide on the glomerular afferent arteriole and the inhibition of $Na^+/K^+$-ATPase are not related, that opens the possibility of three separate filtration lowering mechanisms: constriction of the afferent arteriole, dilation of the efferent arteriole, and proximal tubular reabsorption (Fig. 23). Although a reduction in the filtered electrolyte load could also reduce reabsorption, it will not in itself affect the fractional reabsorption (as seen in Study III), and it cannot explain the findings on the isolated proximal tubular cells.

![Figure 23](image-url)

**Figure 23** Three plausible, separate mechanisms by which C-peptide may decreases diabetes-induced glomerular hyperfiltration supported by results presented in this thesis.
As stated in the introduction, a main factor determining acute alterations in the GFR is $P_{\text{net}}$ (Fig. 24 and 25), which is determined by the $P_{\text{cap}}$ and $P_{\text{BS}}$. Changes in $P_{\text{cap}}$ are a result of alterations in the interplay of afferent and efferent arteriolar resistance, which alter the pressure transmitted into the glomerular capillaries. $P_{\text{tub}}$ is mainly determined by proximal tubular reabsorption, and under certain conditions by the hydraulic resistance in the more distal nephron segments, e.g. tubular obstruction. Since $P_{\text{tub}}$ will affect $P_{\text{BS}}$, and thus is one of the determinants of $P_{\text{net}}$, changes in the proximal reabsorption rate have the potential to influence the GFR.

$$P_{\text{net}} = P_{\text{cap}} - P_{\text{tub}}$$

*Figure 24* Relationship between net filtration pressure ($P_{\text{net}}$), glomerular capillary pressure ($P_{\text{cap}}$) and early proximal tubular pressure ($P_{\text{tub}}$).

GFR is also influenced by differences in $\Delta \Pi_{\text{once}}$ (Fig. 25). $\Delta \Pi_{\text{once}}$ is determined by differences in the protein concentration between the blood in the glomerular capillaries and the primary urine in Bowman’s space.
The main intrarenal factor influencing acute changes in $\Delta \Pi_{\text{onc}}$ is the FF$^{90, 91}$. A reduced FF will decrease the difference between the blood in the glomerular capillaries and the primary urine, thereby decreasing the protein concentration in the blood that leaves the glomerulus through the afferent arteriole. Thus, FF can reduce the driving force for reabsorption along the proximal part of the nephron into the peritubular capillaries$^{92}$. The decrease in FF seen in Study III supports the involvement of reabsorption in the mechanism of C-peptide-reduced reduction of GFR.

No reduction was seen in total Na\(^+\) excretion after C-peptide, a finding that is consistent with previous studies$^{36}$. However, the reduction in tubular Na\(^+\)-load, due to reduced GFR after C-peptide, will conceal the inhibitory effect on total urinary Na\(^+\) excretion. The calculated fractional Na\(^+\) excretion increased in the diabetic animals after C-peptide administration, which shows that C-peptide directly inhibits Na\(^+\) reabsorption exclusively in the
diabetic rats. The observed differences in the reduction of $P_{\text{stf}}$ and $P_{\text{ff}}$ in this study may also indicate a component of proximal tubular reabsorption in the decrease in GFR.

Renal glomerular arteriolar vascular tone and blood flow

Renal glomerular arterioles exert critical of $P_{\text{cap}}$, thereby influencing GFR. However, a pronounced constriction of the afferent arterioles alone would reduce GFR, FF and RBF, and concomitantly increase RVR. Indeed, C-peptide reduced the elevated GFR and FF in the present study, but without altering RBF whatsoever. Although an unaltered RBF is consistent with previous reports, these results suggest that C-peptide reduces hyperfiltration partly via constriction of the afferent glomerular arteriole, but without affecting renal blood flow, findings that imply there must be a simultaneous dilation of the efferent arteriole that counterbalances the effect on RBF. The finding reduced RVR and $P_{\text{stf}}$ after C-peptide administration even indicates a net dilation of the efferent arteriole in vivo.

Cellular C-peptide signaling

The vasoconstrictive effect of C-peptide takes considerable time to develop (10 minutes), compared to angiotensin II or norepinephrine. An explanation could be a sequential cascade of intracellular events leading to vasoconstriction, e.g. through the activation of a receptor with subsequent effects on protein synthesis. Although it seems clear that C-peptide possesses a reducing effect on diabetic hyperfiltration, there is still no effector, no receptor, and no downstream signalling cascade reported for this phenomenon. So what causes this effect of C-peptide on hyperfiltration?

C-peptide and Rho-kinase

The G-protein Rho and its downstream effector Rho-kinase play important roles in mediating vasoconstriction in the kidney. They regulate glomerular blood flow and GFR, as well as the function and structure of renal cells such as tubular epithelial cells and mesangial cells. Inhibition of Rho-kinase in constricted renal afferent arterioles, as well as in other vessels, causes a relaxation. Rho/Rho-kinase seems to act in the tonic phase of constriction. Although this phase is stimulated by $\text{Ca}^{2+}$ influx and Rho/Rho-kinase is activated by $\text{Ca}^{2+}$, vasoconstriction is maintained at modestly elevated $\text{Ca}^{2+}$ levels, a phenomenon referred to as $\text{Ca}^{2+}$-sensitization. Except for $\text{Ca}^{2+}$ influx or $\text{Ca}^{2+}$ sensitization Rho-kinase activity has also been shown to be stimulated independently of $\text{Ca}^{2+}$.
We show in this study that the Rho-kinase inhibitor Y-27632 prevents the vasoconstrictive effects of C-peptide *in vitro*. These results are in line with previous data by Zhong *et al.*, showing translocation and binding of RhoA by C-peptide in renal tubular cells. A C-peptide-induced activation of Rho-kinase could be attributable to an increase in Ca\(^{2+}\) sensitivity.

However, the preventive effects of Y-27632 on C-peptide-induced vasoconstriction of isolated afferent arterioles do not necessarily imply that C-peptide activates Rho-kinase. C-peptide is known to increase intracellular Ca\(^{2+}\) in human tubular cells, smooth muscle cells, and aortic endothelial cells, and it has been reported that C-peptide activates PKC via increased intracellular Ca\(^{2+}\) in opossum proximal tubular cells. C-peptide also activates MAPK through a PKC-dependent mechanism. Importantly, PKC and MAPK both mediate contraction, e.g. via phosphorylation of CPI-17 and caldesmon, respectively. Additionally, the MAPK cascade has been linked to phosphorylation of the regulatory subunit of the myosin light chain in the same location as myosin light chain kinase. Inhibition of Rho-kinase will allow for greater myosin phosphatase activity, which in turn decreases the phosphorylation status of the regulatory subunit of the myosin light chain. Therefore, it is possible that C-peptide induces phosphorylation of the myosin light chain in a non-Rho-A-dependent manner, such as PKC or MAPK (Fig. 26).
Figure 26  A. Vasoconstriction is dependent on the phosphorylation of the myosin light chain, which is controlled by myosin phosphatase, mitogen-activated protein kinase (MAPK) and myosin light chain kinase (MLCK). B. The myosin phosphatase activity is regulated by Rho kinase and protein kinase C (PKC).
C-peptide and oxygen consumption

Electrolyte transport via Na\(^+\)/K\(^+\)-ATPase activity accounts for approximately 80% of total renal Q\(_{O_2}\). Therefore, a diabetes-induced increase in Na\(^+\)/K\(^+\)-ATPase activity is likely to influence total renal Q\(_{O_2}\), possibly limiting renal O\(_2\) availability\(^{46, 47}\). The finding of increased cellular Q\(_{O_2}\) in diabetic rats in Study III is in good agreement with previous reports\(^9, 111\). However, Study III shows that C-peptide directly inhibits Q\(_{O_2}\) in isolated proximal tubular cells, but the in vivo total kidney Q\(_{O_2}\) did not differ before and after C-peptide administration, even though T\(_{Na}\) decreased by approximately -17%. A possible explanation can be a shift of Na\(^+\) reabsorption to other sites along the nephron after the inhibition of proximal tubular Na\(^+\)/K\(^+\)-ATPase activity. The Q\(_{O_2}\) per T\(_{Na}\) in the proximal tubulus is significantly less compared to more distal parts of the nephron, since a notable part of the Na\(^+\) reabsorption in the proximal tubulus is achieved via paracellular transport\(^{112}\). This explanation would account for the lack of reduced Q\(_{O_2}\) in vivo in C-peptide-treated diabetic rats.

Ouabain inhibits Na\(^+\)/K\(^+\)-ATPase, although complete inhibition cannot be achieved in rats\(^{113, 114}\). Ouabain-treatment reduced cellular Q\(_{O_2}\) in both investigated groups, but C-peptide had no further effect on ouabain-treated cells. This strongly indicates that C-peptide reduces Na\(^+\)/K\(^+\)-ATPase activity. Notably, this would not be unique for C-peptide, since insulin also has been shown to influence tubular electrolyte transport\(^{115}\).

Sustained hyperglycemia is closely associated with a reduced nitric oxide bioavailability, which might influence Q\(_{O_2}\). A possible mechanism for the observed reduction in Q\(_{O_2}\) might therefore be C-peptide-mediated effects on NO production. However, in Study III, inhibition of NO production by L-NAME had no effect on the C-peptide-induced decrease in Q\(_{O_2}\) by the isolated tubular cells.

Potential tissue or state-specificity of C-peptide

Although the beneficial effects of C-peptide in diabetes mellitus have been observed in various tissues\(^{116, 117}\), no complete mechanism, receptor, or downstream signalling cascade has yet been proposed to fully explain these effects, and existing data seem contradictory. In this thesis, C-peptide induced a constriction of the renal afferent arteriole. Blood flow seems to be differentially affected in the kidney compared to peripheral tissues in the diabetic state\(^{118-120}\), and when previous studies have showed normalizing effects of C-peptide on diabetes-impaired blood perfusion and blood cell velocity\(^{116, 121}\), C-peptide has had a normalizing effect on diabetes-induced decreases in blood perfusion\(^{122-124}\), indicating a dilating effect. In renal ves-
sels, sustained diabetes will cause increased blood flow\textsuperscript{118}. Thus, it is likely that C-peptide exerts tissue-specific effects.

Data sometimes seem contradictory also on the molecular level. Neurons, heart and skeletal muscles from diabetic animals, as opposed to diabetic proximal tubules, display reduced Na\textsuperscript{+}/K\textsuperscript{-}-ATPase activity\textsuperscript{117, 125}. C-peptide has been reported to improve autonomic nerve function via stimulation of Na\textsuperscript{+}/K\textsuperscript{-}-ATPase activity\textsuperscript{126, 127}, and seems to increase erythrocyte Na\textsuperscript{+}/K\textsuperscript{-}-ATPase in diabetes\textsuperscript{128, 129}. A diabetes-induced decrease in Na\textsuperscript{+}/K\textsuperscript{-}-ATPase activity also occurs in the renal medulla after long-term hyperglycemia\textsuperscript{130}, and C-peptide seems to stimulate medullary Na\textsuperscript{+}/K\textsuperscript{-}-ATPase activity after an extended period of hyperglycemia\textsuperscript{131}. In glomerular tissue from diabetic rats, Na\textsuperscript{+}/K\textsuperscript{-}-ATPase activity may decrease as well as increase as a result of long-term hyperglycemia\textsuperscript{132, 133}. Taken together, most studies report pronounced effects of C-peptide on the Na\textsuperscript{+}/K\textsuperscript{-}-ATPase activity, but C-peptide seems to have tissue-specific effects during insulinopenic diabetes. Furthermore, most of the previous studies on C-peptide-induced effects on Na\textsuperscript{+}/K\textsuperscript{-}-ATPase activity in the kidney, have been conducted during normoglycemia and by applying pharmacological doses\textsuperscript{86, 131}. Thus, it cannot be excluded that the effects are crucially dependent on hyperglycemia or the length of C-peptide deficiency.

Lack of effect on normoglycemic animals

In neither Study I, nor III was there any effect of C-peptide on normoglycemic animals with normal GFR. In Study II, the afferent arterial diameter was unaffected by C-peptide until the very last measurement. The fact that normoglycemic mice and rats did not respond to C-peptide is consistent with previous studies\textsuperscript{23, 134, 135}. Also, as reported previously, perfusion with scrambled C-peptide did not produce any effect\textsuperscript{27, 38, 117, 136, 137}.

The lack of effect in normoglycemic animals can be explained by C-peptide, or a residual effect of C-peptide, being present in these animals before the start of exogenous administration. This explanation is supported by the observation that the full effect of C-peptide is obtained already at nanomolar concentrations\textsuperscript{136}.

C-peptide-induced reduction of blood glucose

In Study I, C-peptide as well as EVARQ reduced blood glucose concentrations in diabetic rats. The effect of C-peptide on blood glucose has previously been described\textsuperscript{134}. Sato and co-workers have reported that EVARQ increases glucose turnover to the same extent as intact C-peptide\textsuperscript{37}, which
suggests that the active site within the C-peptide that mediates these metabolic effects is located within the EVARQ sequence.

**Intradermal insulin treatment**

Good glycemic control is essential in order to minimize the risk of diabetes-induced complications\(^1\)\(^7\). Study IV shows no disadvantage using the intradermal patches as compared to the standard, subcutaneous delivery route. Subcutaneous injections are known to cause a substantial variability in dose delivered\(^5\)\(^3\). Intradermal delivery showed a controlled distribution to the systemic circulation, and even displayed a trend towards lower variability, possibly due the size of the infusion area, which minimizes the influence of the infusion site. The novel, patch-like microneedle-assisted intradermal infusion, showed a pronounced effect on blood glucose before the effect on blood glucose was obvious using subcutaneous infusion. This could also be due to the larger infusion area of the microneedle array, compared to the single-needle subcutaneous infusion.

Previously, few data exist from *in vivo* studies on diabetic animals, and never on animals given the time to develop the skin characteristics of diabetes mellitus\(^1\)\(^3\)\(^8\). Previous studies have been performed on small groups of anaesthetized rats without facilitated breathing, compensation for fluid losses or monitoring of hematocrit and blood pressure to guarantee physiologically relevant conditions\(^1\)\(^3\)\(^9\). No previous reports describe an integrated system where the microneedles are attached to a drug dispenser, and there is little previous research on active microneedle delivery, where insulin can be administered in a controllable fashion. The results for Study IV show that the newly developed device has all these characteristics.

It is not at all unlikely that compliance to insulin treatment would be higher if the treatment procedure was simple and painless, as with this patch-like microneedle device\(^6\)\(^1\). In parallel with avoiding multiple injection regimens, needle anxiety, and social difficulties associated with self-injecting, this novel administration route requires minimal training and attention. Thus, the patch-like microneedles are a feasible option to improve glycemic control, and might reduce the development of long-term diabetes complications.

Study IV presents a possibility of controllable and patient-friendly insulin delivery. The results indicate that microneedle arrays used as intradermal patches should be used clinically, and might introduce a more efficient treatment for diabetic patients. This microneedle technology could also prove to be efficient in systemic as well as local delivery of other macromolecular drugs, such as DNA vaccines, and endocrine substances\(^1\)\(^4\)\(^0\)-\(^1\)\(^4\)\(^4\).
Summary and conclusions

Taken together, the results from these studies support the conclusion that C-peptide affects diabetes-induced glomerular hyperfiltration via several seemingly different mechanisms. These include constriction of the afferent arteriole and dilation of the efferent arteriole, but possibly also reduction of the proximal tubular electrolyte reabsorption, all factors influencing GFR.

The results from Study I show that the in vivo effects of C-peptide can be mimicked by its carboxy-terminal penta-fragment EVARQ. The infusion of EVARQ decreased filtration to the same extent as the intact C-peptide. This suggests at least one active site within the EVARQ region of C-peptide.

The main new findings from Study II, are that C-peptide has a pronounced vasoconstrictive effect on afferent arterioles from diabetic mice. This effect seems to be dependent on Rho-kinase, since the Rho-kinase inhibitor Y27632 prevented any C-peptide effect.

In Study III, acute administration of C-peptide reduced GFR in diabetic rats via possibly two separate mechanisms. First, C-peptide caused a net dilation of the efferent arteriole. Secondly, C-peptide inhibits tubular Na\(^{+}\) reabsorption in diabetic rats, likely via a direct effect on the Na\(^{+}\)/K\(^{+}\)-ATPase.

Study IV showed that intradermal microneedle insulin administration reduced plasma glucose and increased plasma insulin comparably to standard, subcutaneous administration, indicating that the microneedle approach should be tested clinically, and might provide a more efficient treatment of diabetic patients.
Future perspectives

The results from these studies display several opportunities for new drug targets and research directions.

Since it theoretically should be simpler to develop agonists to a pentadecapeptide than a 31-amino acid peptide, the \textit{in vivo} effect of EVARQ may simplify the development of agonists for the clinical settings. Research should aim at investigating the potential of EVARQ treatment via less invasive delivery systems, such as preparations for inhalation, intradermal delivery or oral administration. Finally, the development of antagonists against C-peptide targets could provide valuable tools in the search for the pathophysiological mechanisms mediating the development of diabetic nephropathy.

Since the reducing effect of C-peptide on diabetes-induced glomerular hyperfiltration is achieved without affecting blood flow, further studies should be undertaken, investigating the effect of C-peptide on the intricate interplay between the afferent and the efferent arteriole and on renal Na\textsuperscript{+} reabsorption, e.g. on expression and trafficking of Na\textsuperscript{+}-transporters.

Concomitantly, the effectiveness of C-peptide on the prevention and reversal of diabetic complications, should be more thoroughly investigated in human subjects. To achieve this, long-term, large-scale studies should be conducted in order to evaluate safety and health benefits of long-term administration of C-peptide or a C-peptide derivate.

Finally, this thesis shows that the newly developed microneedles are a possible treatment strategy for the commonly used, fast-acting insulin lispro. Further studies should aim at investigating the microneedle device in terms of increased flow rate, biocompatibility and develop devices with incorporated blood glucose sensing and self-controlled insulin rate.

\textit{Meum cerebrum nocet}
Sammanfattning på svenska


Njurskador vanliga vid diabetes

Ofta drabbas diabetiker av komplikationer som ytterligare försämrar deras hälsa. Båda typerna av diabetes leder under årens lopp många gånger till livshotande följder, t.ex. njurskador. Så mycket som 30% av de diabetiker som haft sjukdomen i 10-20 år uppvisar påtagliga njurskador.

Njuren spelar en mycket viktig roll inte bara för utsöndringen av diverse skadliga ämnen från kroppens ämnesomsättning, utan också för regleringen av kroppens blodtryck. Denna reglering sker på så sätt att kroppens vätskevolym bestämmer blodtrycket. Ju större vätskevolym, desto högre blodtryck. I njurarna filtreras allt blod kontinuerligt över från små kapillärer, glomeruli, till ett rörsystem, tubuli. Regleringen av kroppens blodtryck sker sedan genom att njurarna reglerar hur mycket vätska som tas tillbaka till kroppen igen och hur mycket som ska stanna kvar i tubuli för att så småningom ledas ut ur njuren till urinblåsan.

Idag vet man att minutiöst övervakade blodsockernivåer minskar riskerna för skador och att själva skadeprocessen kan bromsas med noggrann insulinbehandling och en aggressiv blodtryckssänkande behandlingsstrategi. Någon egentlig bot finns dock fortfarande inte och heller ingen annan behandling som i längden kan bromsa sjukdomsförloppet. Detta medför inte bara mycket lidande för de drabbade, utan kostar även sjukvården och samhället i övrigt stora summor pengar, eftersom patienterna ofta tvingas till sjukpension och omfattande vård.

Inte bara insulin


Ett av de första tecknen på att njurarna påverkats hos en diabetespatient är att njurarna tillväxer och att de filtrerar alltför mycket, något som belastar njuren och de kapillärer, glomeruli, där filtrationen sker. Senare övergår denna ökade filtration istället till en underfiltration, och proteiner börjar läcka ut till urinen. Alla dessa parametrar anses vara viktiga markörer för begynnande njurskador. Det är inte klart vad som orsakar dessa fenomen, men tidigare studier har visat att C-peptid kan minska den ökade filtrationen till normala nivåer hos både försöksdjur och diabetiska patienter. Mekanismen bakom detta är dock okänd.

Ett steg i att kartlägga C-peptids verkningsmekanism och bana vägen för nya läkemedel, är att undersöka vilken del av molekylen som ger effekt. I avhandlingsarbetets första studie undersökte vi om en kortare bit av C-peptid, EVARQ, kan ge samma effekt som hela molekylen. Studien visade att EVARQ sänker den ökade filtrationen hos diabetiska råttor lika effektivt som hela C-peptidmolekylen. På sikt skulle detta kunna underlätta utvecklingen av nya läkemedelsmolekyler som, till skillnad från C-peptid, inte bryts ned i magtarmkanalen och därmed kan tas i exempelvis tablettform.
C-peptid skyddar


Sammantaget antyder försöken att C-peptid minskar belastningen på njurens nefron genom att dra ihop de tillförande kärlen, vidga de frånledande
samt eventuellt skapa ett mottryck via en hämmande effekt på Na⁺/K⁺-ATPas.

En sorts plåster med miniatyrnålar

För att förebygga uppkomsten av njursjukdom hos diabetiker krävs att blodsockernivåerna noggrant hålls stabila, något som kräver ständigt förbättrade behandlingsformer och att patienten är följsam och använder sina läkemedel på ett optimalt sätt. I den fjärde och avslutande studien testade vi en sådan ny behandlingsform, en sorts plåsterliknande beredning med mycket korta och tunna, smärtfria mikronålar på undersidan, som bara är en tiondels millimeter tjocka.

I våra studier sänkte mikronålarna blodsockret mer förutsägbart än traditionell behandling. Resultaten visade att mikronålarna mycket väl borde kunna användas för tillförsel av insulin till diabetiker. Eftersom sådana smärtfria ”nålplåster” inte gör ont och alltså torde vara mindre obehagliga att använda än de injektioner diabetiker är beroende av idag, borde följsamheten också öka, så att fler patienter stabiliserar sina blodsockernivåer och på så sätt minskar risken för följdsjukdomar. Dessa nålar öppnar dessutom för ett helt nytt sätt att tillföra peptidbaserade läkemedel, såsom insulin och C-peptid.

Ett fortsatt vetenskapligt arbete kan ge en fördjupad insikt i de sjukdomsprocesser som ligger bakom de njurskador och därmed också hjärtkärlsjukdomar som ofta uppstår hos diabetespatienter. I bästa fall kan de bidra till att bana vägen till nya behandlingsstrategier. Kanske skulle följdsjukdomar kunna stoppas om insulinbehandlingens förändrades samtidigt som C-peptid gavs redan vid insjuknandet, allt som en naturlig del av behandlingen.
Acknowledgements

This work was performed at the department of Medical Cell Biology, Uppsala University, Sweden. It was financially supported with grants from The Swedish Medical Research Council, The Swedish Heart and Lung Foundation, The Swedish Society for Medical Research, The Fredrik and Ingrid Thuring Foundation, The Marcus and Amalia Wallenberg Foundation, The Magnus Bergvall Foundation, NIH K-99 grant (DK-077858), The Wallenberg Foundation, The Swedish Foundation for Strategic Research (SFF), The Ingabritt and Arne Lundberg Foundation, The Wenner-Gren-Foundation, Medartuum, Stiftelsen Sveriges Farmaceutiska Sällskap, Svensk Njurmedicinsk Förening, Stiftelsen Petersenska Hemmet, Fredrika Bremer Stiftelsen, Håkanssons stipendiefond, Rönnows stipendiefond, Kvinnliga Akademikers Förening (KAF), Rektors Wallenbergmedel, The Swedish Pharmaceutical Society, and The Scandinavian Physiological Society.

Sincere thanks and gratitude to everyone who, in different ways, has contributed to this thesis by helping, encouraging, guiding, nodding or shaking your heads during these years. I utterly hope you already know how much I have appreciated your support, but if I haven’t showed it enough, don’t ever think I didn’t notice!

In particular, I would like to thank:

My supervisor, Mats Sjöquist, for introducing me to research, and for inspiring me to think independently. Thanks also to my co-supervisor, Peter Bergsten, for enthusiastic, bold ideas.

Erik Persson, for thoughtful and invaluable help. Leif Jansson for sharing much needed wisdom and inspiration, Britta Isaksson, for always taking the time to help, and for helping to perfection. It simply wouldn’t have been possible without you. Angelica Fasching, for untiring help, for encouragement, and for laboratory skills beyond belief. All the other corridor seniors; Peter Hansell, Johanna Henrikznäs, Lena Holm, Annika Jägare, Örjan Källskog, Mia Phillipson, Göran Såhl, Mats Wolgast et al. For your vast knowledge in practical as well as theoretical aspects of research, for challenging and stimulating discussions, for surströmming, kräftskivor, and november-
fester. I have enjoyed working with you. A special thanks to Astrid Nordin for your excellent technical assistance.

A great thank-you also to my co-authors and contributors: To Enyin Lai for excellent skills and company and for being a good friend, to Niclas Roxhed for changing faculty over night and becoming a life scientist instead of being a civil engineer. Erika Moe for making it worth it in the very begin-
ning, Russell Brown for unimaginable speed, Göran Stemme for making the insulin-patch project a reality, Patrick Griss for turning Swiss fondue and wine into science, and of course to Marco Seiz for cooking it in the first place.

My warmest thanks to Carolyn Ecelbarger for welcoming me to your lab and for involving me in your research. Thanks also to Swasti Tiwari, Shahla Riazi and Xinqun Hu for fun and hard-working summers at the lab, for in-
spiring collaboration, and for taking the time to share your experience.

Thanks also to the C-peptide group at Karolinska Institute, Stockholm. For rat C-peptide and scrambled C-peptide kindly provided from the Dr Ekberg lab. To Emma Lindahl, the catcher in the rye. And of course to profes-
sor John Wahren for helpful discussions and encouragement.

Birgitta Klang, Gunno Nilsson, and all students for making teaching inter-
esting, uncomplicated, worthwhile, and fun. Agneta Bäfwe, Kärstin Flink, Marianne Ljungkvist, Karin Öberg, and Gun-Britt Lind, for all your help with the practicalities regarding employment, conferences, leaves of ab-
sence, courses, et cetera. I am seriously impressed by your organizing skills.

Thanks also to the BMC Animal Facility for taking good care of my rats and mice, to the BMC Reception for numerous new key cards, and to BMC Foto for helping me make posters and prints, even though I was always late and often confused.

Daniel Damper Färnstrand, for untiring, kind and skillful revision of my at times slightly overcomplicated Swenglish language. For taking away most of the Who Dunnit? from the manuscripts. Thank you.

Joel, Mattias and Tommy, Johan Fiasko, Olerud, Andrei, Professor Enyin, Zofu, Jenny, Malou-Malou, Olof, Lazlo Deputy Brown, Micke Fluff, Louise, Nina, Sara, Åsa, Ulrika and Gustav: Thank you all for help, coffee breaks, conference professionalism, relaxed Friday beers, spontaneous partying and lab intrigues. And of course to Magnus – best friend and PhD-student in arms.
A special thought to Göran Odmark for forcing me to think: you are truly one of a kind, and without you, I would probably have ended up at a completely different faculty.

Crull, Thérese, Emil, Malin, e-Rolf and Tanya: thanks for nothing but headaches, you bastards. And possibly some friendship.

To M., Odli, HMS T. and the Winers; closer than thee. And to Emelie F-C, for sometimes spontaneously pulling me away for a fast gallop.

For helping me through statistics, setbacks and endless lab sessions: my frank gratitude to Dionne Warwick, Aretha Franklin, Maria Callas, Georg Friedrich Händel, Dolly Parton, Marvin Gaye, Kasey Chambers, Monica Zetterlund, Eric Cartman, Georges Bizet, Björn&Benny, Diana Ross, Antonio Carlos Jobim, Roger Whittaker, Lill Lindfors and, of course, Stevie Wonder.

For the essentials of life: wet kisses to Daniel, Erica, Isabelle, Lilla Gris, Mårten, Saris, Snibben, Tossa (as well as Naken-Janne and Metro-Christer), Åsan, Aida, and all other friends. Thank you for your support, friendship, and for valuing one another for everthing but academic merits. I cannot believe I am finally here! Thank you too, Mårten, even though you discuss science and urine samples after midnight.

Mams och paps, Saris och Larz: thank you for your unconditional love, handiwork, midnight korvmackor, patience and support. Moreover, Saris, sorry for all the lattes and Sven Dufva lunches I ran away from to get back to being boring. Kommissarie Rex next week? Och så klart en stor kram till världens bästa mormor och morfar!

Soon, the storm has finally faded. Thank you, Fredrik, for being constructive when I was not. For challenges, for encouragement, and for keeping my feet on the ground. For covered-up Trollhål. For sharing your life with me. For making me happy. For loving me back.

In utter panic, Uppsala, October 2007

Ad astra per alas porci
References


83. I. Bab, H. Gavish, M. Namdar-Attar, A. Muhlrad, Z. Greenberg, Y. Chen, N. Mansur, A. Shteyer, and M. Chorev, Isolation of mitogenically


vasoconstriction in the porcine palmar lateral vein, J Pharmacol Exp Ther 311(2), 742-7 (2004).


*Estne tibi forte mus magnus albus planissimus??*

*Ubi est mea anaticula cumminosa?*
A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)