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Renal Effects of C-peptide in Experimental Type-1 Diabetes Mellitus

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Schopenhauer

Till Cilia, Johan, Ida och Maria

LIST OF ORIGINAL PAPERS

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

- I.** **Samnegård B**, Jacobson SH, Jaremko G, Johansson BL, Sjöquist M. Effects of C-peptide on glomerular and renal size and renal function in diabetic rats. *Kidney Int* 2001 Oct;60(4): 1258-65.
- II.** **Samnegård B**, Jacobson SH, Johansson BL, Ekberg, K, Isaksson B, Wahren J, Sjöquist M. C-peptide and captopril are equally effective in lowering glomerular hyperfiltration in diabetic rats. *Nephrol Dial Transplant* 2004 Jun;19(6):1385-91.
- III.** **Samnegård B**, Jacobson SH, Jaremko G, Johansson B-L, Ekberg K, Isaksson B, Eriksson L, Wahren J, Sjöquist M. C-peptide prevents glomerular hypertrophy and mesangial matrix expansion in diabetic rats. *Nephrol Dial Transplant* 2005 Mar;20(3):532-8.
- IV.** **Samnegård B**, Jacobson SH, Jaremko G, Johansson B-L, Ekberg K, Isaksson B, Cooper ME, Jandeleit-Dahm K, Giunti S, Wahren J, Sjöquist M. Influence of combined C-peptide and captopril treatment on glomerular structure and function in streptozotocin diabetic rats. *In manuscript*.

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ABSTRACT

Renal Effects of C-peptide in Experimental Type-1 Diabetes Mellitus

by

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The incidence and prevalence of diabetic nephropathy are increasing continuously, mainly due to the increase in type-2 diabetes. In contrast, the risk of diabetic nephropathy for the individual patient has decreased considerably in recent decades due to improved glycemic control, successful treatment of hypertension and hyperlipidemia and the use of renin angiotensin system inhibitors, which have protective effects beyond those on blood pressure. However, optimal glycemic control and normal blood pressure offer only incomplete protection from diabetic nephropathy. Thus, other factors are likely to be involved. In type-1 diabetes, one such factor may be proinsulin C-peptide. C-peptide has been thought to lack metabolic effects but in the past decade several studies have shown that C-peptide itself can be beneficial in preventing diabetic complications.

The aims of the studies in this thesis have been to further evaluate the functional and morphological effects of C-peptide in the kidneys in experimental diabetes. Furthermore, the aim was to compare the C-peptide effects with those of an angiotensin converting enzyme inhibitor (ACEI) and to evaluate the effects of combined C-peptide and ACEI treatment.

Studies were performed in streptozotocin diabetic Sprague-Dawley and Wistar rats and the results demonstrate that C-peptide prevents or reduces diabetes-induced glomerular hyperfiltration by 17-42% ($p < 0.01$) and urinary albumin excretion and urinary albumin/creatinine ratio. Furthermore, C-peptide reduces glomerular and renal hypertrophy by 19-32% ($p < 0.01$), glomerular mesangial matrix fraction by 37% ($p < 0.001$), glomerular expression of type-IV collagen by 42% ($p < 0.001$) and prevents diabetes-induced over-expression of receptors for advanced glycation end products (RAGE). In addition, C-peptide and captopril are equally effective in preventing glomerular hyperfiltration, albuminuria and glomerular RAGE expression, besides having additive effects in lowering glomerular type-IV collagen by 90% ($p < 0.001$) and glomerular filtration fraction ($p < 0.05$). C-peptide does not affect renal blood flow significantly more than placebo.

In conclusion, in experimental type-1 diabetes, C-peptide beneficially affects risk factors or manifestations of diabetic nephropathy such as glomerular hyperfiltration, albuminuria, glomerular hypertrophy, mesangial matrix expansion and glomerular expression of type-IV collagen and RAGE. There may also be potentially beneficial effects from combining C-peptide and an ACEI in preventing this complication in type-1 diabetes.

Key words: Diabetic nephropathy, C-peptide, captopril, albuminuria, glomerular hyperfiltration, glomerular hypertrophy, glomerular mesangium expansion, type-IV collagen, RAGE, renal blood flow

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ABBREVIATIONS

ACEI	angiotensin converting enzyme inhibitor
AGE	advanced glycosylation end products
Ang II	angiotensin II
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
ARB	angiotensin receptor blocker
C-peptide	proinsulin connecting peptide
CTGF	connective tissue growth factor
DAG	diacylglycerol
DDACE	double deletion angiotensin converting enzyme
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal regulated kinase
ESRD	end stage renal disease
ET	endothelin
FITC	fluorescein-5-isothiocyanate
GBM	glomerular basement membrane
GFR	glomerular filtration rate
GLUT-1	glucose transporter 1
IGF-1	insulin-like growth factor 1
iNOS	inducible nitric oxide synthase
JNK	C-Jun N-terminal kinase
MAP	mean arterial blood pressure
MAPK	mitogen activated protein kinase
NF κ B	nuclear factor kappa B
NO	nitric oxide
PAS	periodic acid Schiff
PDGF	platelet derived growth factor
PG	prostaglandins
PKC	protein kinase C
PLC	phospholipase C
RAGE	receptor for advanced glycosylation end products

RAS	renin angiotensin system
RFR	renal functional reserve
RIA	radioimmunoassay
ROS	reactive oxygen species
RPF	renal plasma flow
RRT	renal replacement therapy
SEM	standard error of the mean
Smad	small mothers against decapentaplegic
TGF- β	transforming growth factor beta
UAER	urinary albumin excretion rate
VEGF	vascular endothelial growth factor

INTRODUCTION

In the first edition of his well-known textbook in 1916, Joslin wrote that renal complications of diabetes were unimportant [1]. He soon realized his mistake; in the second edition, from 1917, he stated that with the prolongation of life resulting from modern diabetes therapy, renal complications would deserve attention [2]. He was indeed right.

In the early 1960s chronic hemodialysis treatment became possible thanks to the Scribner shunt, which partially solved the problems of blood access. Unfortunately, diabetic patients gained little or nothing from this new therapy. In an article “The sad truth about hemodialysis in diabetic nephropathy”, Kolff’s group showed that this group had a one-year mortality of 78% and that it seemed impossible to identify the patients who might survive for longer [3]. For many years uremic diabetics were looked upon as the pariahs of medicine and nobody wanted to treat them. Nephrologists declined on account of the diabetes and diabetologists on account of the renal failure.

With more adequate diabetes treatment and renal replacement therapy, the results for diabetic patients have improved considerably. Today, these patients are no longer denied renal replacement therapy (RRT) and diabetes mellitus is now the leading, and still growing, cause of end stage renal disease (ESRD) in the Western world [4], generating a rapidly growing need for RRT in this patient group. This largely reflects an increasing incidence of diabetes mellitus type-2, in addition to longer survival and more liberal indications for RRT in diabetes. Of new ESRD patients, 45% are diabetics in the US [5] and 26% in Sweden [6]. Of prevalent ESRD patients, 37% are diabetics in the US compared with 19% in Sweden. Fortunately, the individual diabetic patient runs a lower risk of nephropathy today than just a decade ago. Earlier, the cumulative incidence of overt diabetic nephropathy after 25 years of diabetes was 25-30% but recent studies have shown a dramatic reduction, probably reflecting improved glycemic control and more aggressive blood pressure reduction [7]. In one study of type-1 diabetic patients the cumulative incidence of nephropathy after 25 years disease duration was only 8.9% [8]. The annual incidence of diabetic nephropathy is highest 10-20 years after diabetes onset and declines sharply thereafter [9, 10]. Patients without proteinuria after 20-25 years of diabetes have an annual risk of developing nephropathy of only about 1% [9], indicating that these patients are less vulnerable than those developing proteinuria early.

Natural course of diabetic nephropathy

The natural course of diabetic nephropathy has been divided by Mogensen [11] into five stages. The first describes the immediate changes after diabetes onset. About 50% of the patients present with renal hypertrophy and glomerular hyperfiltration with a glomerular filtration rate (GFR) 20-40% above that of age-matched controls. Renal plasma flow is also increased, by 9-14%. At this stage insulin therapy may reduce, but usually not normalize GFR. Stage 2 is a clinically silent phase usually lasting for 5-10 years. Urinary albumin excretion rate (UAER) and blood pressure are in most patients still normal. GFR is often still increased compared with the healthy population. Electron microscopy of the glomerulus may reveal early signs of glomerular basement membrane (GBM) thickening and increased mesangial volume fraction after 2-4 years. Stage 3, also called the phase of incipient nephropathy, follows about 5-15 years after diabetes onset; GFR is at this stage usually reduced to normal and there is microalbuminuria, i.e. UAER of 20-200 $\mu\text{g}/\text{min}$ or 30-300 $\text{mg}/24\text{h}$. The occurrence of microalbuminuria is usually associated with rising blood pressure and the morphological changes described above become more pronounced. There are also signs of arteriolar hyalinosis. Stage 4, overt diabetic nephropathy, follows in some patients characterized by a positive urinary albumin dipstick ($>300 \text{ mg}/24\text{h}$) and increasing UAER, in some cases leading to a nephrotic syndrome. A widely accepted definition of diabetic nephropathy is persistent overt proteinuria in a diabetic patient with retinopathy and no other renal disease [10]. Renal biopsy is usually not needed for diagnosis but when done it shows that at this stage the morphological lesions have become unmistakable. Hypertension is common and GFR is gradually declining, a decrease correlated with increasing blood pressure. With no intervention, GFR declines by approximately 10 mL/min per year. Stage 5, usually 25 years or more after diabetes onset, is the ESRD stage, with an absolute need for dialysis or renal transplantation.

Pathophysiology of diabetic nephropathy

With a growing prevalence of diabetic nephropathy it has become increasingly important to determine the pathophysiological mechanisms responsible for this disorder in attempts to find effective treatment options. It is proposed that the renal involvement in diabetes results from a complex interplay between metabolic and hemodynamic factors [12]. This seems to involve a range of growth factors, cytokines and intracellular signalling molecules (Fig. 1).

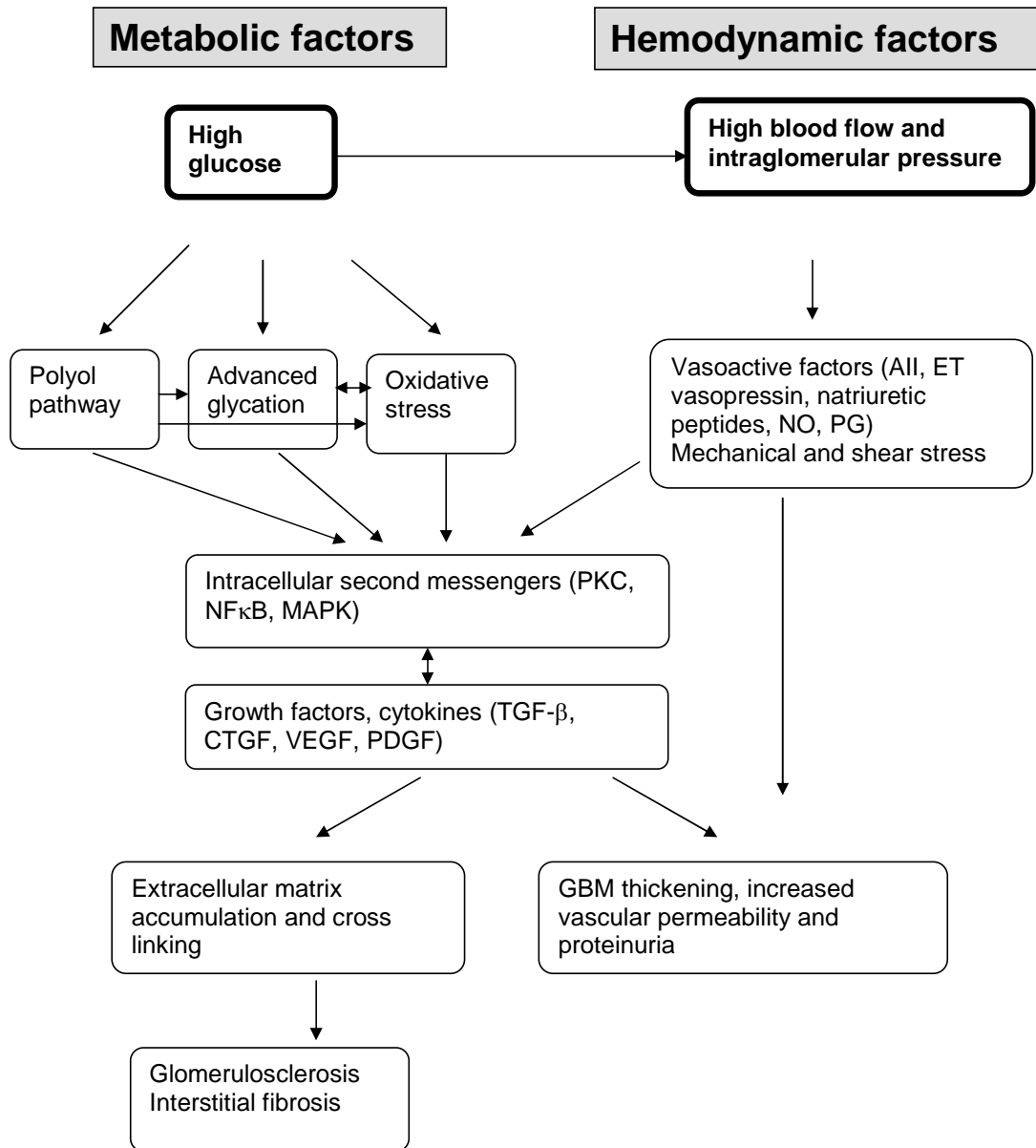


Figure 1. Schematic overview of hemodynamic and metabolic factors contributing to diabetic nephropathy.

Hemodynamic factors

It has been suggested that the earliest risk predictor for the development of diabetic nephropathy is glomerular hyperfiltration, especially in type-1 diabetes. In early type-1 diabetes the glomerular hyperfiltration is closely associated with glomerular hypertrophy and increased renal size [13]. The hypertrophy is a recognized prognostic marker [14], while the prognostic role of glomerular hyperfiltration is still under debate. Originally, Hostetter et al. presented the concept that increased glomerular hydraulic pressure actually plays an important role in the initiation and progression of diabetic nephropathy [15] and this has been confirmed later [16]. However, at the time of these studies, data on the non-hemodynamic effects of ACEI were not available. The importance of hyperfiltration is further supported by one prospective study of hyperfiltration in type-1 diabetic patients: the patients presenting with a GFR above 125 mL/min had a risk of developing microalbuminuria within 8 years of approximately 50% versus only 5% in patients with a lower GFR [17].

In experimental diabetes in animals it has been shown that the hyperfiltration results from a predominantly afferent dilatation of the arterioles that raises intraglomerular pressure and renal blood flow [18]. Micropuncture studies have also shown that, as a result of the afferent dilatation, in experimental diabetes there is an elevation of intraglomerular pressure even in the absence of systemic hypertension [16]. Thus, the renal autoregulatory response to blood pressure changes is not as effective as in non-diabetic individuals, which may explain why not just a normal but rather a low normal blood pressure seems to be beneficial in the diabetic patient. The reasons for these hemodynamic changes causing increased intraglomerular pressure are not fully understood but several factors appear to contribute: *a.* Increased sodium reabsorption along with glucose in the proximal tubules decreases distal delivery of sodium which in turn via the tubuloglomerular feedback mechanism in the macula densa raises GFR via dilatation of the afferent arteriole [19-21]. *b.* By largely unknown mechanisms, advanced glycosylation end products (AGE) can induce increases in renal plasma flow (RPF), GFR and intraglomerular blood pressure [22]. *c.* Accumulation of sorbitol via the polyol pathway seems to be important. Administration of an aldose reductase inhibitor lowers GFR towards normal in hyperfiltering type-1 diabetic patients [23]. *d.* Insulin-like growth factor (IGF-1) can in normal subjects replicate the renal vasodilatation and elevation in GFR seen in diabetic patients [24] and a somatostatin analogue (octreotide) can reverse the renal hypertrophy and glomerular hyperfiltration in early diabetes [25]. *e.* Still under debate is the exact role of the renin angiotensin system (RAS) in the diabetic patient. Studies on renal tubular cells [26] and

renal mesangial cells [27] have indicated increased RAS activity locally in the kidney, which may increase the intraglomerular pressure. In contrast, systemic renin levels are usually normal or low in diabetes. *f.* Hyperglycemia per se induces hyperfiltration via the induced volume expansion which increases the level of the vasodilating atrial natriuretic peptide (ANP). Thus, start of insulin treatment lowers GFR towards the normal range [28]. An increased or decreased influence of other vasoactive hormones has also been postulated to mediate progressive renal injury, for example vasoconstrictors such as endothelin and vasopressin and vasodilators such as NO, ANP and prostaglandins [12].

Another controversial aspect of hemodynamics in diabetes is the renal functional reserve (RFR), defined as the difference between basal GFR and the maximally stimulated GFR. In healthy subjects with normal RFR, a protein load leads to increased GFR. In diabetic subjects this physiological response is impaired [29-31]. This probably implies that the residual nephrons are already in a state of maximal GFR and RFR is then an indicator of workload per nephron [32]. Thanks to this compensatory mechanism, the GFR can remain at a normal level. Loss of RFR is, in these cases, an earlier marker of loss of nephrons than is GFR [33].

Regardless of the underlying mechanisms, the increased glomerular pressure is deleterious in that it increases the mechanical strain on the capillary walls. Besides the direct mechanical damage to the endothelium and the podocytes, this strain enhances the production of matrix components and growth factors [34], the deposition of macromolecules [35] and eventually leads to proteinuria. The damage propagates to the tubulointerstitium as proteinuria further enhances inflammation caused by increased local production of chemokines [36].

Metabolic factors

Hyperglycemia activates several metabolic factors, such as advanced glycation end products (AGE), polyol formation and oxidative stress.

Non-enzymatic glycation of proteins is greatly enhanced by hyperglycemia. The process is reversible initially but conversion into AGEs with strong cross-links occurs over months or years, whereafter the reaction is irreversible [37]. This process is faster in diabetic than in non-diabetic patients and the AGEs accumulate in patients with impaired renal function [38]. Mesangial and basement membrane accumulation of AGE correlates with the degree of nephropathy in diabetic subjects [39]. The AGE cross-links affect the function of glycosylated proteins directly and also affect AGE interaction with binding proteins such as receptors for

advanced glycosylation end products (RAGE) and soluble RAGE. This causes a downstream increase in the production of transforming growth factor beta (TGF- β), platelet derived growth factor (PDGF), type IV collagen and reactive oxygen species (ROS) [40, 41]. This, in turn, leads to fibrosis [42]. Prevention of AGE formation with aminoguanidine treatment and the use of AGE cross-link breakers have been shown to prevent albuminuria and to reduce renal fibrosis in experimental diabetes [42, 43].

Accumulation of polyols has been proposed to be involved in the development of diabetic nephropathy [44]. In the polyol pathway, glucose is converted to sorbitol by aldose reductase and in the next step to fructose by sorbitol dehydrogenase. Compared with the healthy individual, the relative importance of this pathway increases in the diabetic patient, leading to accumulation of sorbitol. This leads to induction of oxidative stress. It also causes increased ROS production via a stimulatory effect of fructose on AGE production. Treatment with aldose reductase inhibitors reduces protein kinase C (PKC) activation and TGF- β production in mesangial cells [45]. Aldose reductase inhibitors have also been shown to reduce hyperfiltration in type 1 diabetic patients [46]. So far, however, the ability of aldose reductase inhibitors to prevent various expressions of diabetic nephropathy in humans have unfortunately been rather disappointing [47].

Both the polyol pathway and the AGEs play important roles in the induction of oxidative stress, which is proposed to be an important component in the pathogenesis of diabetic complications [48]. However, there is still a controversy over whether oxidative stress is the link between hyperglycemia and complications rather than a consequence of the primary pathogenetic mechanisms. Thus, it has been shown that ROS activates AGE-dependent pathways [49] but also that AGE leads to ROS generation [50]. Inhibition of ROS in cultured endothelial cells blocks PKC activation, nuclear factor kappa B (NF κ B) activation and AGE formation [49]. The link between ROS and PKC seems to be bi-directional, like the ROS-AGE interaction [51].

Downstream and intracellular signalling

The hemodynamic and the metabolic factors activate a range of intracellular second messengers such as mitogen activated protein kinase (MAP) [52], PKC [53], NF κ B and growth factors like the pro-sclerotic TGF- β [54] and the angiogenic vascular endothelial growth factor (VEGF) [55].

PKC- β seems to be an important mediator of diabetes-induced vascular dysfunction [53]. PKC- β inhibitors attenuate glomerular hyperfiltration and albuminuria, possibly by reducing the expression of TGF- β [56]. Angiotensin converting enzyme inhibitors (ACEI) and aminoguanidine (an AGE inhibitor) prevent PKC activation in diabetes [57]. PKC- β seems to be a critical downstream messenger that is similarly important for many different pathways in the pathogenesis of diabetic nephropathy.

NF κ B in the cytoplasm is also activated by several different stimuli, including glucose [58], via a PKC dependent pathway. NF κ B activates angiotensinogen, NOS, cytokines and adhesion molecules, all of which are important in the development of diabetic nephropathy [59]. Inhibition of NF κ B has been shown to prevent fibrosis in non-diabetic models [60]. Elevated NF κ B has been correlated with proteinuria and interstitial cell infiltration [61].

The MAPK family has three main groups: the extracellular signal regulated kinase (ERK), C-Jun-N-terminal kinase (JNK) and p38MAPK. They play central roles in transducing extracellular signals to intracellular responses, including cell growth, differentiation and apoptosis [62]. Glucose activates MAPK via a PKC-dependent pathway [63] and it has been hypothesized that MAPK could play a central role in the pathogenesis of diabetic nephropathy [64]. MAPK also play an important role in mediating the pro-sclerotic effect of TGF- β [65].

Effects of the cytokine TGF- β are often modulated and mediated by other signalling pathways, including PKC, NF κ B, MAPK and Smad proteins [66]. TGF- β mediates collagen formation in the kidney [67] and thus is the most important pro-sclerotic cytokine. It stimulates the production of matrix proteins but also inhibits matrix protein degradation. TGF- β expression is stimulated by hyperglycemia, AGE, mechanical stretch, angiotensin II (AngII), and products of oxidative stress. Accordingly, TGF- β is an important link between the hemodynamic and metabolic pathways. Antagonists to TGF- β could therefore be preventive in diabetic nephropathy and it has been shown that antibodies against TGF- β lead to reduced extracellular matrix accumulation in type-2 diabetic mice. However, it did not affect proteinuria [68].

In summary, the pathogenetic mechanisms underlying increased albumin leakage, extracellular matrix accumulation, glomerulosclerosis and tubulointerstitial fibrosis are indeed complex and still incompletely understood.

Genetic factors in diabetic nephropathy

Diabetic nephropathy often occurs in familial clusters, which suggests a genetic basis in both type-1 and type-2 diabetes, and these phenomena have been shown to be the most important determinants of risk for diabetic nephropathy [69]. Searches are in progress for important genetic loci for nephropathy susceptibility. The method is genomic screening and candidate gene approaches. Some, but not all, studies have shown an association between the double deletion (DD) ACE genotype and diabetic nephropathy [70, 71]. Moreover, in these patients the course of diabetic nephropathy may be accelerated [72]. ACE gene polymorphism may also determine the ACEI effects in the individual diabetic patient. Other genes that have been suggested to contribute are the glucose transporter 1 (GLUT-1) gene [73] and inducible nitric oxide synthase (iNOS) [74]. There is now focus on several other genes that may be similarly important. To summarize, the genetic contribution to diabetic renal complications is not completely understood but several genes are probably involved.

Morphology of diabetic nephropathy

A constellation of lesions, such as mesangial matrix expansion, thickening of the GBM and arteriolar hyalinosis, is specific to diabetic nephropathy [75] (Fig 2).

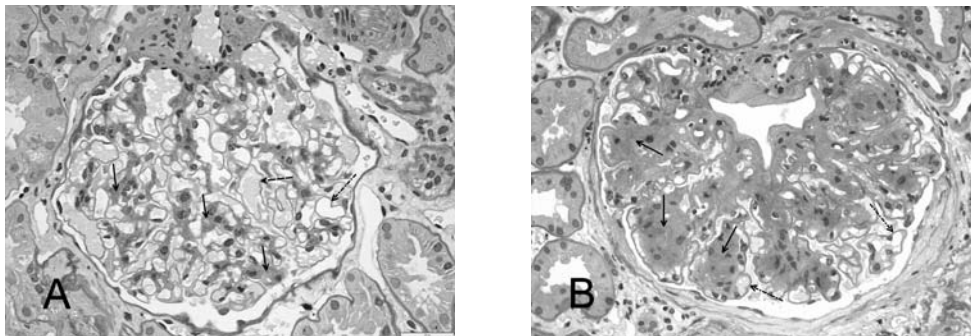


Figure 2. Characteristic appearance of moderate (A) and severe (B) diabetic nephropathy with GBM thickening (dashed arrows) and mesangial expansion (whole arrows).

The first hallmark of a diabetic influence on the kidneys is renal and glomerular hypertrophy, which in contrast to other chronic renal diseases partly persists when renal failure progresses. The capillary luminal volume and filtration surface increases, which is probably a prerequisite for glomerular hyperfiltration. The first microscopically observable structural damage is thickening of the GBM, which can occur within 2 years after diabetes onset [76]. GBM thickening is related to the albumin excretion rate but not to GFR or hypertension. After a few years, with or without concomitant hypertension, arteriolar hyalinosis occurs in both the afferent and the efferent arterioles, the latter being specific for diabetic nephropathy; mesangial expansion follows, although usually not earlier than 5-10 years after diabetes onset [75, 76]. The subsequent glomerulosclerosis can be diffuse or nodular (Kimmelstiel Wilson nodules).

Mesangial expansion is the structural parameter that correlates most closely with the clinical changes in diabetic nephropathy. Thus, it correlates with proteinuria and hypertension [75]. The mesangial expansion also restricts the space for other structures, resulting in a reduced filtration surface [77]. Consequently, mesangial expansion and GFR are inversely correlated. At this late stage the podocytes' foot processes widen and the filtration slits shorten [78]. There is also interstitial expansion, fibrosis, tubular atrophy and cell vacuolisation in the diabetic kidney. However, it is still not clear whether these changes are consequences of the initial glomerular changes [79] or an independent contribution to the progress of nephropathy resulting from different pathogenetic mechanisms [80].

Microalbuminuria and albuminuria

Normal albumin excretion is less than 30 mg/day (20 µg/min). The earliest laboratory sign of renal damage in diabetes is microalbuminuria [81], defined as an albumin excretion of 30-300 mg/day (20-200 µg/min). Leakage of more than 300 mg/day is considered to represent overt albuminuria. The lifetime risk of persistent proteinuria is 33 % in type 1 diabetes [82] and proteinuria is a strong predictor of early death in diabetic patients [83]. Microalbuminuria is reversible with adequate treatment of hypertension, especially when RAS blocking therapy is used [84].

There are today two main theoretical explanations for albuminuria. The classical view sees the size and charge selectivity of the filtration barrier as crucial [85]. According to this theory, the barrier consists of four different layers, namely the endothelial glycocalyx, the endothelial

cells, the glomerular basement membrane and the podocyte slit diaphragm. Thus, when albuminuria develops, the number of large pores increases and the amount of the negatively charged heparan sulphate is decreased [86, 87]. The latter is proposed to be an important charge barrier. The exact constitution of the filtration barrier and the relative importance of the mechanisms mentioned above is, however, still controversial [88]. In recent years, much interest has focused on the podocytes and more specifically on the filtration slit between their foot processes in which nephrin is an important molecule. Nephrin deficiency, known to occur in diabetes [89] as well as in acquired human nephrotic syndrome [90], is associated with proteinuria. There is also increasing focus on the 300 nm thick glycocalyx covering the endothelial cells. This consists of plasma proteins such as orosomucoid and negatively charged glycosaminoglycans and proteoglycans and is proposed to be an important contributor to the filtration barrier [91-93].

The second theory for albumin leakage is the albumin retrieval theory, claiming that the filtration barrier is far more permeable than previously believed. According to this theory, a large amount of albumin is filtered over the barrier and then either reabsorbed by a rapid transtubular pathway or directed to tubular cell lysosomes and degraded to albumin fragments, which are excreted in the urine. These fragments are overlooked by the common methods for detecting albuminuria [88]. According to the albumin retrieval theory the albuminuria is rather a consequence of decreased reabsorption of albumin or decreased lysosomal activity in the proximal tubules. A possible explanation for the impaired lysosomal activity is the increased TGF- β levels resulting from both the hemodynamic and the metabolic pathways [94].

While the exact mechanisms for albuminuria are still unclear, the importance of microalbuminuria as an early marker of diabetic nephropathy is indisputable, making early intervention possible [95]. Important data show that proteinuria per se is deleterious for the kidney as increased reabsorption of albumin induces local production of cytokines and chemokines, leading to recruitment of leukocytes and consequently inflammation [36].

Prevention and treatment of diabetic nephropathy

The major therapeutic approaches that have been investigated in diabetic nephropathy are glycemic control, blood pressure control (with focus on inhibitors of the RAS system), lipid lowering therapy and dietary protein intake restriction.

Of these factors, the most important in preventing diabetic nephropathy in its early stages is strict glycaemic control, which can partially prevent glomerular hypertrophy and hyperfiltration [96], delay the development of albuminuria [8, 97] and lower the risk of progression from microalbuminuria to overt albuminuria [98]. The drawback to this strategy is the heightened risk of hypoglycaemic events [97, 98]. This risk is eliminated in patients who have undergone pancreas transplantation and thus have a normalized glucose handling. A study showed an improved glomerular structure 10 years after the transplantation [99]. Furthermore, an animal study showed that when a diabetic kidney with mesangial expansion was transplanted to a healthy animal, the histological lesions were reversed [100]. Pancreas transplantation has also been shown to reduce albuminuria in diabetic nephropathy [101]. Combined kidney and pancreas or pancreatic islet transplantation resulted in prevention of albuminuria after six years compared with kidney transplantation alone [102]. The authors of the latter two studies conclude that these results are probably due to improved glycaemic control but also possibly to restored C-peptide levels. Pancreatic transplantation or pancreatic islet transplantation is probably the most effective treatment option for diabetic patients when possible.

There is a strong correlation between diabetes and hypertension [103]. Therefore, therapeutic strategies also include reduction of the systemic and intraglomerular hypertension seen in diabetes. Several studies have shown that treatment of systemic hypertension has a major effect in slowing the progress of proteinuria and GFR loss [104]. Also, in view of the impaired renal autoregulation in diabetes, the target blood pressure in diabetic patients differs from that for other hypertensive patients and should be less than 130/80 [105]. Furthermore, it is now widely accepted that inhibition of the RAS is beneficial independently of its effects on systemic blood pressure [106-109]. These effects have to do with the lowering of intraglomerular pressure, besides acting directly through lower AII levels. This important hormone is an inducer of mesangial cell growth and collagen IV synthesis [110]. Dual blockade of the RAS system by ACEI and angiotensin receptor blockers (ARB) has proven even more effective in lowering proteinuria and blood pressure compared with each treatment alone [111]. Furthermore, 40% of the patients on ACEI or ARB develop a secondary increase in aldosterone despite RAS blockade, referred to as aldosterone escape [112]. Addition of a low dose of spironolactone, a competitive aldosterone receptor blocker, further decreases albuminuria in such patients [113].

Dietary protein restriction not only aims to ameliorate uremic symptoms in advanced renal failure but also, at an earlier stage, to reduce the intraglomerular pressure which in turn has been shown to slow the decline in GFR [114-116]. The latter effect is still controversial, since other studies failed to verify it [117]. A major problem in the performance of these studies is poor compliance with the dietary restrictions. The risk of aggravating malnutrition also has to be considered in this patient group. Lipid lowering therapy may also play an important role for renoprotection in diabetes [118] and giving up smoking is probably beneficial, too [119]. Recently, in type-2 diabetes patients, a multi-intervention strategy proved effective in reducing the risk of cardiovascular and microvascular events by about 50% [120].

C-peptide, by-product or hormone?

As described above, improved glycemic control has been of incontrovertible importance in minimizing late diabetic complications and the long-term prognosis is accordingly much better today than just ten years ago [8]. Even so, a large number of patients still suffer from retinopathy, neuropathy, nephropathy and other vascular disorders. Thus, other factors than glycemic and blood pressure control are probably involved in these processes. Accordingly, research activity today aims at further clarifying the mechanisms responsible for the late complications in order to find complementary treatment options to insulin. C-peptide has been proposed to be one such factor [121].

Diabetes type 1 is characterized by β -cell damage in the pancreas, causing insulin deficiency. Insulin replacement therapy is essential to survive this disease but the deficiency in proinsulin connecting peptide (C-peptide) has not attracted much attention since C-peptide has been thought to lack physiological effects. C-peptide and insulin result from the cleavage of proinsulin in the β -cells. The two peptides are released to the circulation in equimolar amounts [122, 123]. The C-peptide or connecting peptide links the insulin A- and B-chains together in the proinsulin molecule. This facilitates the formation of disulfide bridges between the two insulin chains. After its discovery in 1967 by Steiner, C-peptide was evaluated for physiological effects, primarily insulin-like effects, but none were found [124, 125]. It was later shown that the C-peptide molecule differed considerably between species [126] and it became widely accepted that C-peptide was merely a by-product of insulin synthesis. In the past decade, however, studies of C-peptide treatment in patients with type-1 diabetes have revealed clear, positive physiological effects of the peptide itself.

C-peptide metabolism

C-peptide is an acidic peptide containing 31 amino acids. The inter-species variability in the amino acid sequence is larger than usual for bioactive peptides but similar to that for relaxin or parathyroid hormone. However, C-peptide shows partial structural conservation, particularly in mammals, suggesting that the residues Glu 3, Glu 11 and Glu 27 are important for the bioactivity of the peptide [127-129].

After the C-peptide is released from the β -cells it escapes hepatic retention, unlike insulin [130]. Accordingly, C-peptide is extracted by extrasplanchnic tissues only. The kidneys account for about 50% of the C-peptide extraction, mainly by peritubular uptake and degradation and to a lesser extent by urinary excretion [131, 132]. Renal fractional extraction of C-peptide and insulin is about 15% and 20%, respectively, in the fasting state. After a glucose load there is an increase in the fractional extraction of insulin but not of C-peptide [133]. The differences in the endogenous handling of C-peptide and insulin made C-peptide a more suitable molecule as a marker for insulin production than insulin itself. For many years, therefore, interest in C-peptide was limited to its role as a marker for residual insulin production in type-1 diabetes [134].

C-peptide binding to cell surfaces

Specific binding of C-peptide has been reported to renal tubular cells, skin fibroblasts and endothelial cells [135]. Neither scrambled C-peptide (with the same amino acids assembled in a randomized order) nor the D-enantiomer of C-peptide, insulin, proinsulin or IGF-1 could displace C-peptide from its binding site on the cellular surface, attesting to the specificity of the binding. Likewise, C-peptide does not displace insulin from its receptor. However, the C-terminal pentapeptide did displace the intact C-peptide molecule, indicating an important role for this pentapeptide in the interaction of C-peptide and the cell membrane. The binding curve for C-peptide shows saturation of the binding sites at about 1 nM, i.e. within the physiological range. This offers a probable explanation for the consistent lack of metabolic effects of exogenous administration of C-peptide in non-diabetic subjects [125, 136].

Intracellular signalling following C-peptide binding

Binding of C-peptide to the cell surface (Figure 3) leads to an increased intracellular Ca^{2+} concentration [128, 137]. This increase is prevented by addition of a calcium channel blocker or a calcium-free medium, which supports the view that there is a Ca^{2+} influx from the extracellular space following the C-peptide cell membrane interaction [138, 139]. Pertussis

toxin also blocks this mechanism, indicating that a G-protein is involved in this pathway [128, 138]. Following the binding and calcium influx, phospholipase C (PLC) is activated and this in turn stimulates protein kinase C ϵ (PKC ϵ), protein kinase C δ (PKC δ) and the small GTPase RhoA. The result is phosphorylation of the MAPKs ERK and JNK, which have been shown to be of importance in the C-peptide signal transduction [140, 141] and which activate transcription factors. Furthermore, the ERK activation and the calcium influx per se lead to increased expression of endothelial nitric oxide synthase (eNOS) and subsequently to an increased release of nitric oxide (NO) from aortic endothelial cells [139, 141, 142] and increased Na⁺, K⁺-ATPase activity in renal tubular cells and collecting duct cells [137, 140, 143, 144]. Similar findings have also been reported in neural tissue [145, 146]. A C-peptide related increase in ERK activity in cultured podocytes has been shown to inhibit TGF- β mediated up-regulation of type-IV collagen. An ERK inhibitor abolished this effect [147].

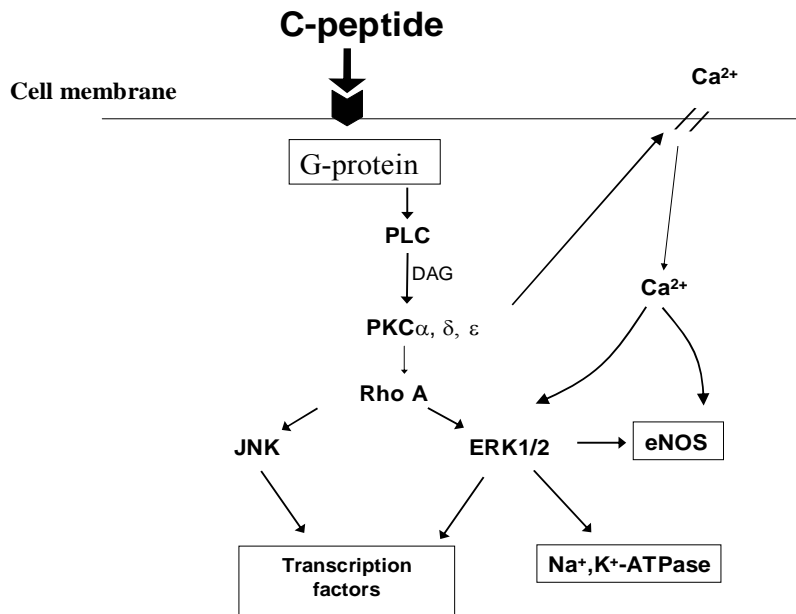


Figure 3. Pathways activated by the C-peptide binding to the cell surface.

C-peptide effects in diabetic neuropathy

Diabetic neuropathy is a group of disorders affecting the peripheral and autonomic nervous systems. The prevalence of neuropathy is 10% after one year of diabetes and 50% after 25 years [148] and eventually almost every patient develops this complication. The underlying causes are multiple. They include hyperglycemia and insulin deficiency, causing metabolic and molecular abnormalities via several pathways [149, 150]. Aldose reductase inhibitors and inhibitors of AGE formation have been used in attempts to avoid or retard the development of neuropathy, albeit unsuccessfully [151, 152]. As yet, there is no effective therapy for diabetic neuropathy.

C-peptide administration for 3 hours and for 3 months resulted in improved autonomic nerve function in type-1 diabetic patients [153, 154]. Recently, it was shown that C-peptide administration for 3 and 6 months also resulted in positive neural effects, namely improved sural nerve conduction velocity (1-2.7 m/s) [155, 156]. Impaired Na⁺, K⁺-ATPase activity and eNOS activity are possible underlying mechanisms of the early nerve dysfunction. As described earlier, C-peptide has been shown to partially correct these abnormalities without influencing glycemic control [139, 145, 146, 157, 158].

C-peptide effects in diabetic nephropathy

The improvements in glycemic control and the introduction of renin angiotensin system inhibitors have considerably decreased the incidence of nephropathy in diabetes. Nevertheless, diabetes as a cause of end stage renal disease (ESRD) is still increasing due to the rising number and age of type-2 diabetic patients. Also, there are still type-1 diabetic patients who progress to ESRD in whom other factors than poor metabolic control clearly must be involved. About a decade ago, Johansson and co-workers started to evaluate the renal effects of C-peptide. Specifically, effects on glomerular hyperfiltration and albuminuria were evaluated. Short-term effects (2 hours) of C-peptide were studied in type-1 diabetics. During the infusion of C-peptide, GFR decreased and renal plasma flow increased [159]. In a double-blind study, type-1 diabetes patients with incipient nephropathy received C-peptide for 4 weeks [160], again resulting in lowered GFR and reduced albumin excretion. When the duration of treatment was extended to 3 months in a cross-over study in which the patients were treated with insulin alone for three months and with a combination of insulin and C-peptide for three months in a randomized order, a significant reduction of albumin excretion (35%) was demonstrated in the C-peptide treated groups [153].

In streptozotocin diabetic rats, a short-term infusion (90 minutes) of human C-peptide abolished the glomerular hyperfiltration seen immediately before this infusion. The RFR was also restored and urinary protein leakage decreased [161]. Scrambled C-peptide had no effects on these variables. The effects on GFR and albuminuria have later been shown to be dose-dependent [162] in diabetic animals, with no effects in healthy animals.

The mechanisms for the renal effects of C-peptide are largely unknown. C-peptide has been shown to stimulate Na^+ , K^+ -ATPase activity in proximal convoluted tubule segments [137] and in medullary thick ascending limb [143]. However, other studies in renal tissue have shown that the Na^+ , K^+ -ATPase activity is increased rather than decreased in diabetic subjects [163, 164]. Under these circumstances it may be asked whether increased Na^+ , K^+ -ATPase activity is beneficial. Furthermore, there is evidence of C-peptide induced stimulation of eNOS in aortic endothelial cells [158], which could be a mechanism for the C-peptide effects also in the kidney.

AIMS

The aim of this thesis was to evaluate the renal effects of C-peptide in experimental type-1 diabetes mellitus. Specifically, the studies have evaluated:

- effects of C-peptide administration on renal function (GFR, RFR, RBF and albuminuria) and structure (hypertrophy, mesangial expansion, GBM thickness, AGE, RAGE and type-IV collagen).

- interaction between C-peptide and an angiotensin converting enzyme inhibitor with regard to renal effects.

METHODS

Animals

Eight-week-old male Sprague-Dawley rats (Møllgaard, Copenhagen, Denmark) weighing about 200 g at the beginning of the study, were used in studies I, II and IV. In study III, the rats were eight-week-old male Wistars with an initial weight of 250 g. The animals had free access to tap water and were fed with a standardized chow (R36, Lactamin) throughout the study periods.

Diabetes induction

Diabetes was induced by injecting the potent alkylating agent streptozotocin intravenously in the tail in a dose of 60 mg/kg body weight in studies I and III and 55 mg/kg in studies II and IV. The rats were consistently considered to be diabetic when blood glucose reached 15 mmoles/l. Blood glucose concentrations were analyzed by means of Accutrend® (Boehringer Mannheim GmbH, Mannheim, Germany) when sampled from the rat's tail (studies I and III) and by a glucose oxidase method (Glucose Analyzer, Yellow Spring Instruments, USA) when obtained at the end of the final experiment in studies I, III and IV. In studies II and IV, blood glucose concentrations obtained from the rat's tail were analyzed using a MediSense Pen® and Precision Plus Electrodes® (Abbot Scandinavia AB, MediSense Produkter, Solna, Sweden).

Metabolic cages

Before streptozotocin treatment and at specified intervals after diabetes onset (Table 1) all rats in studies I, III and IV were kept in metabolic cages, one animal per cage (Tecniplast Gazzada 3701MO-000, Buguggiate, Italy), for 24 hours. Daily intake of water and food, body weight and excretion of urine and faeces were measured. Urine samples were collected for analyses of albumin, sodium and potassium excretion and osmolality. Blood samples for blood glucose measurements were taken from the tip of the tail.

Table 1: Summary of methodological characteristics in the studies.

	Study I	Study II	Study III	Study IV
Rat strain	Sprague-Dawley	Sprague-Dawley	Wistar	Sprague-Dawley
Groups and numbers	Normal n=7 D-placebo n=7 DCp n=7	C-ACEI n=5 D-placebo n=7 DCp n=13 D-ACEI n=9 D-ACEICp n=8	N-early n=9 D-early n=7 N n=9 D n=11 DCp n=11	N n=10 (8) D n=12 (8) DCp n=13 (8) DACEI n=13 (7) DCpACEI n=16 (8)
Streptozotocin dose	60 mg/kg	55 mg/kg	60 mg/kg	55 mg/kg
Treatment drugs and route of administration	Saline iv C-peptide iv	Saline iv C-peptide iv Captopril iv	Saline sc C-peptide sc	Saline sc C-peptide sc Captopril po
Treatment time	2 weeks	60 minutes	4 weeks	8 weeks
Metabolic cages	Days 0, 4, 8, 12		Weeks 0, 1, 2, 3, 4	Weeks 0, 2, 4, 6, 8
Anesthesia	Inactin® 120 mg/kg	Inactin® 120 mg/kg	Inactin® 70 mg/kg	Inactin® 120 mg/kg
GFR method	FITC-labelled inulin	[³ H]-inulin	[³ H]-inulin	[³ H]-inulin
Kidney fixation	Formaldehyde		Formaldehyde	Formaldehyde and freezing
Morphology	Light microscopy		Light and electron microscopy	Light microscopy
Analyses of special interest in diabetic nephropathy	GFR and RFR Albuminuria Renal weight Glomerular volume	GFR RBF MAP	GFR Albuminuria Glomerular volume Mesangial and mesangial matrix volume GBM thickness	GFR Albuminuria Glomerular volume Immunohistochemistry for type-IV collagen, AGE and RAGE

Surgical preparation

After 2 weeks (studies I and II) and 8 weeks (studies III and IV) of diabetes, respectively, the rats were anesthetized for the final experiments by an intraperitoneal injection of Inactin® (sodium 5-sec-butyl-5-ethyl-2-thiobarbiturate; RBI Natick, MA 01760 USA), 120 mg/kg body weight (except in study III on Wistar rats, in which 70 mg/kg was given) and then placed on a servo-controlled heating pad to maintain the body temperature at about 37.5°C. To make spontaneous breathing easier, a cannula was inserted into the trachea. The right femoral artery was cannulated for blood sampling and for measurements of the arterial blood pressure. The right femoral vein was cannulated for intravenous infusions. The bladder was catheterized via a suprapubic incision.

In study II, thereafter, the left kidney and renal artery were exposed via a flank incision and dissected free of connective tissue and fat before the kidney was placed in a cup and covered with oil. Subsequently, the ureter on the same side was ligated as distally as possible and catheterized in order to collect the urine from the left kidney. This procedure enabled GFR measurements from each individual kidney. The renal artery was exposed and an ultrasound recorder probe (Transonic® T 206, Transonic Syst. Inc., Ithaca, New York, USA) was positioned for continuous measurement of renal blood flow.

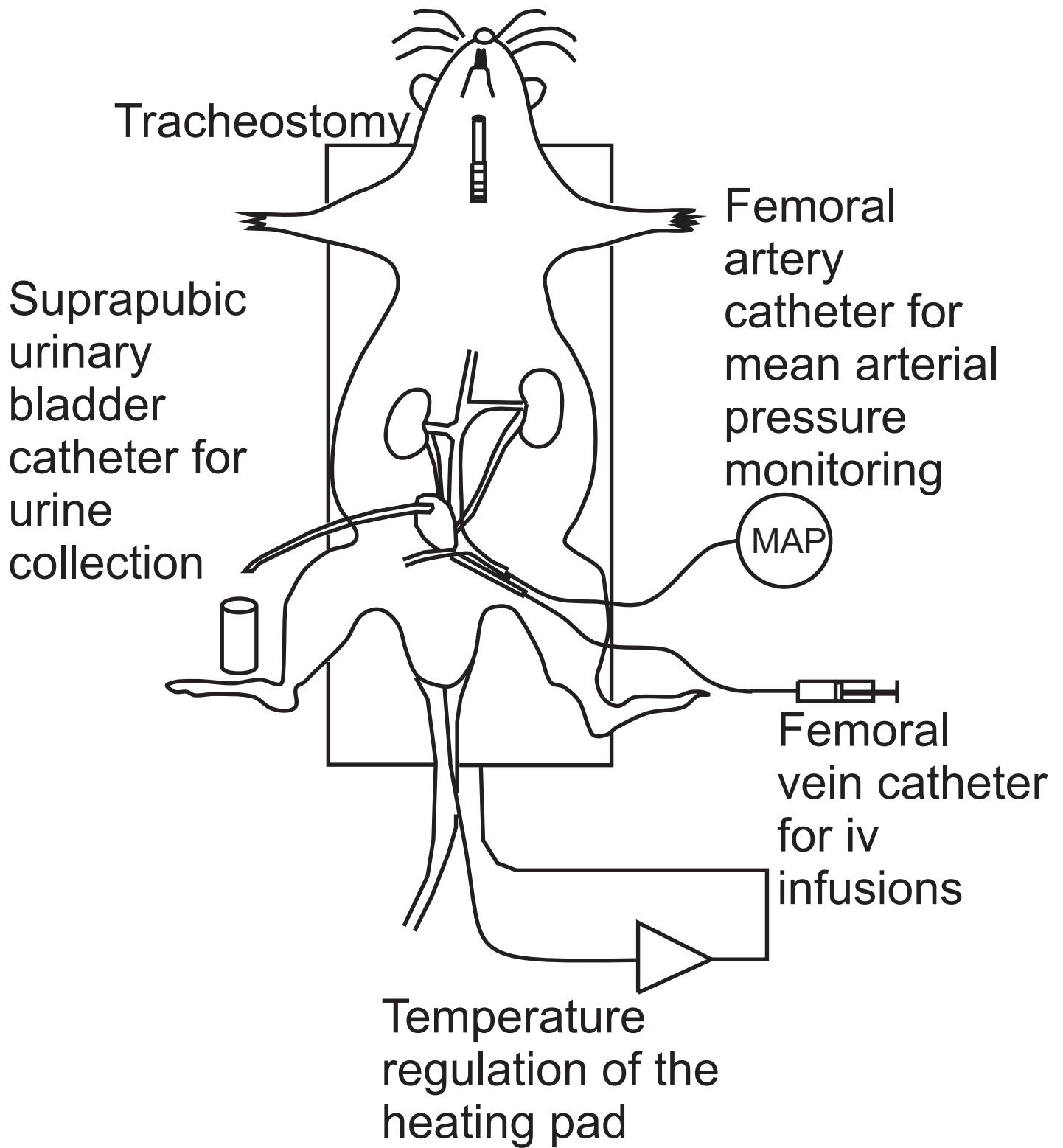


Figure 4. Rat prepared for GFR experiments.

Determination of GFR and RFR

Study I

Immediately after surgery, arterial blood was drawn to determine blood glucose, sodium and potassium concentrations. Thereafter, an infusion of a Ringer solution containing 129 mM NaCl, 25 mM NaHCO₃, 2.5 mM KCl, 0.75 mM CaCl₂ and fluorescein isothiocyanate (FITC)-labelled inulin (Pharmacia AB, Uppsala, Sweden) 4 mg/ml was started. Following a bolus dose of 1 ml, the infusion rate was 5 ml x hr⁻¹ x kg⁻¹ body weight. When glycine was infused, the Ringer solution was also used as a vehicle.

After about 45 min, when steady state had been reached, urine samples were collected at 15 min intervals for analyses of urine volume, osmolarity, albumin excretion rate and sodium and potassium concentrations. For analyses of FITC-labelled inulin, plasma samples (~60 µl) were obtained at the midpoint of each 15 min urine collection period, thus making calculation of GFR possible. After two 15 min periods, when basal GFR was measured, infusion of glycine (0.22 mmol x kg⁻¹ x min⁻¹) was started for determination of the available RFR. This infusion was maintained for the remaining 60 min of the experiment.

Studies II, III and IV

Inulin clearance was also included in these three studies, though isotonic saline containing [³H]-inulin was used instead of Ringer solution with FITC-labelled inulin. In studies III and IV, GFR was measured at 20 min intervals. RFR was not measured in studies II, III and IV.

Fixation of the kidney (studies I, III and IV)

When GFR and RFR measurements had been completed, blood samples were taken for determination of C-peptide, sodium and potassium concentrations. The sampled volume was immediately replaced by an equal volume of Ringer solution (study I) or isotonic saline (studies III and IV) to maintain adequate renal perfusion. Thereafter, the left kidney, the renal vessels and aorta were exposed via a subcostal incision. The catheter inserted earlier via the femoral artery was adjusted to the level of the left renal artery. The aorta was subsequently ligated both distally and proximally to the left renal artery. Immediately after the ligation the left kidney was perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).

The perfusion pressure was adjusted to the mean arterial blood pressure just before the perfusion was started. In study IV the right kidney was also removed and quickly frozen in liquid nitrogen.

Analyses

Inulin

FITC labelled inulin (study I) was analyzed in a Ploemopak fluorescence microscope (Leitz Dialux 22 EB, Germany) equipped with a photometer (Leitz MPV compact, Germany). Measurements were made from the central portion of the sample after it had been transferred to constant-bore capillaries (0.5 µl; Microcaps, Drummond, USA) [24]. [³H]-inulin in plasma and urine (studies II, III and IV) was determined by liquid scintillation counting (PW 4700, Philips, Holland). The sample (1 µl of urine or 10 µl of plasma) was mixed with 1 ml of water; whereafter 3 ml of scintillation fluid (Pico-Flour 40TM, CiAB, Chemical Instruments AB, Lidingö, Sweden) was added.

C-peptide

Plasma concentrations of C-peptide were measured by radio immuno assay (RIA, Linco Research Inc., USA).

Urine variables

The urine volume was determined gravimetrically. Urinary potassium and sodium concentrations were determined in a flame photometer (IL 543; Instrumentation Lab., Milano, Italy) and urine osmolality by a freezing point depression method (Model 3 MO; Advanced Instruments, MA, USA). Urine albumin concentration was measured by nephelometry (studies I, III and IV).

Blood pressure

In all studies the blood pressure during the final experiment was measured continuously by a transducer (GOULD Statham P23XL, Millar Instruments, Houston, TX, USA) connected to a printer (BBC Goertz Metrawatt SE 460, Brown Boveri, Sweden). Mean arterial blood pressure (MAP) was calculated ($1/3 \times (\text{systolic pressure} - \text{diastolic pressure}) + \text{diastolic pressure}$).

Light microscopy

The mean glomerular volume (studies I, III and IV) was estimated by Cavalieri's principle [165]. The formaldehyde-fixed kidney was first weighed and then cut into slices 1.4 mm thick, using a device with parallel razor blades. From each rat, two systematically and randomly sampled slices were embedded in glycol methacrylate (Technovit 7100, Kulzer, Werheim, Germany). Then, the plastic blocks were cut into consecutive sections 3.0 μm thick, which were stained with periodic acid schiff staining (PAS). Fifteen superficial glomeruli were analyzed in each kidney. At 15- μm intervals, the profile areas of glomerular tufts completely included within the serial sections were point counted using an ocular grid with 32.1 μm (= d) between each point (= p) at tissue level (X420). The sum of the profile areas (= A SUM) of a glomerulus was determined according the formula $A \text{ SUM} = p_{\text{TOT}} \cdot d^2$, where p_{TOT} is the sum of the points hitting all the sampled profile areas of the same glomerulus.

For the final calculation of glomerular volume, the mean of the actual section thickness was measured by confocal microscopy as described by Brismar et al. [166]. In brief, the x-z profile of a section is scanned by confocal microscopy. The full width half maximum of the intensity profile in the axial direction of the x-z profile is then used as an estimate of the actual section thickness. A SUM was thereafter multiplied by the distance between the analyzed sections, i.e. 5 times the actual section thickness.

Electron microscopy

For electron microscopy (study III), cortical tissue was post fixed in 1% osmium tetroxide and embedded in Epon by standard procedures. Ultrathin sections were stained with uranylacetate and lead citrate and studied in a JEM 100S electron microscope (Jeol, Tokyo, Japan). From each rat, 4-5 glomeruli were analyzed. The reference space of the glomerular tuft was defined as in reference [167]. At 3,000X, sets of 8-14 micrographs per glomerulus were taken in a systematic random manner by moving the specimen stage between predetermined points. The mesangial volume fraction and the mesangial matrix volume fraction were analyzed by point counting, using a superimposed square lattice grid with approximately 3 μm between the points at tissue level. The basement membrane thickness was estimated using the orthogonal intercept method of Jensen and co-workers [168] on a separate set of 6-9 micrographs taken from each glomerulus at 7,500 X. The final magnification was corrected using a grating grid with 2160 lines per mm.

Immunohistochemistry

Four-micrometer paraffin sections of kidneys were used to stain for collagen type IV, AGE and RAGE. The primary antibodies included a polyclonal goat anti-collagen type IV antibody (Southern Biotechnology, Birmingham, AL; diluted 1:1500), a polyclonal rabbit anti-AGE antibody (AGE 4G9, which recognizes the AGE carboxymethyllysine; diluted 1:1000), and a polyclonal goat anti-RAGE antibody (Chemicon, Temecula, CA; diluted 1:500). In brief, sections for AGE and RAGE were dewaxed, hydrated, and quenched with 3% H₂O₂ in TBS (pH 7.6) to inhibit endogenous peroxidase activity. This was followed by incubation in Protein Blocking Agent (Lipshaw-Immunon, Pittsburgh, PA) for 60 min at room temperature. The sections were then incubated with anti-AGE and anti-RAGE antibodies overnight at 4°C in a humid atmosphere. Sections for collagen IV were dewaxed, hydrated and quenched with 3% H₂O₂ in TBS. In addition, sections were digested with 0.4% pepsin (Sigma Chemical Co) in 0.01 M HCl at 37°C for two min. Subsequently, sections were incubated with the primary antibody anti-collagen IV overnight at 4°C followed by avidin/biotin blocking. Thereafter, biotinylated anti-rabbit Ig (Vector Laboratories) diluted 1:500 for AGE, and biotinylated anti-goat Ig (Vector Laboratories) diluted 1:500 for collagen type IV and RAGE were applied as the secondary antibodies, followed by horse-radish peroxidase-conjugated streptavidin (VECTASTAIN Elite ABC Staining Kit, Vector Laboratories). Peroxidase conjugates were subsequently visualized using 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co) in 0.08% H₂O₂/TBS as the chromogen. Finally, sections were counterstained with Mayer's hematoxylin, dehydrated and mounted. All sections were examined under light microscopy (Olympus BX-50, Olympus Optical) and digitized with a high-resolution camera. For the quantification of the proportional area of staining, 20 views were randomly located in the renal cortex (Optimas 6.2-Video Pro-32; Bedford Park, SA, Australia). All assessments were performed in a blind manner.

Study groups and treatment regimens

Study I

Three groups were studied: normal rats treated with isotonic NaCl (normal group, n=7), diabetic rats treated with isotonic NaCl (D-placebo group, n=7) and diabetic rats treated with rat C-peptide-2 (Genosys Biotechnologies, U.K.) (D-C-p group, n=7). Treatment with either C-peptide (50 pmol x kg⁻¹ x min⁻¹) or NaCl (which was also the vehicle for C-peptide) was initiated at diabetes onset (blood glucose >15 mmol/l) and administered as a continuous

intravenous infusion for 2 weeks by an osmotic pump (type 2002, Alzet, U.S.) which was attached to a right jugular vein catheter. The pump was placed in the subcutaneous tissue of the neck. The rats were not treated with insulin.

Study II

Four diabetic groups were treated with either isotonic NaCl (D-placebo group, N=7), rat C-peptide-2 (D-Cp, N=13; Genosys Biotechnologies, UK), captopril (D-ACEI, N=9; Sigma-Aldrich Sweden AB, Stockholm, Sweden) or C-peptide and captopril (D-Cp-ACEI, N=8). We also studied a healthy control group treated with captopril (C-ACEI, N=5). The experiments were performed at 2 weeks after induction of diabetes. During these two weeks no treatment was given. When the basal GFR had been measured, infusion of either saline as placebo, C-peptide ($50 \text{ pmol} \times \text{kg}^{-1} \times \text{min}^{-1}$), captopril ($3 \text{ mg} \times \text{kg}^{-1} \times \text{h}^{-1}$) or a combination of C-peptide and captopril was started and maintained for the remaining 60 min of the experiment. GFR was measured individually for each kidney, a prerequisite for calculations of the filtration fraction changes on the left side where the blood flow was measured.

Study III

Three groups were studied for eight weeks: non-diabetic placebo treated rats (N group, n=9), diabetic placebo treated rats (D group, n=11) and diabetic rats treated with rat C-peptide-2 (Genosys Biotechnologies, U.K.) (DCp group, n=11). In two additional groups, designated the normal early group (N-early, n=9) and the diabetic early group (D-early, n=7), GFR was studied earlier, namely after four weeks, for comparison with the three groups above. Treatment with C-peptide ($50 \text{ pmol} \times \text{kg}^{-1} \times \text{min}^{-1}$) dissolved in isotonic saline in the DCp group or with saline alone in the other two main study groups was initiated four weeks after onset of diabetes and administered as a continuous subcutaneous infusion for four weeks by an osmotic pump (type 2002, Alzet, U.S.) placed in the subcutaneous tissue of the neck.

Study IV

Five groups were studied: normal placebo treated rats (N group, n=10), diabetic placebo treated rats (D group, n=12), diabetic rats treated with rat C-peptide-2 (Genosys Biotechnologies, U.K.) (DCp group, n=13), diabetic captopril (Sigma-Aldrich, Stockholm, Sweden) treated rats (DACEI, n=13) and rats treated with a combination of C-peptide and captopril (DCpACEI, n=16). Osmotic pumps continuously infused C-peptide ($50 \text{ pmol} \times \text{kg}^{-1} \times \text{min}^{-1}$) solved in isotonic saline in the DCp and DCpACEI groups or just saline in the other study groups during the study period of eight weeks. The first pump was replaced after 4

weeks since it could not contain enough C-peptide or placebo for the whole study period. In addition, the DACEI and DCpACEI groups had captopril (Sigma-Aldrich, 90 mg/l) added to the drinking water.

In a subgroup of these rats, morphological and immunohistochemical data were studied: normal placebo treated rats (N group, n=8), diabetic placebo treated rats (D group, n=8), diabetic rats treated with rat C-peptide-2 (Genosys Biotechnologies, U.K.) (DCp group, n=8), diabetic captopril (Sigma-Aldrich, Stockholm, Sweden) treated rats (DACEI, n=7) and rats treated with a combination of C-peptide and captopril (DCpACEI, n=8).

Statistical methods and presentation of data

Data in the text, figures and tables are presented as means \pm SEM.

Study I

Significant differences in metabolic data, glomerular volume, renal size and basal GFR were calculated by analysis of variance (ANOVA) followed by Tukey's post-hoc test. RFR for each time interval and albuminuria (in the latter after the values had been logarithmically transformed) were compared to the basal state using ANOVA for repeated measurements within each group followed by Tukey's post-hoc test. For comparisons of available RFR between groups, the mean percentage increases in GFR during the glycine infusion period were compared by ANOVA followed by Tukey's post-hoc test.

Study II

Analysis of variance followed by Tukey's post-hoc test was used to detect changes within and between groups before and after treatment.

Study III

Differences in metabolic data, glomerular volume, renal size, GFR, mesangial volume and GBM thickness were evaluated by ANOVA followed by Tukey's post-hoc test. Albuminuria (after the values had been logarithmically transformed) was compared to the basal state using ANOVA for repeated measurements within each group followed by Tukey's post-hoc test.

Study IV

Significant differences in metabolic data, glomerular volume, GFR, and type-IV collagen, AGE and RAGE levels were calculated by ANOVA followed by Tukey's post-hoc test. The final albumin/creatinine ratio was compared with the basal state within the same group by ANOVA for repeated measurements followed by Tukey's post-hoc test. In the immunohistochemical analyses, outliers (>2 SD) were excluded.

Ethical considerations

The study protocols for each individual study were reviewed and approved by the local animal ethics committee.

RESULTS

Study I

In summary, this study shows that C-peptide administration, in physiological doses, to rats with streptozotocin induced diabetes, prevents glomerular hyperfiltration, glomerular and renal hypertrophy and albuminuria and preserves the RFR.

Metabolic data

Before diabetes induction, the groups were, as expected, similar in blood glucose, body weight, food intake, water intake, faeces weight, urine volume, urine osmolarity and urinary excretion of sodium and potassium. Twelve days after diabetes induction by streptozotocin, the two diabetic groups had similar and high glucose concentrations. Thus, blood glucose was 36.7 ± 1.3 mmol/l in the D-placebo group, 34.0 ± 1.7 mmol/l in the D-C-p group and 8.8 ± 0.8 mmol/l in the normal group. As a result of their diabetes, the D-placebo and D-C-p groups also showed statistically significant polydipsia, polyuria, polyphagia, reduced growth rate and increased faeces excretion. All these changes were statistically significant when compared within groups to the basal state, and also in comparison to the normal group 12 days after diabetes onset. Except for a higher faeces excretion in the D-placebo group, these variables did not differ significantly between the diabetic groups. The excreted amounts of sodium and potassium were elevated and urine osmolarity was markedly reduced in the two diabetic groups compared with the normal group.

The C-peptide concentration was significantly lower in the D-placebo group (0.15 ± 0.02 nmol/l) than in the normal group (1.3 ± 0.3 nmol/l). The D-C-p group had physiological C-peptide levels (1.5 ± 0.4 nmol/l) that did not differ from the normal group.

Glomerular volume and renal weight

The glomerular volume was $0.92 \pm 0.05 \times 10^6 \mu\text{m}^3$ in the normal group, $1.50 \pm 0.06 \times 10^6 \mu\text{m}^3$ or 63% larger ($p < 0.001$ vs. normal) in the diabetic placebo treated group and $1.13 \pm 0.06 \times 10^6 \mu\text{m}^3$ (non significant vs. normal) in the diabetic C-peptide treated group. The D-placebo group presented a 32% ($p < 0.001$) larger glomerular volume than the D-C-p group (Fig. 5). Renal weight did not differ significantly between the D-C-p group and the normal group; in the D-placebo group, on the other hand, renal weight was 31% higher than in the normal group ($p < 0.05$) (Fig. 5).

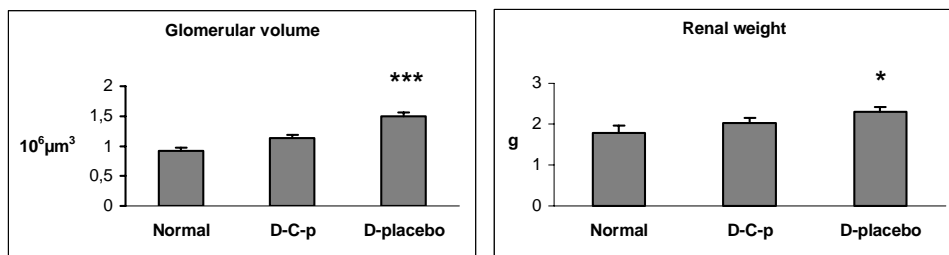


Figure 5. Glomerular volume and renal weight in the study groups 14 days after start of the study. The diabetic placebo treated group but not the D-C-p group had significantly increased glomerular volume and renal weight compared with the normal group; * $p < 0.05$, *** $p < 0.001$.

GFR and RFR

Basal GFR in the normal rats was 1.72 ± 0.12 ml/min after 14 days. The D-placebo group had marked hyperfiltration, with a GFR of 3.73 ± 0.19 ml/min or 117% higher than in the normal group ($p < 0.001$) and 73% higher than in the C-peptide treated diabetic group ($p < 0.001$). GFR in the D-C-p group was 2.16 ± 0.16 ml/min, which was not significantly higher than in the normal group (Fig. 6).

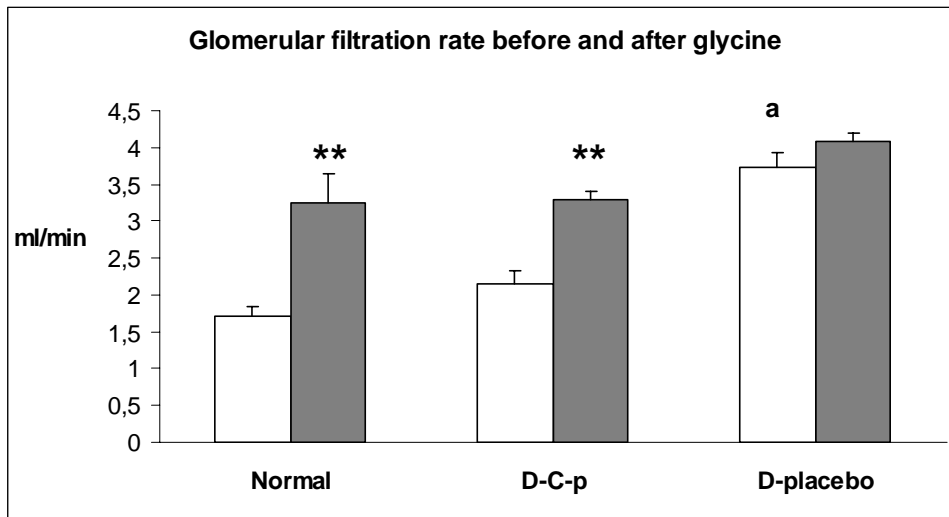


Figure 6. GFR in the study groups 14 days after start of the study before (open bars) and after (closed bars) glycine infusion. Statistical difference between the groups in the basal state is indicated by **a**; $p < 0.05$. Asterisks indicate significantly increased GFR from the basal state within each group; ** $p < 0.01$.

GFR in the normal rats increased by $93 \pm 25\%$ ($p < 0.01$), from 1.72 ± 0.12 ml/min in the basal state to an average of 3.25 ± 0.39 ml/min during the glycine infusion. This change represents RFR. In the D-placebo group, GFR increased by $10 \pm 4\%$ (non-significant), from 3.73 ± 0.19 ml/min in the basal state to 4.08 ± 0.11 ml/min during the glycine infusion, and in the D-C-p group GFR increased by $57 \pm 13\%$ ($p < 0.01$), from 2.16 ± 0.16 ml/min in the basal state to 3.30 ± 0.10 ml/min during the glycine infusion. RFR in the D-C-p group did not differ significantly from that of the normal group, whereas it was absent in the D-placebo group (Fig. 6).

The D-placebo group did not present a statistically significant increment in GFR at any measured time interval during glycine infusion compared with the basal state. In contrast, the D-C-p group had a significantly increased GFR at all measured time intervals and the normal group at all time intervals from 45 minutes onwards.

Albuminuria

During the study neither the D-C-p group nor the normal group showed any statistically significant increase in albumin excretion compared to the basal state but in the D-placebo group the urinary albumin excretion was significantly increased from the basal state on days 4 ($p<0.05$), 8 and 12 ($p<0.01$) after treatment start. Thus, the D-placebo group, but not the D-C-p group, had higher albumin excretion in the urine than the normal group, (Fig 7).

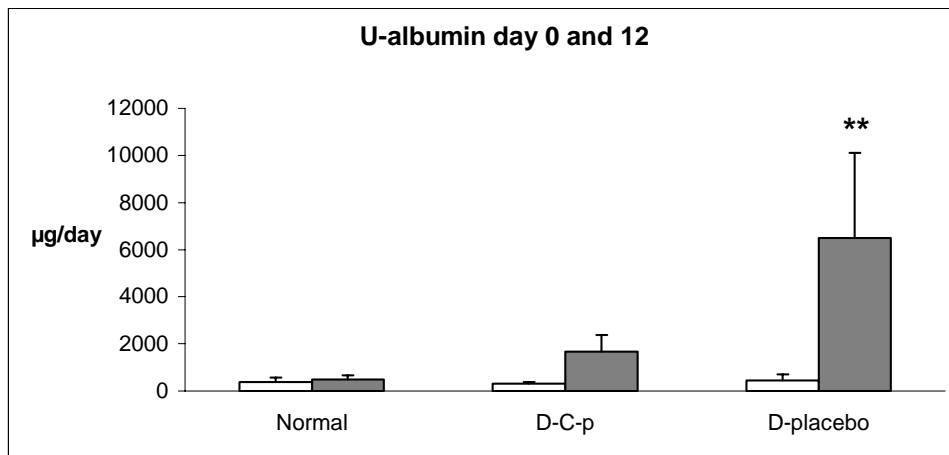


Figure 7. Urinary albumin excretion on days 0 (filled bars) and 12 (open bars). The diabetic placebo treated group, but not the D-C-p group, had significantly increased albumin excretion after 12 days; ** $p<0.01$.

Study II

In summary, this study shows that C-peptide and captopril are equally effective in lowering glomerular hyperfiltration in rats with experimental type-1 diabetes mellitus. Captopril, but not C-peptide, increased renal blood flow more than placebo. Combination treatment with C-peptide and captopril, but not treatment with each substance alone, lowered the filtration fraction more than placebo.

Basal data and urine variables

Table 2 shows the basal data at the time of the experiments and the urine flow and osmolality during the experiments. The mean glucose levels in the four diabetic groups ranged from 20.6 to 22.6 mmol/L. Body weight did not differ significantly between the groups. The C-peptide levels at the end of the experiment in the D-placebo and D-ACEI groups were 0.46 ± 0.12 nmol/L and 0.19 ± 0.03 nmol/L, respectively. The C-ACEI group had a physiological C-peptide level of 1.25 ± 0.14 nmol/L. The D-Cp and D-Cp-ACEI groups had C-peptide levels after treatment of 46 ± 13 nmol/L (range 0.3-120 nmol/L) and 50 ± 20 nmol/L (range 10-160 nmol/L), respectively.

During the experiment the urine flow was, as expected, significantly higher in the diabetic groups than in the non-diabetic group and it decreased significantly after treatment in all groups, while urinary osmolality increased in all study groups.

Blood pressure

MAP was similar in the study groups in the basal state and fell significantly in every group after treatment. In the D-placebo group MAP decreased by $7 \pm 2\%$, in the D-Cp group by $9 \pm 2\%$ and in the C-ACEI, D-ACEI and D-Cp-ACEI groups by $15 \pm 4\%$, $13 \pm 1\%$ and $14 \pm 3\%$, respectively. The relative fall in blood pressure was slightly greater in the ACEI-treated groups, but not significantly so.

Table 2. Data 2 weeks after diabetes onset before and after treatment in each study group: Healthy captopril-treated rats (C-ACEI), diabetic placebo-treated rats (D-placebo), diabetic C-peptide-treated rats (D-Cp), diabetic captopril-treated rats (D-ACEI) and diabetic rats treated with the combination of C-peptide and captopril (D-Cp-ACEI). Asterisks show statistically significant changes from the basal state (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Group	C-ACEI		D-placebo		D-Cp		D-ACEI		D-Cp-ACEI	
	Basal	After treatment	Basal	After treatment	Basal	After treatment	Basal	After treatment	Basal	After treatment
Blood glucose mmol/l	6.7±0.3		20.6±0.7		22.6±0.6		22.3±0.5		21.8±0.3	
C-peptide nmol/l		1.25±0.14		0.46±0.12		46±13		0.19±0.03		50±20
Body weight g	280±14		265±7		254±7		264±8		240±12	
Urine flow µl/min	16±2	9±2*	46±8	29±5**	59±6	33±3***	57±5	40±3**	68±6	43±3***
Urine osmolality mOsm/kg	852±60	1325±161*	1056±45	1250±52**	1014±45	1215±31***	981±31	1172±29***	875±32	1066±30***

GFR

All the diabetic groups presented with statistically significant glomerular hyperfiltration in the basal state compared with the healthy C-ACEI group (Fig. 8). GFR decreased significantly following treatment with either C-peptide or ACEI or a combination of the two in the diabetic groups, while GFR in the C-ACEI group and the D-placebo group did not change significantly after treatment. Thus, GFR in the D-Cp group decreased by $17\pm 4\%$ ($p < 0.01$), in the D-ACEI group by $14\pm 5\%$ ($p < 0.01$) and in the D-Cp-ACEI group by $18\pm 7\%$ ($p < 0.01$). Consequently, after treatment, GFR in these groups was normalized. There was no statistically significant relationship between the plasma concentration of C-peptide and the changes in GFR or blood flow, respectively, within each C-peptide treated group.

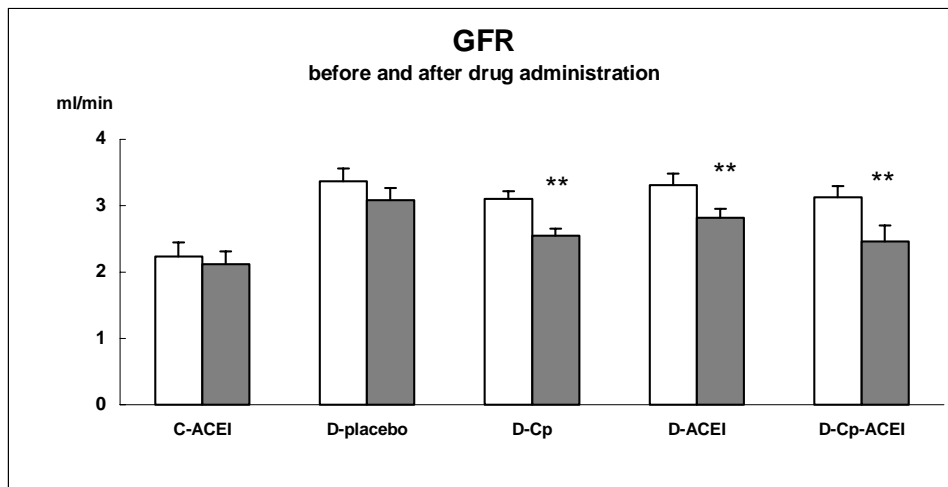


Figure 8. GFR before (open bars) and after (filled bars) treatment in each study group at 2 weeks after induction of diabetes: healthy captopril-treated rats (C-ACEI), diabetic placebo-treated rats (D-placebo), diabetic C-peptide-treated rats (D-Cp), diabetic rats treated with captopril (D-ACEI) and diabetic rats treated with the combination of C-peptide and captopril (D-Cp-ACEI). Statistically significant differences, within each group, from the basal state are shown, $**p < 0.01$.

Blood flow and filtration fraction

Renal blood flow increased in all study groups, in the C-ACEI group by $26\pm 3\%$ ($p<0.01$), in the D-ACEI group by $27\pm 4\%$ ($p<0.001$) and in the D-Cp-ACEI group by $32\pm 6\%$ ($p<0.001$). Blood flow in the D-Cp group and the D-placebo group increased by $5\pm 2\%$ ($p<0.05$) and $5\pm 1\%$ ($p<0.01$), respectively. The blood flow changes in the three groups receiving captopril were significantly greater than those in the D-placebo and D-Cp groups. In contrast, blood flow did not increase more in the D-Cp group than in the D-placebo group.

The filtration fraction decreased significantly in all study groups (Fig. 9); in the C-ACEI group by $23\pm 9\%$, in the D-placebo group by $12\pm 5\%$, in the D-Cp group by $20\pm 4\%$, in the D-ACEI group by $31\pm 5\%$ and in the D-Cp-ACEI group by $36\pm 7\%$. Only in the latter, combination treated, group was the fall significantly greater than in the D-placebo group ($p<0.05$).

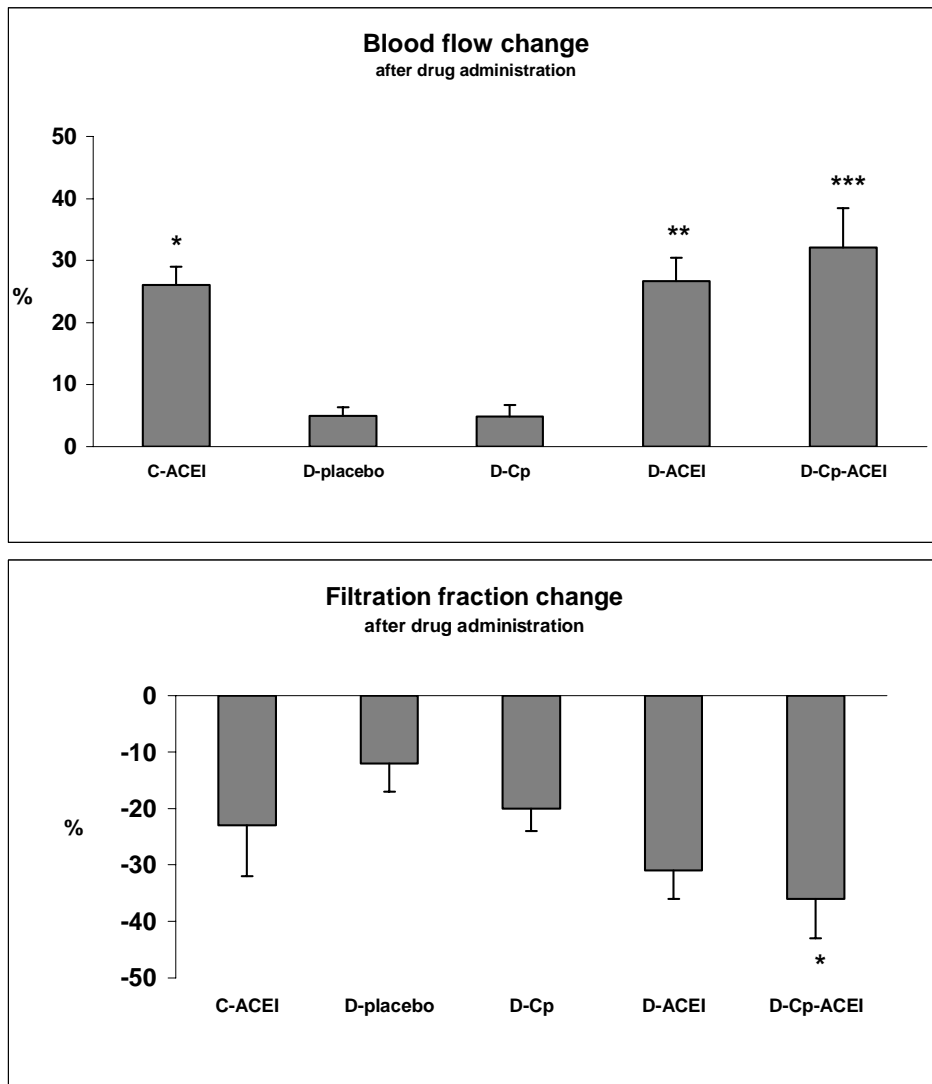


Figure 9. Relative renal blood flow and filtration fraction changes after drug administration in each study group; healthy captopril-treated rats (C-ACEI), diabetic placebo-treated rats (D-placebo), diabetic C-peptide-treated rats (D-Cp), diabetic rats treated with captopril (D-ACEI) and diabetic rats treated with the combination of C-peptide and captopril (D-Cp-ACEI). Statistically significant differences from the D-placebo group are shown; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Study III

Study III shows that C-peptide administration to streptozotocin diabetic Wistar rats prevents glomerular hypertrophy and mesangial matrix expansion in the post-hyperfiltration phase of the disease. No effect on GBM thickness was detected.

Metabolic data

Before streptozotocin treatment there were no statistically significant differences between the study groups in body weight, water intake, urine volume, food intake, faeces excretion, urine osmolality, or urinary sodium and potassium excretion. At 8 weeks the diabetic groups showed reduced weight gain, polyuria, polydipsia and polyphagia. The D group but not the DCp group also showed lower urine osmolality than the N group. At 8 weeks there were no significant differences in these variables between the D and DCp groups.

Blood glucose and C-peptide levels

Table 3 shows the average blood glucose levels during weeks 1-4 (before C-peptide or placebo treatment) and weeks 5-8 (during treatment). The C-peptide treatment did not affect the glucose levels. In the DCp group, the plasma C-peptide concentration was 1.6 ± 0.2 nmol/L, statistically not significantly different from that in the non-diabetic group (1.7 ± 0.2 nmol/L). In contrast, the diabetic group not receiving C-peptide showed a low C-peptide level of 0.1 ± 0 nmol/L, as expected in streptozotocin induced diabetes.

GFR

After 4 weeks of diabetes, GFR was 5.23 ± 0.78 mL/min in the D-early group and 2.59 ± 0.37 mL/min ($p < 0.001$) in the N-early group, indicating glomerular hyperfiltration in the diabetic group at this stage. Results for the other groups show that after 8 weeks of diabetes there was no longer a statistically significant hyperfiltration in the diabetic placebo-treated group, although GFR still tended to be higher ($p = 0.051$). Thus, GFR in the D group was 3.39 ± 0.24 mL/min and in the N group 2.47 ± 0.08 mL/min; in the DCp group GFR was 2.77 ± 0.31 mL/min, not significantly different from the N-group (Fig.10)

Table 3. Blood glucose levels presented as an average of the first four and last four weeks, respectively, and C-peptide, measured at the end of the study. Non-diabetic early group (N-early), diabetic early group (D-early), non-diabetic placebo treated group (N), diabetic placebo treated group (D) and diabetic C-peptide treated group (DCp). Asterisks indicate significant differences from the N group at the same time point; *** $p < 0.001$.

Group	Blood glucose weeks 1–4, i.e. before treatment (mmol/L)	Blood glucose weeks 5–8, i.e. during treatment (mmol/L)	C-peptide concentration after 8 weeks (nmol/L)
N-early	6.2±0.2		
D-early	22.7±0.4***		
N	5.7±0.2	5.7±0.2	1.7±0.2
D	24±0.8***	24.5±0.4***	0.1±0***
DCp	25.4±0.6***	24.8±0.4***	1.6±0.2

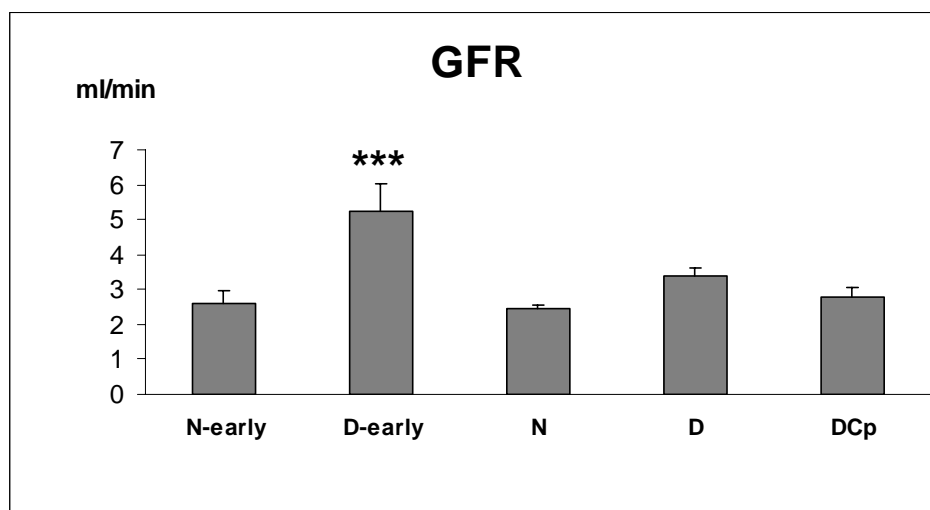


Figure 10. GFR was measured after 4 weeks of diabetes without treatment in the normal early (N-early) and the diabetic early (D-early) groups. In the normal placebo treated group (N), the diabetic placebo treated group (D) and the diabetic C-peptide treated group (DCp), GFR was measured 8 weeks after diabetes onset, thus, 4 weeks after start of treatment. Asterisks indicate statistical difference compared with the N-early group after 4 weeks; *** $p < 0.001$.

Glomerular volume

The average glomerular volume was $1.34 \pm 0.03 \times 10^6 \mu\text{m}^3$ in the D group, which was 33% larger than in the N group ($1.01 \pm 0.05 \times 10^6 \mu\text{m}^3$, $p < 0.001$). The glomerular volume in the DCp group ($1.09 \pm 0.02 \times 10^6 \mu\text{m}^3$) was 20% smaller than that of the D group ($p < 0.001$) and similar to that of the non-diabetic animals (Fig. 11).

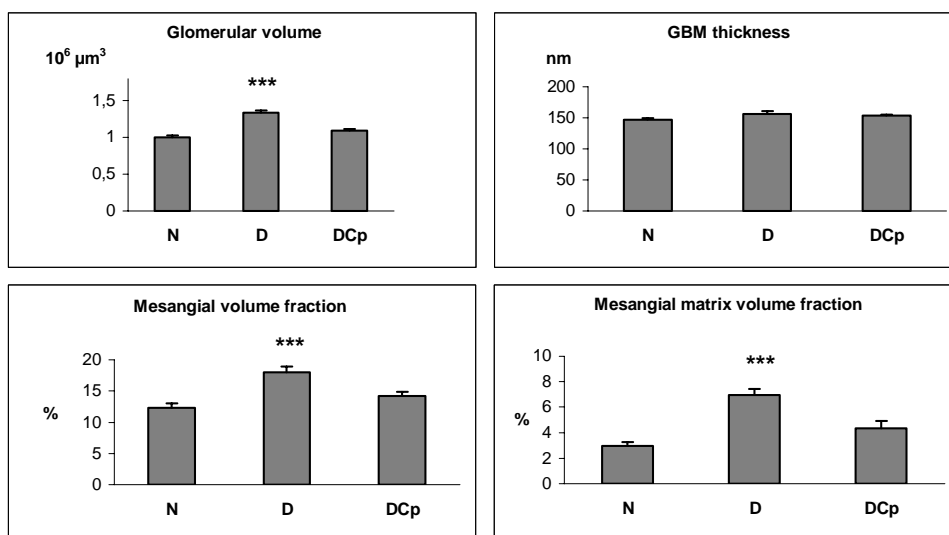


Figure 11. Glomerular, mesangial and mesangial matrix volume fractions and GBM thickness after 8 weeks (4 weeks after treatment start) in each study group: the normal placebo treated group (N), the diabetic placebo treated group (D) and the diabetic C-peptide treated group (DCp). Asterisks indicate statistically significant differences from the N group; *** $p < 0.001$.

Mesangial and mesangial matrix volumes

The mesangial volume fraction (Figs. 11 and 12) of the glomerular volume was $12.3 \pm 0.7\%$ in the N group, $18.0 \pm 0.9\%$ in the D group and $14.2 \pm 0.7\%$ in the DCp group. Thus, the D group showed a mesangial volume fraction that was 46% larger than in the N group ($p < 0.001$) and 27% larger than in the DCp group ($p < 0.01$). In contrast, the diabetic C-peptide treated group (DCp) did not differ significantly from the normal (N) group.

The relationships for the mesangial matrix volume fraction (Figs. 11 and 12) were similar to those for the total mesangial volume fraction. Thus, the mesangial matrix volume fraction of the total glomerular volume was $3.0 \pm 0.3\%$ in the N group, $7.0 \pm 0.5\%$ in the D group and

4.4±0.6% in the DCp group. The D group showed a mesangial matrix fraction that was more than twice that in the N group ($p<0.001$) and 59% larger than in the DCp group ($p<0.01$). The DCp group did not differ significantly from the N group. The mesangial cell volume fraction (data not shown) of the glomerular volume did not differ significantly between the study groups.

GBM thickness

The thickness of the GBM did not differ significantly between the study groups. Thus, GBM thickness was 147±2 nm in the N group, 156±6 nm in the D group and 153±2 nm in the DCp group (Fig. 11).

Interstitial tissue

There were no signs of interstitial expansion, fibrosis or tubular atrophy in any of the study groups.

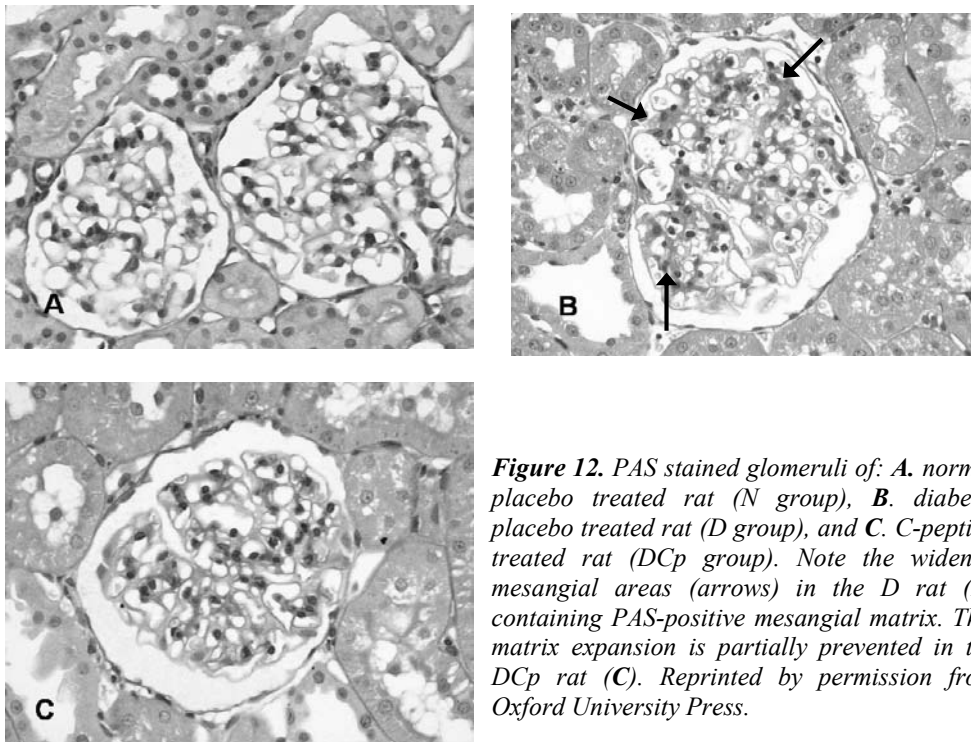


Figure 12. PAS stained glomeruli of: **A.** normal placebo treated rat (N group), **B.** diabetic placebo treated rat (D group), and **C.** C-peptide treated rat (DCp group). Note the widened mesangial areas (arrows) in the D rat (**B**) containing PAS-positive mesangial matrix. This matrix expansion is partially prevented in the DCp rat (**C**). Reprinted by permission from Oxford University Press.

Albuminuria

There was a large dispersion in the urinary albumin excretion rate in all study groups. The excretion rate in the healthy N group after four and eight weeks was 109 ± 23 $\mu\text{g/day}$ and 239 ± 67 $\mu\text{g/day}$, respectively. In the D group the albumin excretion was 546 ± 133 $\mu\text{g/day}$ ($p<0.05$ vs. the N group) after four weeks and 591 ± 212 $\mu\text{g/day}$ after eight weeks (n.s. vs the N group). The DCp group showed an albumin excretion of 441 ± 126 $\mu\text{g/day}$ and 698 ± 113 $\mu\text{g/day}$ after four and eight weeks, respectively (both n.s. vs the normal group).

Study IV

This study shows additive effects of C-peptide and captopril with regard to inhibition of glomerular type-IV collagen. C-peptide, but not captopril, in single therapy also lowered type-IV collagen. C-peptide, captopril and the combination of the two effectively prevent glomerular hypertrophy, albuminuria and glomerular RAGE expression.

Metabolic data

Before diabetes induction, all groups were similar in body weight, water and food intake, urine and faeces excretion, urine osmolality and excretion of sodium and potassium in the urine. All diabetic groups presented with severe hyperglycemia, low weight gain, polydipsia, polyuria and low urine osmolality compared with the N group. Urinary potassium excretion was increased in the ACEI treated groups and urinary sodium excretion in the DCpACEI group compared with the N group. In these metabolic respects there were no significant differences between any of the diabetic groups.

C-peptide levels

The C-peptide levels in the D (75 ± 11 pmol/l) and the DACEI (117 ± 30 pmol/l) groups were low compared with the N group (1970 ± 228 pmol/l), as expected in streptozotocin induced diabetes mellitus. In contrast, the C-peptide levels in the C-peptide treated diabetic groups, i.e. the DCp (1052 ± 536 pmol/l) and the DCpACEI (1135 ± 279 pmol/l) groups, were in the physiological range and did not differ statistically significantly from the N group.

GFR

GFR levels are shown in (Fig. 13). There were no significant differences in GFR between the study groups after 8 weeks, showing that C-peptide and captopril alone or in combination did not affect GFR at this stage of diabetes.

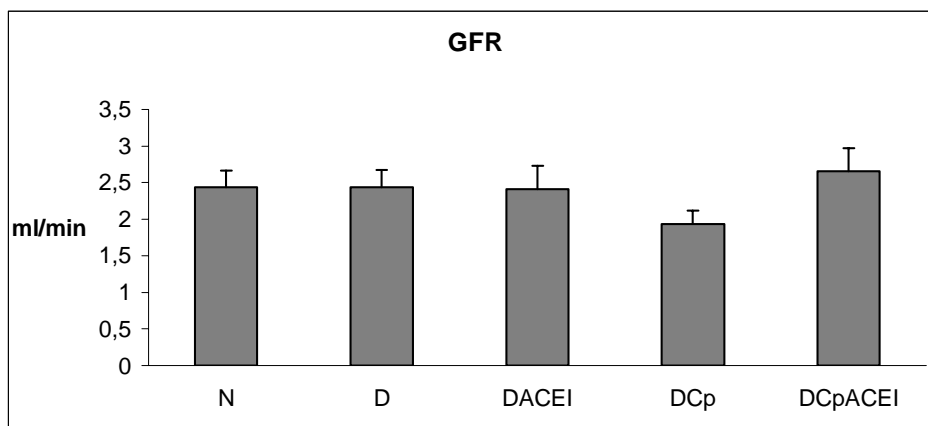


Figure 13. GFR at 8 weeks after induction of diabetes and treatment start. Healthy placebo treated rats (N), diabetic placebo-treated rats (D), diabetic C-peptide-treated rats (DCp), diabetic rats treated with captopril (DACEI) and diabetic rats treated with the combination of C-peptide and captopril (DCpACEI). There were no statistically significant differences between the groups.

Blood pressure

The mean arterial blood pressure (MAP) after 8 weeks during anaesthesia was 138 ± 4 mm Hg in the N group. All the diabetic groups had MAP in the range 106-111 mm Hg, significantly lower than in the N group. There was no statistically significant difference between the diabetic groups.

Albumin/creatinine ratio

The albumin/creatinine ratio increased, in absolute terms, in all diabetic study groups during the study but only in the untreated diabetic group (D group) was the change statistically significant (Fig. 14), indicating a protective effect on albuminuria from C-peptide, captopril or a combination of the two. The final ratio was 1.6 ± 0.4 in the N group, 17.3 ± 6.2 in the D group, 12.9 ± 5.9 in the DACEI group, 6.4 ± 2.3 in the DCp group and 3.5 ± 1.3 in the DCpACEI group.

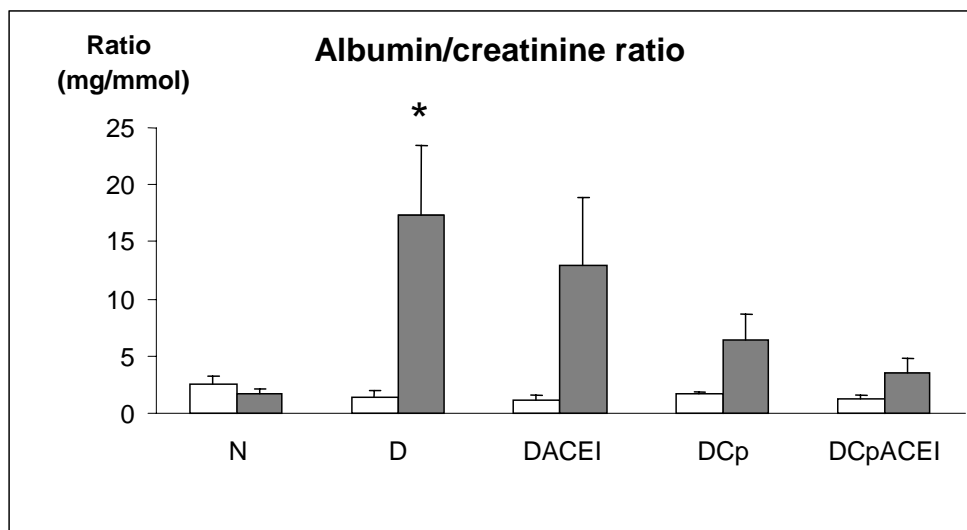


Figure 14. Albumin/creatinine ratio before and after 8 weeks of treatment. Healthy placebo treated rats (N), diabetic placebo-treated rats (D), diabetic C-peptide-treated rats (DCp), diabetic rats treated with captopril (DACEI) and diabetic rats treated with the combination of C-peptide and captopril (DCpACEI). Statistically significant differences, within each group, from the basal state are shown, * $p < 0.05$.

Glomerular volume

The placebo treated diabetic (D) group presented with statistically significant glomerular hypertrophy ($1.50 \pm 0.08 \times 10^6 \mu\text{m}^3$) compared with the normal (N) group ($1.13 \pm 0.04 \times 10^6 \mu\text{m}^3$, $p < 0.01$). This hypertrophy was prevented in the captopril treated diabetic (DACEI) group ($1.28 \pm 0.08 \times 10^6 \mu\text{m}^3$), in the C-peptide treated diabetic (DCp) group ($1.15 \pm 0.05 \times 10^6 \mu\text{m}^3$) and in the combination treated (DCpACEI) group ($1.19 \pm 0.05 \times 10^6 \mu\text{m}^3$). None of these three groups differed significantly from the N group (Fig. 15). Furthermore, the DCp and DCpACEI groups had significantly smaller glomeruli than the D group ($p < 0.05$), while the observed difference between the D and DACEI groups did not reach statistical significance ($p = 0.10$).

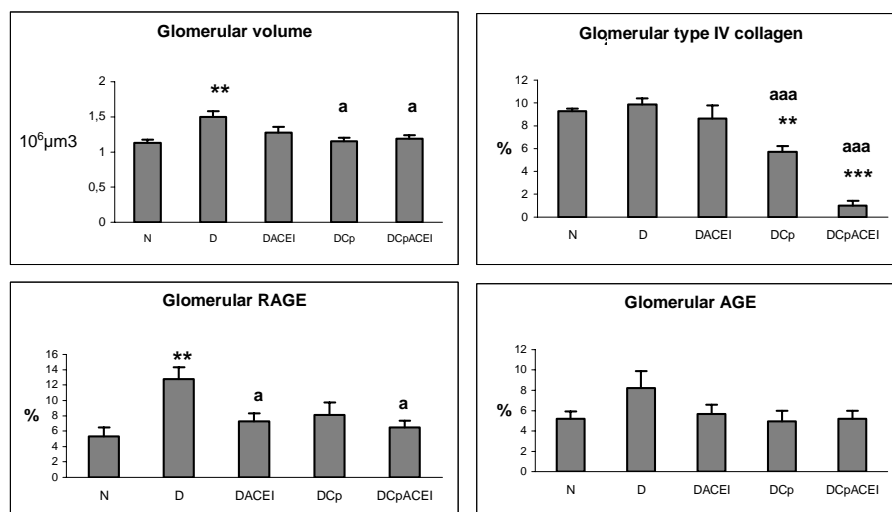


Figure 15. Glomerular volume, type-IV collagen, RAGE and AGE at 8 weeks after induction of diabetes and treatment. Healthy placebo treated rats (N), diabetic placebo treated rats (D), diabetic C-peptide treated rats (DCp), diabetic rats treated with captopril (DACEI) and diabetic rats treated with the combination of C-peptide and captopril (DCpACEI). Asterisks show statistically significant differences from the normal N group; ** $p < 0.01$, *** $p < 0.001$. Letter a shows statistically significant difference from the D group ^a $p < 0.05$, ^{aaa} $p < 0.001$.

Type IV collagen expression

There was no statistically significant difference in type IV collagen expression between the N (9.24 ± 0.25 %), the D (9.85 ± 0.58 %) and the DACEI (8.15 ± 1.13 %) groups. However, the DCp group (5.74 ± 0.47 %) had significantly lower collagen expression than the N ($p < 0.01$), D ($p < 0.001$) and DACEI ($p < 0.01$) groups. In the DCpACEI group, type IV collagen expression (0.98 ± 0.43 %) was significantly lower than in any other group ($p < 0.001$), (Fig. 15).

AGE and RAGE

RAGE was significantly ($p < 0.01$) increased in the placebo treated D group ($12.75 \pm 1.57\%$) compared with the healthy N group ($5.36 \pm 1.09\%$), while the DACEI ($7.27 \pm 1.02\%$), DCp ($8.09 \pm 1.60\%$) and the DCpACEI ($6.50 \pm 0.83\%$) groups did not differ significantly from the healthy N group (Fig. 15). Furthermore, the DACEI and DCpACEI groups expressed significantly less RAGE than the D group ($p < 0.05$), while the observed difference between the D and DCp groups did not reach statistical significance ($p = 0.095$). For AGE there were no significant differences between the groups (Fig. 15). However, there was a tendency towards increased AGE expression in the placebo treated D group versus the N group.

DISCUSSION

It used to be thought that C-peptide has no physiological effects [124, 125]. In the past decade, however, this view has gradually changed on account of the accumulation of data demonstrating that C-peptide binds specifically to cell membranes, shows intracellular signalling pathways distinct from those of insulin, stimulates Na^+ , K^+ -ATPase, eNOS and transcription factors, resulting in amelioration of diabetic neuropathy [153-155, 157] and nephropathy [153, 159-162]. This thesis adds further information to support the view that C-peptide is a bioactive peptide with metabolic effects that may prove important in the prevention of nephropathy in type-1 diabetes.

Hyperfiltration, RFR, RBF and glomerular hypertrophy

Glomerular hyperfiltration is the earliest indication of renal involvement in diabetes. During the first five years of diabetes, about 50% of the type-1 diabetic patients present a GFR that is 25-50% above normal [18]. The most important hemodynamic mechanism underlying hyperfiltration is a decrease in glomerular afferent and efferent arteriolar resistances. The dilatation is most prominent on the afferent side [15, 16]. This afferent dilatation, which entails loss of renal autoregulation to blood pressure changes, is probably caused by a variety of mechanisms, such as tubuloglomerular feedback signalling and increased levels of AGE, sorbitol and IGF-1. Other candidates are increased ANP and altered levels of prostaglandins and NO. The pathogenetic importance of hyperfiltration is still being debated. It has been shown that patients with hyperfiltration have an increased risk of developing microalbuminuria at a later stage of the diabetic disease [17].

This thesis shows that Sprague-Dawley rats with streptozotocin-induced diabetes present with glomerular hyperfiltration at 2 weeks after diabetes induction (studies I and II). The hyperfiltration phase has passed after 8 weeks (study IV). Wistar rats in study III present with glomerular hyperfiltration after 4 weeks of diabetes but not after 8 weeks, attesting to the transitory nature of the glomerular hyperfiltration in diabetes.

Study I shows that C-peptide treatment for 14 days prevents glomerular hyperfiltration in streptozotocin-induced diabetes, indicating that this effect, demonstrated earlier in an acute-phase study [161], is not just transient. In study II, an acute infusion of C-peptide lowered GFR by 17%, similarly to captopril and to the combination of captopril and C-peptide. ACEI

is known to lower intraglomerular pressure by dilating the arterioles, predominantly the efferent ones. The resultant increase in renal plasma flow does not usually compensate for the lowered glomerular pressure, the net result being a decrease in GFR [16]. Besides reduced AII, an increased level of bradykinin may contribute to this ACEI effect. This view is supported by the fact that angiotensin receptor blockers do not influence GFR to the same extent as ACEI [169].

It is conceivable that C-peptide exerts its effects of GFR by the same mechanisms as ACEI. To test this hypothesis, renal blood flow changes were measured in study II. Blood flow increased with ACEI and with the combination of ACEI and C-peptide but not with C-peptide alone. These findings are in keeping with an earlier study in rats in which human C-peptide was administered [162]. Accordingly, possible C-peptide effects on the renal arterioles must be in opposite directions in the afferent and the efferent arterioles, respectively. An efferent arteriolar dilatation matched by an afferent constriction could result in a combination of unchanged renal blood flow and lower intraglomerular pressure. This could be a possible explanation for the rapid GFR lowering effect of C-peptide seen earlier [161] and in study II in this thesis. It is also possible, as proposed earlier [162], that C-peptide affects the other major determinant of the filtration fraction, namely the glomerular ultrafiltration coefficient which, in turn, is determined by the filtration surface area and capillary wall permeability. This would be a plausible explanation for the reduction of glomerular hyperfiltration in the long term [160] and in study I, since glomerular hypertrophy is simultaneously prevented in the latter study, but it can hardly explain the short-term effects.

While the filtration fraction fell in all study groups in study II, the fall in the group treated with the combination of C-peptide and ACEI was greater than in the other groups. It was only in this group that the decrease in filtration fraction exceeded that in the diabetic placebo treated group. These findings indicate that C-peptide and ACEI exert their effects on GFR by different mechanisms. Micropuncture studies are planned to confirm this.

Renal functional reserve is the difference between basal GFR and GFR maximally stimulated by, for example, an amino acid load [31]. Absence of RFR, seen in diabetes [29, 30], may be an early indicator of risk of diabetic nephropathy since it points to an increased work load per nephron [31]. According to this theory, the residual nephrons in the diabetic patient are already in a state of maximal filtration. Measurement of RFR in study I showed that RFR is absent in untreated diabetic rats. The C-peptide treated group, however, presents with

preserved RFR. These rats' nephrons have thus maintained their ability to respond in a normal physiological way to an amino acid load.

Glomerular and renal hypertrophy often occur simultaneously [13] and the hypertrophy starts soon after diabetes onset [170]. The glomerular hypertrophy is a possible prognostic marker [14]. Glomerular hypertrophy with increased radius of the capillary loop increases the strain on the capillary wall according to Laplace's law. Together with increased intraglomerular pressure, this could result in greater damage to the endothelial cells and podocytes. Study I provided the first evidence that C-peptide prevents glomerular hypertrophy and this was confirmed in studies III and IV. As described above, this could possibly be explained by decreased intraglomerular pressure but it also seems to be at least partly due to reduced mesangial expansion (see below). Furthermore, one might speculate that the number and length of the glomerular capillary loops decrease due to positive C-peptide effects on growth factors and cytokines, e.g. TGF- β . There is also evidence, in study IV, of decreased type-IV collagen content in the glomeruli, which may have an impact on glomerular hypertrophy.

Albuminuria

Microalbuminuria is the earliest laboratory sign of renal damage in diabetes. It offers a possibility to identify patients at risk of progression to overt albuminuria and later decline in GFR. Albuminuria itself also contributes to the renal damage as it induces inflammatory responses when large amounts of albumin are reabsorbed in the tubules [36].

The mechanisms causing proteinuria in diabetes are probably multifactorial. An increased number of large pores [87] in the filtration barrier, a decreased amount of negatively charged molecules such as heparan sulphate [86] in the GBM and in the endothelial glycocalyx, and disturbed podocyte function with nephrin deficiency in the filtration slit [89] are all mechanisms that appear to contribute. C-peptide has earlier been shown to lower urinary albumin excretion in human type-1 diabetic patients [153, 160] and in acute experiments in streptozotocin diabetic rats [161].

Treatment with C-peptide for 14 days to streptozotocin diabetic Sprague-Dawley rats prevented the albuminuria that occurred in the untreated diabetic group (study 1). The ratio between albumin and creatinine in the urine was significantly lower in the C-peptide treated group. The glucose levels were high and similar in these groups. Similar results were

observed in study IV, which was also performed in Sprague-Dawley rats. In this study, albumin excretion in the diabetic placebo treated group was increased after 8 weeks of diabetes compared with at the start of the study, whereas this was not the case in the ACEI, C-peptide or combination treated groups. However, there was a tendency towards increased albumin excretion even in the latter groups. In absolute terms, albumin excretion was lowest in the combination treated group.

Study III differed from the other studies in that albumin excretion showed no statistically significant increase in any of the study groups after 8 weeks of diabetes, although it did tend to rise in the diabetic groups. A possible explanation is the use of a different rat strain; moreover, the dispersion was quite large, which is a well-known problem in rat models of diabetes. Other factors that may have contributed are intraindividual day-to-day variation and methodological factors such as stress in the metabolic cages.

The mechanisms for the C-peptide effects on albuminuria are still poorly investigated. The effect may partly depend on the lowered GFR seen in the C-peptide treated groups, since hyperfiltration can cause a transient albuminuria. However, there is, in study IV, evidence of lower albumin excretion even when the hyperfiltration phase is over and the GFR is similar in the study groups. This effect is probably dependent on the permeability of the filtration barrier. Changes in the charge barrier due, for example, to a decreased amount of heparan sulphate or to other changes in the composition of the endothelial and podocyte glycocalyx are other possible mechanisms. The effect of C-peptide on nephrin in the filtration slit is also as yet unknown. It should be noted that in the early stage of diabetes studied in this thesis the nephrin deficiency is usually not apparent [171].

The tendency, observed in study IV, for the combination of C-peptide and ACEI treatment to be even more effective than ACEI or C-peptide alone is interesting. This is in accordance with the earlier discussion concerning the possibility that the two substances differ in their action mechanisms in the kidney and may point the way to improved prevention of nephropathy in the future. However, as incomplete angiotensin II inhibition is also conceivable in study IV, it could still be the case that C-peptide and ACEI do act via similar metabolic mechanisms.

Renal morphology

As already discussed, the first renal morphological hallmark in diabetes is glomerular hypertrophy. It is usually followed by thickening of the GBM [76] and, as the disease progresses, by arteriolar hyalinosis and mesangial matrix expansion.

Studies I and III show that C-peptide prevents glomerular hypertrophy and study III also demonstrates that this can be explained, at least in part, by reduced mesangial matrix expansion. This new finding appears to be important, since mesangial expansion is the single structural variable best correlated to proteinuria, hypertension and progression of diabetic nephropathy [75].

TGF- β , known to induce production of matrix components and to inhibit matrix protein degradation, may be involved in the C-peptide action in the kidney. It has been shown that C-peptide dose-dependently inhibits TGF- β induced up-regulation of collagen IV in podocytes [147]. Study IV shows that C-peptide substantially reduces glomerular type-IV collagen expression and that a combination of C-peptide and captopril is even more effective in this respect. Long-term studies are needed to explore whether these changes will also prevent glomerular fibrosis.

AGE and RAGE accumulating in the glomerular basement membrane and mesangium correlate with the degree of nephropathy in diabetics [39]. Immunohistochemistry in study IV shows that C-peptide, captopril or the combination of the two prevent a diabetes induced increase of RAGE expression in the glomeruli. For AGE the tendency was the same, though not statistically significant. Further studies of the C-peptide effects on different subclasses of RAGE and AGE may give useful information about the additive effect of C-peptide and captopril on type-IV collagen expression.

The morphological findings in this thesis are of interest in connection with the observation that pancreas transplantation is followed by a reversal of the mesangial matrix expansion in diabetic patients. Moreover, successful pancreatic islet transplantation leads to an improved kidney graft survival rate and decreased urinary albumin excretion [172]. These effects are probably due in part to improved glycemic control [99]; in the latter study, however, restored levels of C-peptide were proposed as another possible mechanism behind these positive renal findings after transplantation.

Study III also aimed to examine C-peptide's effects on GBM thickening. However, this could not be done since no sign of GBM thickening could be detected in the diabetic placebo treated group at this stage of diabetes in Wistar rats. This was surprising since GBM thickening in diabetic patients usually occurs before the onset of mesangial matrix expansion. However, the time at which GBM thickening occurs in Wistar rats varies greatly in the literature, from 8 weeks to 6 months.

Methodological considerations

Originally evaluated as an antibiotic drug, streptozotocin was soon found to cause pancreatic beta cell damage and diabetes induction. The streptozotocin model has been widely used as a type-1 diabetic experimental model and as such is well established. The timing of diabetes onset after the streptozotocin injection is quite predictable and reproducible [173]. However, the model has been criticized for streptozotocin's non-specific toxicity and its mutagenic effect [174]. The latter aspect is not an important issue in this thesis since the follow-up time is too short for any tumours to occur. But the unspecific toxicity in the kidney [175] could have influenced the results. This risk for misinterpretation of data is low, however, since in this context the toxic effects of streptozotocin, such as tumours and tubular abnormalities, do not mimic early diabetic nephropathy [176]. Furthermore, all treatment groups in this thesis have been compared to a diabetic control group that received the same amount of streptozotocin. The major disadvantage with the streptozotocin model of diabetes is that these rats do not develop advanced renal insufficiency at later stages of nephropathy than studied in this thesis. Another rat model should be chosen for such studies of long-term effects.

One objection to this model may be that in the present studies the diabetic rats were not treated with insulin. We chose this model to clarify the effects of C-peptide itself and to speed up the development of the diabetic lesions.

GENERAL SUMMARY AND CONCLUSIONS

1. C-peptide administration to rats with streptocotozin induced type-1 diabetes mellitus:

- reduces glomerular hyperfiltration, loss of RFR and albuminuria.
- prevents glomerular hypertrophy and mesangial matrix expansion.
- reduces glomerular RAGE and type-IV collagen expression.

2. C-peptide and captopril administration to rats with streptocotozin induced type-1 diabetes mellitus equally effectively:

- prevents glomerular hyperfiltration and albuminuria.
- reduces glomerular RAGE expression in rats with experimental type-1 diabetes mellitus.

3. C-peptide and captopril administration to rats with streptocotozin-induced type-1 diabetes mellitus exerts additive effects in lowering:

- glomerular type-IV collagen.
- glomerular filtration fraction.

4. C-peptide administration in replacement dose to rats with experimental diabetes exerts beneficial effects on renal function and structure. It is possible that the beneficial effect may be enhanced by co-administration of C-peptide and ACEI.

FUTURE PERSPECTIVES

Since insulin treatment and antihypertensive treatment do not prevent diabetic nephropathy in all patients, factors other than hyperglycemia must contribute to this process. The conclusion in this thesis is that C-peptide is one such factor that may prevent or retard type-1 diabetic nephropathy. ACEI and ARB are documented renoprotective drugs. These drugs are not always sufficient and are difficult to use in some patients on account of side effects and hyperkalemia. C-peptide, an endogenous hormone, is less prone to exert negative side effects and data also suggest that C-peptide may be beneficial in other diabetic complications, such as diabetic neuropathy.

This thesis has presented data supporting additive protective effects of C-peptide and ACEI. Future long-term clinical studies in patients with type-1 diabetes will be required to further clarify whether or not the two drugs have additive effects in the long term. Further efforts to clarify the molecular mechanisms of action of C-peptide are also crucial for determining the role of C-peptide. In particular, effects on pro-sclerotic growth factors such as TGF- β , CTGF and VEGF should be evaluated. Furthermore, the effects must be confirmed in insulin-treated patients and animals.

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