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FOR DEBATE

C-peptide is a bioactive peptide

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Abbreviations

BB/Wor	BioBreeding/Worcester
eNOS	endothelial nitric oxide synthase
ICAM-1	intracellular adhesion molecule 1
MAPK	mitogen-activated protein kinase
NCV	nerve conduction velocity

During the past decade, reports from several laboratories have focused on the physiological effects of C-peptide. Experimental data and clinical studies suggest that that C-peptide is a biologically active peptide. Clinical studies show that C-peptide administration in type 1 diabetes patients, who lack the peptide, results in amelioration of diabetes-induced renal and nerve dysfunction. Molecular studies demonstrate binding to cell membranes, activation of intracellular signalling pathways, and specific end effects of importance for vascular endothelial function. These findings have prompted the hypothesis that C-peptide deficiency in type 1 diabetes may contribute to the development of microvascular complications, and that C-peptide replacement, together with regular insulin therapy, may be beneficial in the treatment or prevention of these complications. In the present article we argue the case in favour of C-peptide as a biologically active peptide based on in vivo data and in vitro findings, as summarised in Table 1.

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Soon after the discovery of insulin biosynthesis in 1967, and the identification of C-peptide and its role in promoting the correct folding of proinsulin, researchers began to investigate whether C-peptide had any insulin-like effects. However, none were found, and interest in the peptide focused instead on its use as a marker of endogenous insulin secretion. Interest in a physiological role for C-peptide persisted, and received support from the clinical observation that patients with type 1 diabetes, who continue to maintain a small endogenous beta cell activity, are less prone to develop long-term complications and have fewer episodes of hypoglycaemia than those who become totally C-peptide deficient [1, 2]. It was also noted that islet or pancreas transplantation in type 1 patients, with restoration of both insulin and C-peptide secretion, often results in amelioration of the functional and structural abnormalities that accompany diabetic neuropathy and nephropathy [3, 4]. These considerations gave rise to a series of studies involving the administration of a replacement dose of C-peptide to type 1 diabetes patients.

Beneficial effects on renal function and structure in type 1 diabetes

Clinical studies Glomerular hyperfiltration, a risk factor for the development of nephropathy, is an early abnormality in type 1 diabetes that is not corrected by insulin therapy. This

Table 1 Summary of clinical, in vivo animal and in vitro cellular effects of C-peptide

Effect	Reference
In vivo effects	
Renal	
Functional reserve ↑	[7]
Glomerular hyperfiltration ↓	[5, 7]
Urinary albumin excretion ↓	[6]
Structural abnormalities ↓	[8]
Nerve	
Conduction velocity ↑	[9, 10, 12, 13, 37]
Vibration perception ↑	[10]
Blood flow ↑	[13, 20]
Na^+/K^+-ATPase activity ↑	[12, 37]
Hyperalgesia ↓	[14]
Structural abnormalities ↓	[12, 15]
Circulation	
Muscle blood flow ↑	[16]
Skin blood flow ↑	[19]
Myocardial blood flow and contraction rate ↑	[17, 18]
Myocardial ejection fraction ↑	[17, 18]
QT interval ↓	
In vitro effects	
Membrane interaction	
Specific binding in nanomolar range	[22, 23]
Intracellular signalling	
G-protein involvement	[25–29]
Intracellular Ca^{2+} ↑	[27, 30]
PKC, MAPK and PI-3Kγ ↑	[26, 28, 31]
NFκB, PPARγ, Bcl2, c-Fos, ZEB ↑	[29, 36, 46]
End effects	
eNOS activity and protein levels ↑	[30, 33, 34]
Na^+/K^+-ATPase activity and protein levels ↑	[25, 31, 36]
Cell growth ↑	[40]
Apoptosis ↓	[29, 40]
Insulinomimetic effects	[32]
Anti-thrombotic effects	[21]
Other	
Disaggregation of insulin hexamers	[41]

PI-3Kγ, phosphatidylinositol 3-kinase γ; *PKC*, protein kinase C; *ZEB*, zinc finger homeodomain enhancer-binding protein

prompted studies of the effects of C-peptide on kidney function. Short-term C-peptide infusion in young patients without signs of manifest nephropathy was shown to decrease GFR and to modestly increase renal plasma flow [5]. Subsequently, it was established that both GFR and urinary albumin excretion were diminished following C-peptide replacement therapy for 3 months in patients with early stage nephropathy. Specifically, in a double-blind, placebo-controlled, crossover study in patients with no other medication except insulin, there was a 40% reduction in albumin excretion during 3 months of C-peptide replacement [6]. The results were seen as a direct effect of C-peptide, since both systemic blood pressure and glycaemic control were similar during the C-peptide and placebo treatment periods.

Animal studies C-peptide administration in streptozotocin-induced diabetic rats for periods of up to 4 weeks resulted in a concentration-dependent correction of glomerular hyperfiltration, augmented renal functional reserve and diminished or abolished urinary albumin excretion [7, 8]. The specificity of the C-peptide effect was verified in control studies involving infusion of scrambled C-peptide—a peptide with the same residues but in random order—that showed no effect. Microscopic examination of the kidneys showed glomerular hypertrophy in the diabetic animals and a substantial reduction of the glomerular expansion after C-peptide replacement. Further examination of the glomeruli revealed that it was primarily the hypertrophy of the mesangial matrix that was inhibited by C-peptide [8]. The results thus support the notion that C-peptide administration exerts beneficial renal effects in type 1 diabetes.

C-peptide in replacement doses reduces glomerular hyperfiltration, diminishes urinary albumin excretion and retards or reverses renal structural abnormalities in type 1 diabetes.

Amelioration of nerve dysfunction and structural abnormalities

Clinical studies The effects of C-peptide on nerve dysfunction in type 1 diabetes were evaluated in a double-blind, placebo-controlled, 3-month study, including 46 patients with relatively short diabetes duration (10 years) and reduced sensory and motor nerve conduction velocity (NCV) but no overt signs of neuropathy [9]. Sensory (sural) NCV increased progressively in the group that received C-peptide treatment; the increase after 3 months was 2.7 m/s, corresponding to 80% correction of the initial NCV deficit. Vibration perception thresholds decreased during treatment, consistent with improved sural nerve function. These observations have been extended in a subsequent double-blind, placebo-controlled, clinical trial involving 161 type 1 patients with manifest diabetic peripheral neuropathy [10]. Sensory NCV improved in the C-peptide treated patients, and the number of positive responders was greater among the patients receiving C-peptide than those given placebo ($p<0.03$). The improvement compared with placebo was most marked (1 m/s) in the least severely diseased half of the patients ($p<0.02$), emphasising the benefit of early intervention. Neurological impairment score and vibration perception also improved in the C-peptide treated group. Changes in glycaemic control could not account for the improved nerve function.

There is also evidence suggesting that autonomic dysfunction in type 1 patients can be ameliorated by C-peptide administered at replacement doses. Short-term infusion of C-peptide is reported to significantly increase both heart rate variability during deep breathing and the heart rate brake index after tilting [11]. A similar but less marked improvement has been reported after 3 months of C-peptide administration [6].

Animal studies In BioBreeding/Worcester (BB/Wor) rats showing spontaneous development of type 1-like diabetes, administration of a replacement dose of C-peptide prevented diabetes-induced deterioration of NCV [12]. In addition, 3 months of C-peptide administration elicited a significant increase in NCV when treatment was commenced at 5 months after onset of diabetes, by which time nerve dysfunction had become established [12]. Similarly, in streptozotocin-induced diabetic rats receiving C-peptide from 6 to 8 weeks after induction of diabetes, a significant increase in both sensory (saphenous) and motor (sciatic) NCV was seen [13]; scrambled C-peptide had no effect. C-peptide replacement in diabetic rats reduced paranodal swelling by 60% at 2 months and resulted in near total prevention of axoglial dysjunction and paranodal demyelination at 8 months [12]. Marked improvements in structural abnormalities were also observed when C-peptide was given at 5–8 months after disease onset: axoglial dysjunction and paranodal demyelination improved significantly, axonal degeneration decreased and there was a fourfold increase in nerve fibre regeneration [12]. Replacement of C-peptide in BB/Wor rats is also reported to effectively prevent the development of thermal hyperalgesia and diminish the extent of unmyelinated fiber loss [14]. Furthermore, C-peptide administration in diabetic rats exposed to sciatic nerve crush injury has been found to improve axonal radial growth and elongation of regenerating fibres [15]. The combined evidence thus demonstrates beneficial effects of C-peptide on diabetes-induced functional and structural nerve abnormalities. A schematic representation of possible mechanisms of action is shown in Fig. 1.

C-peptide in replacement doses stimulates nerve Na^+/K^+-ATPase activity, increases endoneurial blood flow and stimulates neurotrophic factors, resulting in improved NCV and prevention or reversal of nerve structural changes.

Circulatory responses to C-peptide

Administration of C-peptide in type 1 diabetes patients results in increased blood flow in several tissues. Skeletal muscle perfusion (forearm) at rest and during exercise is increased in a concentration-dependent manner across the 0–1 nmol/l range, but higher concentrations of C-peptide elicit no further rise in blood flow [16]. In type 1 patients without signs of cardiac disease, C-peptide infusion results in augmented left ventricular blood flow. The patients also show improved rates of myocardial contraction, increased ejection fraction, plus a shortening of the QT interval [17, 18]. There is also augmented skin capillary blood cell velocity and redistribution of blood flow to nutritive capillaries following C-peptide administration [19]. Direct measurements of sciatic endoneurial blood flow in diabetic animals demonstrated augmented nerve perfusion in response to C-peptide administration [13, 20]. It is likely that the observed circulatory effects of C-peptide are mediated by stimulation and increased expression of endothelial nitric oxide synthase (eNOS) (see below), since the effect may be prevented by a NOS inhibitor [13]. An antithrombotic effect of C-peptide in diabetic mice has also been reported [21].

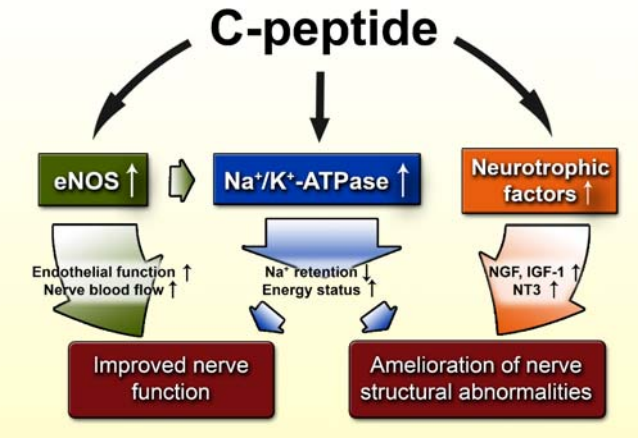

Fig. 1 Schematic representation of mechanisms whereby C-peptide may improve nerve function and ameliorate nerve structural abnormalities secondary to type 1 diabetes. For references, see Table 1. *NGF* nerve growth factor; *NT3* neurotrophin 3

Cell membrane interaction and intracellular signalling

The nature of C-peptide cellular interactions has not been fully determined. Fluorescence correlation spectroscopy has shown the stereospecific binding of C-peptide to renal tubular cells, endothelial cells, skin fibroblasts, mesangial cells and neuroblastoma cells; the association constant was 3×10^9 l/mol and the binding curve indicated saturation at 0.9 nmol/l [22]. C-peptide binding did not show cross-reactivity with insulin, IGF-1 or IGF-2. Binding of the peptide to detergent-solubilised cell fractions and cell lysates has also been demonstrated using fluorescence

correlation spectroscopy and surface plasmon resonance [23], but there are no data based on conventional radioligand binding. It is unlikely that C-peptide interacts directly with lipid cell membrane components [24]. The identity of the cellular binding structure has so far proven elusive and a specific receptor has not been identified.

Figure 2 provides an overview of intracellular responses to C-peptide. Most of the intracellular actions can be blocked by pre-incubation of cells with pertussis toxin, indicating the involvement of a G_i/G_o-linked protein in C-peptide signalling [25–28], as confirmed by direct studies assessing guanosine triphosphate γS binding to a G_i protein after C-peptide exposure [29]. Exposure of renal tubular and endothelial cells to C-peptide at physiological concentrations results in a prompt elevation of intracellular Ca^{2+} concentrations [27, 30]. C-peptide also elicits phosphorylation of several protein kinase C isoforms and phosphatidylinositol 3-kinase [26, 28, 31]. Activation of one or several components of the mitogen-activated protein (MAP) kinase cascade in a concentration-dependent manner is consistently observed in all examined cell types following exposure to C-peptide [26, 28, 32, 33]. C-peptide has also been found to mimic the effects of insulin in muscle cells [32].

Cellular end effects

eNOS C-peptide elicits release of NO in endothelial cells in a concentration- and time-dependent manner [30]. The effect, which occurs within a few minutes, is abolished in Ca^{2+}-free medium and in the presence of pertussis toxin or a NOS inhibitor. In addition, increased expression of eNOS mRNA [34] and protein [33] has been demonstrated after exposure of endothelial cells to C-peptide; eNOS expression is enhanced via MAP kinase-dependent transcriptional activation [33]. The in vitro observations are consistent with the finding that C-peptide administration to type 1 patients and animal models of type 1 diabetes results in concentration-dependent increases in blood flow in several tissues, and suggest that C-peptide replacement in type 1 diabetes improves the diabetes-induced endothelial dysfunction [35]. Specifically, the C-peptide effect on nerve dysfunction may be mediated in part via a NO-sensitive vascular mechanism that elicits vasodilation of the vasa nervorum [13].

Na^+/K^+-ATPase C-peptide exerts a stimulatory effect on Na^+/K^+-ATPase activity and protein expression in renal tubular cells [25, 31, 36]. The effect is concentration-dependent, may be blocked by pertussis toxin, and is dependent on Ca^{2+} [25]. Decreased activity of this enzyme in peripheral nerve is a characteristic abnormality in type 1 diabetes. C-peptide at physiological concentrations prevents or partially corrects the diabetes-induced reduction in nerve Na^+/K^+-ATPase activity, both in streptozotocin-induced diabetic animals [37] and in BB/Wor rats [12]. Moreover, the Na^+/K^+-ATPase activity of red blood cells is diminished in patients with type 1 diabetes; the

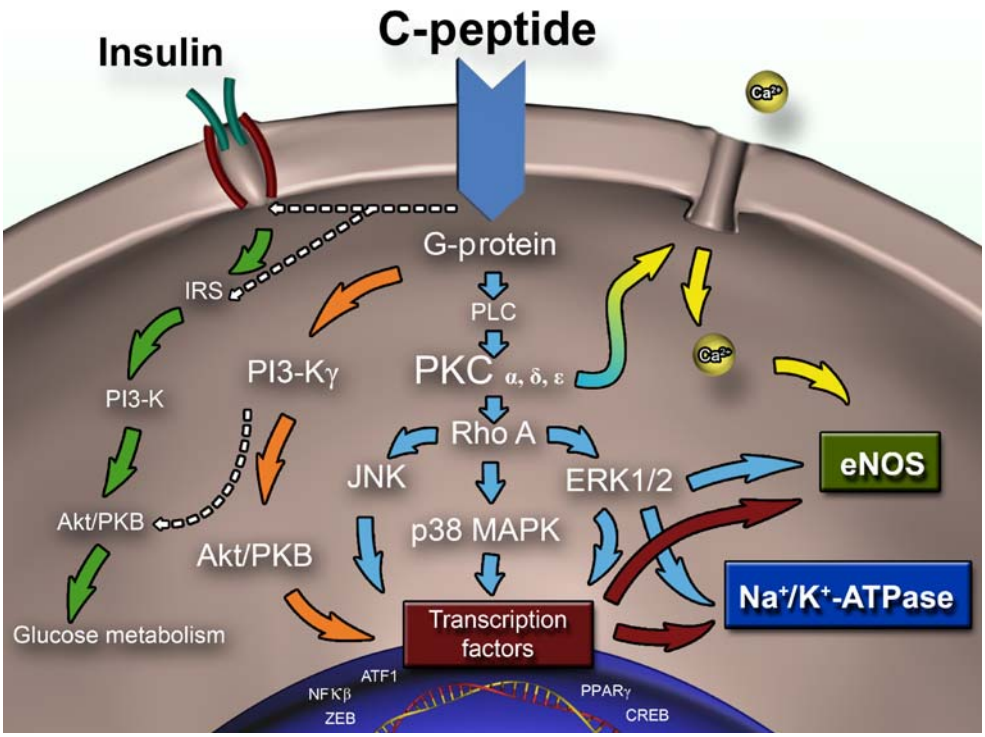

Fig. 2 Preliminary model of C-peptide signalling pathways. The influence of C-peptide on eNOS and Na^+/K^+-ATPase include both activation and induction. *Dashed lines* indicate insulinomimetic signalling demonstrated in muscle cells. *ATF1*, activating transcription factor 1; *CREB*, cyclic AMP response element-binding protein; *ERK1/2*, extracellular signal-regulated kinase 1/2; GTPγS, guanosine triphosphate γS; *JNK*, c-Jun N-terminal kinase; *PI3-K*, phosphatidylinositol 3-kinase; *PKB*, protein kinase B; *PKC*, protein kinase C; *PLC*, phospholipase C; *PPARγ*, peroxisome proliferator-activated receptor γ; *ZEB*, zinc finger homeodomain enhancer-binding protein

reduction is proportional to the decrease in C-peptide levels and can be corrected by C-peptide administration [38]. Secondary effects of the decreased red cell Na^+/K^+-ATPase activity are impaired deformability of the cells and altered rheological properties; these are corrected after exposure to C-peptide [39]. Taken together, the evidence provides support for a direct relationship between C-peptide levels and Na^+/K^+-ATPase activity in renal and nerve tissue and red blood cells under both in vitro and in vivo conditions.

Transcriptional effects C-peptide causes activation and DNA binding of several transcription factors. Increased eNOS mRNA and protein levels [33] in endothelial cells have been discussed above. C-peptide is also reported to elicit decreased surface expression of the cell adhesion molecules P-selectin and intercellular adhesion molecule 1 on vascular endothelium, thereby inhibiting leucocyte–endothelium interactions [34]. A stimulatory influence of C-peptide on cell proliferation has been reported for renal tubular [28] and neuroblastoma cells [40]. In the latter there was activation of phosphoinositol 3-kinase and p38 MAP kinase, resulting in enhanced expression and translocation of NFκB after C-peptide exposure. C-peptide has been found to protect against TNFα-mediated apoptosis of renal tubular cells [29], and the anti-apoptotic effect of insulin on neuroblastoma cells grown at a high glucose concentration is significantly enhanced by C-peptide [40].

C-peptide binds specifically to cell membranes, resulting in G-protein-mediated intracellular signalling and activation of Na^+/K^+-ATPase, eNOS and several transcription factors.

C-peptide–insulin interactions

Interactions between C-peptide and insulin oligomers have been identified using surface plasmon resonance. Unexpectedly, it was discovered that C-peptide influences the disaggregation of insulin, probably by binding to insulin oligomers, with dissociation constants in the micromolar range [41]. Mass spectrometry revealed that insulin hexamers in solution became undetectable in the presence of C-peptide, leading to the conclusion that C-peptide binds to and causes disaggregation of hexameric insulin, increasing the availability of biologically active, monomeric insulin. Accordingly, subcutaneous injection of an insulin and C-peptide mixture in type 1 diabetes patients has been found to result in a more rapid appearance of insulin in plasma and more marked stimulation of glucose utilisation compared with injection of insulin alone [41].

Not your regular peptide

The physiological effects of C-peptide can be demonstrated only in patients or animals deficient in C-peptide and not in healthy individuals. The nature of the C-peptide binding curve may help explain this unusual feature. The curve indicates saturation of cellular binding at approximately 0.9 nmol/l [22], which is within the physiological range. Thus, in healthy subjects and animals with normal beta cell function, receptor saturation is probably achieved at the ambient C-peptide concentration, so that no further effects can be expected in response to exogenous administration.

The structural variability of C-peptide in different species has been put forward as an argument that C-peptide is unlikely to possess biological activity. C-peptide is, however, not unique in this regard. Parathyroid hormone, gastrin-releasing peptide and relaxin all show similar interspecies variability. In addition, among mammalian species, nine residues (Glu1, Glu3, Gln6, Val7, Glu11, Leu12, Leu26, Glu27 and Gln31), localised primarily to the N- and C-terminal segments, show ≥90% conservation [42]. The exact functional correlates for these residues are not apparent, but Glu3, Glu11 and particularly Glu27 are all known to be important for the cellular effects of C-peptide [42].

Are long-term complications different in type 1 and type 2 diabetes?

The DCCT study demonstrated a decreased occurrence of complications in the group of type 1 diabetes patients that received intensive insulin therapy and achieved improved blood glucose control, in keeping with the view that hyperglycaemia is a major culprit in the pathogenesis of microvascular complications. Even though blood glucose levels were close to normal in the group that received intensive treatment, a large proportion of these patients still developed complications: 40% presented with clinical neuropathy or grossly abnormal nerve conduction after 5 years [43], and a similar fraction had developed signs of nephropathy or retinopathy. This suggests that factors in addition to hyperglycaemia may be involved in the development of microvascular complications in type 1 diabetes patients. In view of the data for C-peptide described above, we suggest that lack of C-peptide contributes to the pathogenesis of complications of type 1 diabetes. If this were the case, one would expect that the complications associated with type 1 diabetes would be different from those associated with type 2. Direct comparisons have been made in patients and in animal models of diabetes, particularly with respect to neuropathy. Thus, neuropathy caused by type 1 diabetes occurs more

predictably and progresses more rapidly than in type 2 diabetes. Both patients and experimental animals with type 1 diabetes neuropathy show characteristic structural abnormalities of the nodal and paranodal myelin sheath and ion channel barrier, which affect the large myelinated fibres in particular; these derangements are not seen in type 2 diabetes [44, 45]. In addition, type 1 diabetic animals show progressive axonal degeneration coupled with impaired regenerative capacity, resulting in the gradual loss of nerve fibres, and this is either not present or significantly milder in animal models of type 2 diabetes [45]. Finally, in type 1 diabetes, but not in type 2, key nodal and paranodal molecules, e.g. contactin, caspr, and ankyrin$_G$ are down-regulated [46]. In view of the above data, we propose that, besides hyperglycaemia, C-peptide deficiency is an important factor in the pathogenesis of neuropathy and possibly other microvascular complications of type 1 but not type 2 diabetes. Accordingly, type 1 diabetes should be considered a dual hormone deficiency disease and C-peptide replacement therapy may be beneficial in its treatment.

Time for a new scientific perspective on C-peptide

Why is it that despite the extensive documentation of multifaceted effects (Table 1), C-peptide is not yet generally recognised as a bioactive peptide? The fact that the peptide has no effects when administered to healthy animals or individuals may be partly responsible for this. An explanation for this unusual feature, based on the binding characteristics of the peptide, is presented above. Another complicating factor in this regard may be the lack of immediate or major effects of C-peptide withdrawal, as, for example, at the onset of type 1 diabetes. This may indicate that C-peptide is one of several players in the multifactorial system that regulates tissue microcirculation and endothelial function. The C-peptide contribution may be seen as redundant, at least in the short term. The presence of multiple regulatory mechanisms often signifies the vital importance of the specific physiological process being regulated, and the presence of compensatory regulation may help explain why a lack of C-peptide gives rise only to late manifestations that primarily occur in specific tissues. A third objection has been that the biological and clinical importance of C-peptide has not been established. This objection is refuted on the basis of the wealth of information now presented. Hence, we consider C-peptide to be bioactive.

Clearly, there is much more to learn about C-peptide. Identification of the mechanism whereby C-peptide interacts with cell membranes and further delineation of its intracellular signalling pathways and transcriptional effects in different cell types would enhance our understanding of C-peptide bioactivity. On the clinical side, further studies of long duration will be required to document the robustness of its beneficial effects on the different types of long-term complications to define its possible therapeutic role in the therapy of type 1 diabetes, as also emphasised in the accompanying paper by Luzi et al. [47]. Nevertheless, despite the fact that our knowledge is still incomplete, there are several lines of evidence in support of the notion that C-peptide is a bioactive peptide and that its replacement in type 1 diabetes may be beneficial in the treatment of long-term complications. Specific cellular binding of the peptide, its intracellular signalling characteristics and end effects, including its action on eNOS, Na^+/K^+-ATPase and several transcription factors, are now established for many cell systems and by different investigators. Results from studies in type 1 diabetes patients and animal models demonstrate that, at replacement doses, C-peptide exerts beneficial effects on the early stage functional and structural abnormalities of both the kidneys and the peripheral nerves. Even a cautious evaluation of the available evidence thus presents the picture of a bioactive peptide with therapeutic potential.

Duality of interest The authors (J. Wahren, K. Ekberg, H. Jörnvall) hold shares in Creative Peptides (Stockholm, Sweden), and J. Wahren and K. Ekberg are employed by the company.

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