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C-Peptide as a Therapeutic Tool in Diabetic Nephropathy

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Key Words
C-peptide · Diabetic nephropathy

Abstract
Background/Aims: Insulin is synthesised as a pro-hormone with an interconnecting C-peptide, cleaved during post-translational modification. This review discusses growing evidence which indicates that C-peptide is biologically active, benefiting microvascular complications associated with diabetes. ***Methods:*** To explore the renoprotective role of C-peptide in diabetic nephropathy (DN), we reviewed the literature using PubMed for English language articles that contained key words related to C-peptide, kidney and DN. ***Results:*** Numerous studies have demonstrated that C-peptide ameliorates a number of the structural and functional renal disturbances associated with uncontrolled hyperglycaemia in human and animal models of type 1 diabetes mellitus that lead to the development and progression of nephropathy, including abrogation of glomerular hyperfiltration, reduced microalbuminuria, decreased mesangial expansion and increased endothelial nitric oxide synthase levels. The in vitro exposure of kidney proximal tubular cells to physiological concentrations of C-peptide activates extracellular signal-regulated kinase, phosphatidylinositol 3-kinase, protein kinase C, elevates intracellular calcium, and stimulates transcription factors NF-κB and peroxisome proliferator-activated receptor-γ. ***Conclusion:*** Burgeoning studies suggest that C-peptide is more than merely a link between the A and B chains of the proinsulin molecule and represents a future therapeutic tool in reducing complications of DN.

Introduction

For those individuals with type 1 diabetes mellitus (T1DM) there is an absolute requirement for the exogenous administration of insulin. Although alleviating acute hyperglycaemia, insulin alone is unable to prevent chronic and detrimental changes in renal function. The pro-insulin product, C-peptide, has until recently been regarded as biologically inert, acting to ensure the correct folding of the A and B chains to enable alignment and interchain disulfide bond formation [1]. Cleaved from insulin in secretory granules within the pancreatic β cells, C-peptide is released at equimolar concentrations to insulin, and is used clinically as a marker of insulin release and a measure of β-cell activity. Structural variability and the lack of a conserved sequence between species have led to a general consensus that C-peptide lacks physiological activity. However, growing evidence

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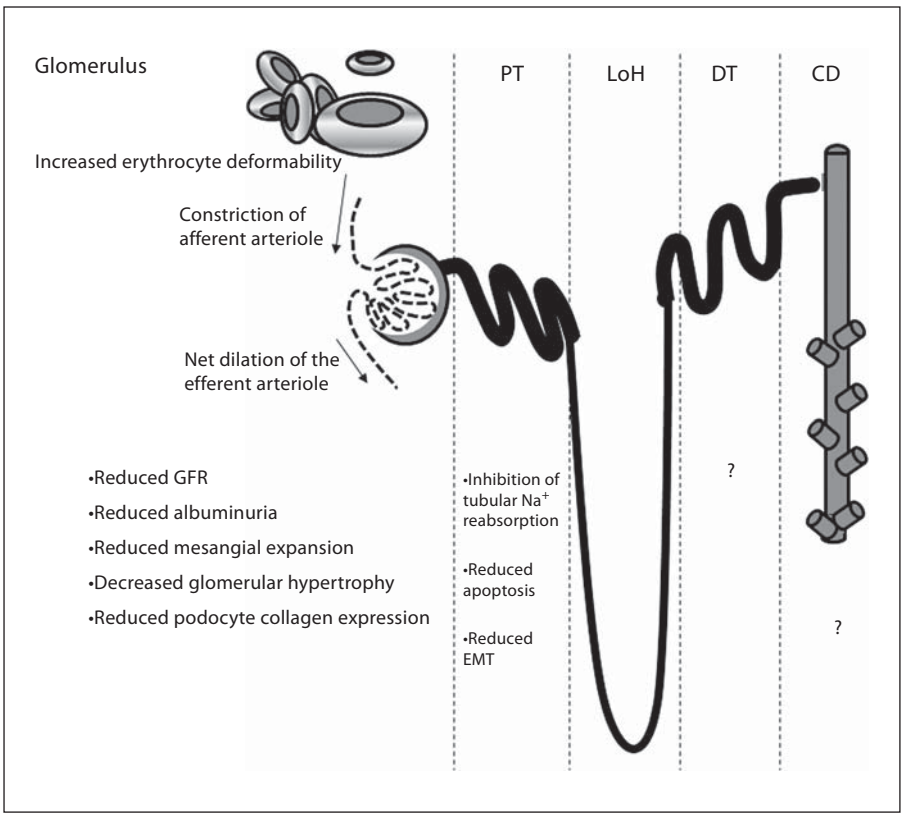

Fig. 1. Current understanding of the protective effects of C-peptide in the glomerulus, the proximal tubule (PT), the loop of Henle (LoH), the distal tubule (DT) and the collecting duct (CD) are illustrated in the schematic above.

supports a role for C-peptide in reducing microvascular complications associated with T1DM, making it much more than just a link between the A and B chains of insulin.

Although C-peptide has no overt beneficial effects in non-diabetic healthy subjects, [2] in both human and animal models of T1DM, C-peptide has demonstrated endocrine-like activity and ameliorates structural and functional disturbances associated with uncontrolled hyperglycaemia salient to the development and progression of renal disease [3, 4]. The ability of C-peptide to reduce renal complications, such as diminished glomerular hyperfiltration [3], reduced microalbuminuria [5], decreased mesangial expansion [6] and increased endothelial nitric oxide synthase (eNOS) [7] (fig. 1), in addition to protecting against tumour necrosis factor-α (TNF-α)-induced apoptosis [8] and transforming growth factor-β (TGF-β)-induced epithelial to mesenchymal transition (EMT) [9], raises the possibility that C-peptide possesses important biological function. In the current review, we examine recent findings supporting a role for C-peptide in reducing/preventing the underlying pathology of diabetic nephropathy (DN) and we discuss the potential for using C-peptide as a future adjunct therapy to insulin in alleviating renal complications associated with T1DM.

Role for C-Peptide in Alleviating the Complications of Diabetic Nephropathy

Worldwide, diabetic nephropathy is the single commonest cause of entry into the renal replacement therapy programme and represents the leading cause of end-stage renal failure in both type 1 (T1DM) and type 2 (T2DM) diabetes mellitus [10]. Of those patients with diabetes, approximately 30–40% will develop DN.

DN refers to a set of structural and functional changes which arise in response to chronic glycaemic assault [11]. Structural abnormalities include hypertrophy, glomerular basement membrane thickening, tubular atrophy and interstitial fibrosis [12]. These changes contribute to increased glomerular filtration rate (GFR), proteinuria, systemic hypertension and loss of renal function [13]. Histologically, DN is characterised by an accumulation of extracellular matrix proteins in the glomerular mesangium

[12, 14] and tubular interstitum. Consequently, an increase in GFR in the early phase of diabetes has been proposed to be related to the development of DN, proteinuria, systemic hypertension and loss of renal function. These changes occur in the face of pathophysiological stimuli and arise as a result of numerous downstream cellular events. Hyperglycaemia is pivotal in the progression of nephropathy and evidence suggests that poor control of blood glucose is a major contributor to the development of albuminuria [15]. High glucose increases formation of reactive oxygen species, activates PKC, TGF-β_1 and G-protein signalling, and alters the expression of numerous proteins including cyclin kinases, matrix proteins, matrix-degrading enzymes and metalloproteinases [16]. Together these events are interconnected and increase deposition of extracellular matrix, a hallmark of DN.

In patients exhibiting DN and undergoing combined islet-kidney transplantation, the survival of renal allografts depends on maintained islet function [17] and the secretion of C-peptide [18]. From these findings one can speculate that C-peptide may act to protect the kidney from glycaemic assault. In accordance with this supposition, the short- and long-term beneficial effects of C-peptide on renal function in patients with T1DM, presenting with low-grade proteinuria, have been studied by Johansson et al. [19]. Patients were entered into a double-blind study in which the acute (2 h) effects of C-peptide were examined using various parameters of renal function [19]. Patients treated with C-peptide exhibited a 7% fall in GFR from 143 ± 3 to 133 ± 4 ml/min/1.73 m^2 whereas the GFR of controls did not change after 60 min of 0.9% saline infusion. This promising outcome prompted further investigation into the long-term effects in patients with either incipient (1-month double-blind study) or mild nephropathy (3-month blind, randomized crossover study). In both studies, under sustained hyperglycaemia C-peptide successfully reduced both GFR by ~6% and albuminuria by 50% (1-month study) as compared to patients on insulin treatment alone [20]. A 40% reduction in urinary albumin excretion from 58 to 34 μg/min was observed at 3 months [21]. Observations that C-peptide evoked renoprotective effects in patients with T1DM were further supported by studies in animal models of T1DM. Streptozotocin (STZ)-induced diabetic rats present with increased glomerular hyperfiltration, protein leakage, and exhibit classic signs of nephropathy. In these animals, C-peptide has been shown to elicit a 20% reduction in GFR with an even more impressive reduction in albumin levels as compared to untreated control

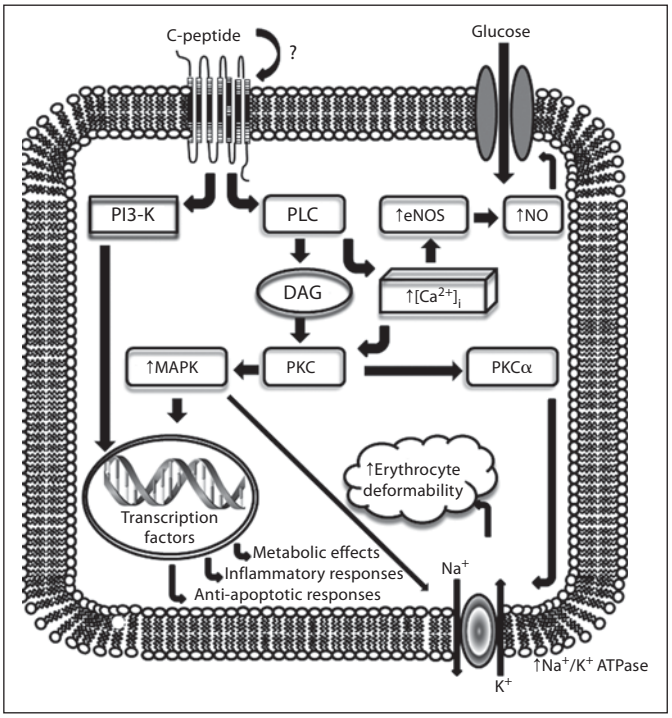

Fig. 2. Cellular effects of C-peptide reputedly involve activation of a pertussis toxin-sensitive G-protein-coupled receptor that stimulates down-stream PLC and PI3-K activity. PLC increases $[Ca^{2+}]_i$, which in turn stimulates both eNOS and PKC. Together with increased DAG synthesis, PKC activates the Na^+/K^+-ATPase via translocation of PKCα to the cell membrane. Stimulation of MAPK also increases Na^+/K^+-ATPase activity and, in addition to elevated PI3-K levels, activates various transcription factors to reduce apoptosis, increase the expression of inflammatory mediators and alter metabolism. These subcellular changes help rectify secondary complications associated with T1DM.

diabetic animals (approx. 70%) [4]. Other groups have used this model to demonstrate improved renal function as measured by reduced GFR, mesangial expansion and diminished albuminuria [3–5]. Elegant studies by Nordquist et al. [22] recently suggested a novel mechanism by which C-peptide may exert its beneficial effects on GFR. The protective effect, mediated by constriction of the afferent glomerular arteriole, net dilation of the efferent arteriole and inhibition of tubular Na^+, reabsorption requires the C-terminal EVARQ fragment of C-peptide [23].

Whilst these findings strongly support the role of C-peptide in alleviating glomerular complications, the effects of this peptide extend far beyond the glomerulus. C-peptide exerts strong anti-apoptotic and anti-fibrotic effects in the proximal kidney through inhibition of

TNF-α-induced apoptosis [8] and TGF-β_1-induced EMT [9]. C-peptide activates a plethora of signalling molecules, including extracellular signal-regulated kinase, phosphatidylinositol 3-kinase, protein kinase C, elevations of intracellular calcium, and stimulation of the transcription factors NF-κB and peroxisome proliferator-activated receptor-γ [reviewed in 24, 25] (fig. 2). Understanding those downstream pathways activated in response to C-peptide is crucial in facilitating strategic targeting for future therapeutic intervention.

Molecular Mechanisms of C-Peptide Action

A putative receptor for C-peptide is yet to be elucidated, however binding studies for C-peptide have demonstrated specific binding sites in various cell types, including pancreatic β-cells [26], human skin fibroblasts and renal tubular cells [27]. Using fluorescently-labelled C-peptide and fluorescence correlation microscopy, it has been suggested that renal tubular cells express 1,000–1,500 binding sites per cell, with 50% occupancy within the physiological range (0.3 nM) and full saturation occurring at a concentration of 0.9 nM [27]. To date, studies have been exclusively performed in patients or animal models with T1DM lacking circulating C-peptide, and it is perhaps of no surprise that supraphysiological supplements of C-peptide in healthy non-diabetic subjects is without effect [28].

Furthermore, binding studies have identified specific regions of C-peptide which confer biological activity. In rat kidney tubule segments the C-terminal pentapeptide of C-peptide, EVARQ, elicited 100% of the Na^+,K^+-ATPase activity of the intact 31 amino acid peptide, whereas the remaining portion of the molecule was completely inactive [29]. The corresponding region of human C-peptide (EGSLQ) elicited 75% activity. The inability of scrambled C-peptide, insulin or insulin-like growth factor to displace C-peptide binding highlights the specificity of the peptide and strongly suggests the existence of a putative receptor [27]. A further clue about the receptor comes from studies where preincubation with pertussis toxin significantly inhibits C-peptide binding at the cell membrane and subsequent downstream signalling events, preventing alterations in cell phenotype that occur in response to ligand binding [8]. The most straightforward explanation of these observations is that the C-terminal pentapeptide binds at the cell membrane and influences cellular signalling events via a G-protein-coupled receptor. These cell signalling events are discussed below.

Na^+/K^+-ATPase, ATP and C-Peptide Resistance in Type 2 Diabetes

A ubiquitous membrane-bound enzyme, the ouabain-sensitive Na^+/K^+-ATPase, helps maintain the ionic gradient across the renal plasma membrane at the expense of ATP hydrolysis. In diabetes, an initial transient rise in ATPase activity is rapidly accompanied by a subsequent decrease in enzyme activity as the condition progresses [30]. Connecting peptide (10–100 nm) stimulates ATPase activity in a pertussis toxin-dependent manner, an effect reliant on conserved homology of the C-terminal pentapeptide sequence [29]. At the molecular level, this activation occurs via PKC-dependent MAPK, enabling phosphorylation of Thr residues on the α subunit by ERK-1/2 [31]. This PI3-K and PKC-dependent phosphorylation relies on the translocation of PKC-α to the membrane fraction [32]. Incubation of human renal tubular cells with the isoform-specific PKC-δ inhibitor rottlerin (20 μM) confirmed PKC-δ to be the predominant isoform required for C-peptide-induced ERK-1/2 phosphorylation [33].

Numerous studies, many of which have been performed under normoglycaemic conditions, have reported a stimulatory role for C-peptide on the Na^+/K^+-ATPase either in vivo [34] or in vitro [32, 35]. Under diabetic conditions, the story in the kidney is quite different. An increase in renal Na^+/K^+-ATPase activity and an increase in total oxygen consumption (Q_{O_2}) in the early phase of glomerular hyperfiltration in STZ diabetes have been reported [36]. These studies suggest C-peptide is able to reduce diabetes-induced hyperfiltration via a net dilation of the efferent arteriole and inhibition of Na^+ reabsorption in the proximal tubule. The reduction in Na^+ reabsorption was matched by reduced Na^+/K^+-ATPase activity, an effect demonstrated by both increased fractional urinary Na^+ excretion and reduced transport, independent of Q_{O_2} [22]. Since electrolyte transport utilises 80% of Q_{O_2}, it is likely that in DM, an increase in Na^+/K^+-ATPase activity is accompanied by impaired renal O_2 availability. These recent findings support a novel role for C-peptide in reducing diabetes-induced hyperfiltration, an effect which will undoubtedly have beneficial effects throughout the kidney and protect against those complications of DN.

Patients with T1DM often exhibit an array of complications arising from microvascular changes [34]. Determined by blood viscosity and the deformability of erythrocytes, a reduction in either will have severe implications for blood flow and oxygen supply. As a result of reduced Na^+/K^+-ATPase activity, erythrocyte deform-

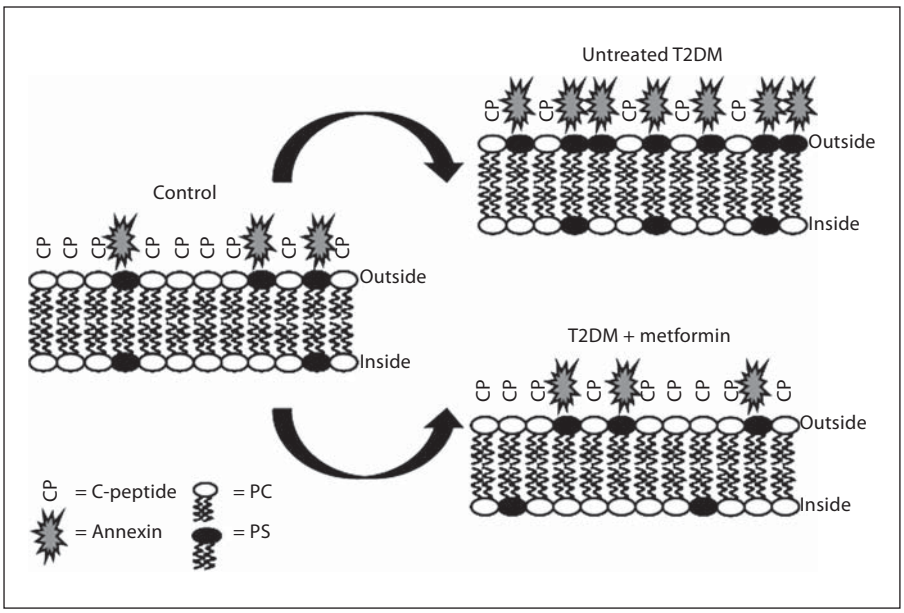

Fig. 3. This diagram illustrates suspected differences in C-peptide binding to erythrocyte membranes in healthy non-diabetic cells versus cells from T2DM, where the membrane exhibits glucose-induced increases in phosphatidylserine (PS) content. PS externalisation increases annexin binding in preference to that of C-peptide, whilst addition of metformin reduces membrane negativity (possibly by charge shielding) and facilitates C-peptide binding at the expense of annexin. For the erythrocyte, the net result is an increase in ATP production. Adapted from Meyer et al. [45].

ability is significantly impaired in T1DM [37]. By stimulating ATPase activity, it is easy to envisage how C-peptide could alleviate deformation of erythrocytes and improve blood flow. The C-terminal fragment of C-peptide has been shown to restore erythrocyte deformability via stimulation of Na^+/K^+-ATPase activity, an effect negated by either pertussis toxin, ouabain or EDTA [38]. Furthermore, stimulation of the enzyme is accompanied by a rise in ATP [39], a recognised stimulus for nitric oxide (NO) production in platelets [40] and endothelium [41]. A potent vasodilator, NO improves erythrocyte deformability by a direct action on the erythrocyte membrane, raising the possibility that C-peptide may improve renal blood flow through ATP-induced NO release [42, 43].

Connecting peptide has been implicated in GLUT-1-mediated glucose uptake and ATP release in erythrocytes [39]. The effect requires C-peptide to be bound to a metal ion such as Cr^{3+} or Fe^{2+}. Meyer et al. [39] hypothesised that this metal ion facilitates C-peptide binding by nullifying repulsion at the negatively charged erythrocyte membrane. To date, clinical studies of C-peptide have been performed exclusively in patients with T1DM, where we can readily envisage how C-peptide replacement may exert beneficial biological effects in the setting of complete deficiency. However, in patients with T2DM, developing insulin resistance is associated with elevated circulating insulin and C-peptide concentrations. Nevertheless, patients with T2DM still develop nephropathy and thus a role for C-peptide is less clear. In 2009, Spence and colleagues [39, 44] extended their observations of C-peptide-induced ATP release in erythrocytes by examining C-peptide resistance in a rat model of T2DM. Using the BBZ DR/Wor rat they examined a role for Zn^{2+}-activated C-peptide in erythrocyte ATP release from cells representing both the healthy and T2DM condition [45]. They revealed that ATP release under non-diabetic conditions was greater than that observed within the diabetic erythrocyte (78.4 ± 4.9 vs. 31.2 ± 4%). Explanation for this is complex, involving hyperglycaemia-induced redistribution of the erythrocyte lipid bilayer. Previous studies have demonstrated that erythrocytes in T2DM exhibit clear structural differences at the cell membrane, reflecting glucose-induced phosphatidylserine (PS) externalisation from the inner leaflet [46]. Since PS has a more negative charge than other components of the lipid bilayer, it seems likely that erythrocytes in T2DM may interact with the negatively charged C-peptide less favourably than those obtained from non-diabetic healthy controls (fig. 3). This observation may pave the way for a greater understanding of C-peptide resistance in type 2 diabetes. Further support for this hypothesis comes from recent studies by Meyer et al. [45] who demonstrated that Zn^{2+}-activated C-peptide-mediated ATP release from erythrocytes from a T2DM model could be normalised to match that of non-diabetic controls following preincubation with the biguanide metformin. Used in the treatment of T2DM, metformin is thought to facilitate C-peptide binding at the erythrocyte membrane by disrupting the

highly negative charge that accompanies PS externalisation. The lipid domains of the cell membrane are believed to be one of the sites where biguanides exert their antihyperglycaemic effect. Other studies demonstrating increased glucose transport [47] and improved blood flow [48] suggest that metformin acts generally to increase membrane fluidity [49]. Decreased binding of annexins (a class of proteins which bind negatively charged phospholipids) at the erythrocyte membrane would also indicate that metformin is able to disrupt the PS lipid bilayer, possibly by charge shielding. These studies not only demonstrate the concept of C-peptide resistance in T2DM and the ability of metformin in sensitizing these effects, but also provide an insight into how C-peptide may alleviate local vascular complications via improved renal blood flow through ATP-induced NO release.

Vasoprotective Effects of C-Peptide – a Role for eNOS

In vivo studies of diabetes have demonstrated that C-peptide can improve both blood flow and oxygen uptake in muscle [19]. These effects are mediated through transcriptional expression of eNOS, a key catalytic enzyme involved in the production of NO [7]. Although the cellular mechanisms behind C-peptide-mediated NO stimulation remain unclear, studies in rat aortic endothelial cells have demonstrated that C-peptide augments NO production through stimulated eNOS expression, an effect dependent on MAPK transcriptional activation [43]. Furthermore, in bovine aortic endothelial cells, C-peptide-induced stimulation of eNOS depends on a rise in intracellular calcium and ERK-1/2 activity [42, 43]. Both of these downstream signals have been implicated in the regulation of eNOS gene transcription and could contribute to enhanced vasodilation in T1DM in response to C-peptide.

Although beneficial in alleviating endothelial related microvascular complications, the role of NO in DN is quite different. Glomerular hyperfiltration associated with early-onset diabetes stems from an increase in GFR as a consequence of inappropriate vasodilation of the afferent arteriole [22]. NO is thought to play a key role in this increased GFR, with increased levels of both eNOS and NO reported in the glomerulus and afferent arteriole [50]. However, signalling cascades controlling eNOS gene transcription in the kidney are negatively regulated in the presence of C-peptide. Administration of C-peptide to the STZ rat negates diabetes-induced NO, preventing hyperfusion in the glomerulus and minimising associated complications. Although the mechanisms by which C-peptide mediates this effect remain to be elucidated, a number of potential physiological mechanisms have been proposed. Increased GFR is a key mediator in the development of DN. The capillary pressure of the glomerulus must be tightly regulated to ensure that the pressure does not exceed that required for efficient filtration. Inappropriate dilation of the arteriole will raise glomerular capillary pressure causing hyperfusion, hyperfiltration and albumin leakage. Pharmacological intervention aimed at lowering glomerular capillary pressure would represent an ideal therapeutic site to slow the progression of renal injury in diabetes. Although previously reported in various tissues to stimulate NO, recent studies have shown that C-peptide counteracts diabetes-induced eNOS levels within the kidney [51]. Ultimately this will reduce inappropriate vasodilation of the arteriole, an effect which fails to diminish renal blood flow [52] and is thus thought matched by dilation of the efferent arteriole. The net effect is an overall reduction in GFR and a reduction in albumin escape. We can hypothesise that C-peptide reduces GFR and albumin excretion through a downregulation of eNOS levels.

Fibrosis, EMT and the Anti-Fibrotic Effects of C-Peptide

The underlying pathology of progressive chronic kidney disease in diabetes is renal fibrosis in both the glomerulus and the tubulointerstitium [16]. Whilst glomerular fibrosis is observed in the early progression from incipient to overt nephropathy, a build-up of fibrotic material in the tubular interstitium accompanies disease progression [53]. The origin of the fibroblasts is not well understood. Progressive renal fibrosis observed in diabetes may, in part, be mediated by the phenotypic changes induced by EMT, the trans-differentiation of tubular epithelial cells into myofibroblasts [54]. This complex process involves loss of cell integrity and decreased expression of proteins involved in the formation of intercellular junctions, in addition to alterations in cell morphology, re-organisation of the cell cytoskeleton and de novo expression of fibroblastic markers [54]. Although multiple signalling pathways have been implicated in EMT, TGF-β1 is thought to be key in the development of renal hypertrophy and accumulation of extracellular matrix, increasing the synthesis of fibronectin, laminin and collagen in glomerular, mesangial, and tubular cells [55]. In models of renal disease and diabetes, TGF-β1 gene expression and secretion are increased. Whilst underlying events mediating the development of fibrotic lesions are poorly understood, TGF-β1 contributes to excessive deposition of fibrotic material through instigation of EMT.

Predominantly mediated via Smad-dependent pathways, TGF-β1 binds to a distinct receptor, the TGF-β1 receptor II (TβRII). This association activates the TβRI kinase prior to phosphorylation of the Smad proteins. Smads are subdivided into three classes: receptor-regulated (R) Smads (Smad 1, 2, 3, 5 and 8), the common (Co) Smads (Smad 4) and the inhibitory (I) Smads (Smad 6 and 7). Following TβRII activation, R-Smads form oligomeric complexes with the common Smad (Co-Smad), prior to translocation and regulation of gene transcription in the nucleus [54]. Smad signalling is stringently controlled in order to protect the cells from any unwanted TGF-β1 responses. Thus, manipulating downstream TGF-β signalling represents a viable therapeutic target in alleviating fibrosis and restoring renal function. A potential anti-fibrotic role for C-peptide has recently been identified. Co-application of C-peptide with TGF-β1 to hPTC prevented TGF-β1-induced upregulation in both TGFβRII and TGFβRI expression, with a concomitant reduction in both Smad 2/3 transcriptional activity and the abolition of Smad 3 phosphorylation. Furthermore, C-peptide negated classical phenotypical and morphological changes associated with TGF-β1-induced EMT, including the inhibition of TGF-β1-induced vimentin expression and the prevention of cytoskeletal reorganisation [9].

TGF-β-induced EMT is a key contributor to fibrotic scar formation as seen in DN. Manipulating downstream TGF-β signalling represents a viable therapeutic target to alleviate fibrosis and restore renal function. More long-term studies of C-peptide administration in animal models of diabetes are now needed to support the use of C-peptide as a potential anti-fibrotic therapy in alleviating complications of DN.

Concluding Comments

The biosynthesis of de novo insulin necessitates the alignment of the A and B chains in order to allow correct folding and appropriate formation of disulphide bonds within the insulin molecule. In man, this role is fulfilled by a 31 amino acid connecting (C)-peptide, which is subsequently cleaved from active insulin during maturation of the secretory granule. Historically, C-peptide has been regarded as biologically inert, however this review suggests that C-peptide is more than merely a link between the A and B chain. The conserved C-terminal fragment of the peptide exerts numerous beneficial effects in the kidney, and it has the potential to reduce and reverse pathological changes often associated with DN. The role of C-peptide should now be re-examined as a potential future therapy in maintaining renal function in diabetes.

Acknowledgements

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