

Renal and vascular benefits of C-peptide: Molecular mechanisms of C-peptide action

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Abstract: C-peptide has long been thought to be an inert byproduct of insulin production, but it has become apparent, and accepted, that C-peptide has important biological properties. C-peptide displays beneficial effects in many tissues affected by diabetic complications, such as increased peripheral blood flow and protection from renal damage. However, the mechanisms mediating these effects remain unclear. C-peptide interacts with cellular membranes at unidentified sites distinctive of the insulin family of receptors, and signals to multiple targets known to play a role in diabetes and diabetic complications, such as Na⁺/K⁺-ATPase and NOS. In general, the physiological and molecular effects of C-peptide resemble insulin, but C-peptide also possesses traits separate from those of insulin. These basic studies have been confirmed in human studies, suggesting that C-peptide may lend itself to clinical applications. However, the molecular and physiological properties of C-peptide are not completely elucidated, and large clinical studies have not begun. In order to further these goals, we critically summarize the current state of knowledge regarding C-peptide's renal and vascular effects and the molecular signaling of C-peptide.

Keywords: C-peptide, insulin, diabetes mellitus, nephropathy, vascular, signaling

Introduction

Diabetes mellitus (shortened as diabetes) is a class of diseases where an individual cannot properly maintain plasma glucose levels. There are two main types of diabetes, type 1 and type 2. Type 1 diabetes is characterized by a lack of insulin due to loss of beta cells in the pancreatic islets of Langerhans, whereas type 2 diabetes is typified by a loss of response to insulin. Type 2 diabetes progresses through two stages. First there is a loss of target tissue insulin action (termed insulin resistance) that then results in increased insulin production (hyperinsulinemia). Eventually the second stage is reached when the islets of Langerhans cannot maintain such a high level of insulin production, and "pancreatic exhaustion" sets in causing insulin production to cease. Although the diseases have unique etiologies, several long-term complications are associated with both types of diabetes. Intensive treatment of hyperglycemia significantly prevents diabetic complications (DCCT 1993), but glycemic control does not offer sufficient protection from the development of complications.

Besides hyperglycemia, other causal factors appear to contribute to the development of diabetic complications. One such factor is the proinsulin connecting peptide, C-peptide. C-peptide is a cleavage product of insulin synthesis created in the pancreas as part of insulin production, and is released into the circulation with insulin. The average physiological blood concentrations of C-peptide are in the low nM levels in healthy individuals (Samnegard and Brundin 2001). When, as in type 1 diabetes and late type 2 diabetes, insulin synthesis is impaired, synthesis of C-peptide is similarly impaired. Exogenous C-peptide has been shown to improve the function of many tissues commonly affected by diabetes complications (Lindstrom et al 1996;

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Johansson et al 2000; Chakrabarti et al 2004; Sima and Li 2006), a development supported by the fact that pancreatic and islet of Langerhans transplantation, restoring not only insulin secretion, but also secretion of C-peptide, will prevent and even reverse diabetic complications (Fiorina et al 2003a, 2003b, 2005a, 2005b; Lee et al 2006; Venturini et al 2006). Hence, C-peptide deficiency has successively emerged as a possible mechanism for the development of the disproportionate burden of complications affecting insulinopenic diabetes patients. In this paper, some of the beneficial renal and vascular effects of C-peptide are reviewed, as well as the current knowledge regarding C-peptide-mediated molecular mechanisms.

Beneficial effects of C-peptide

Circulatory benefits

It has long been known that diabetes mellitus patients suffer an increased risk of developing, as well as accelerating atherosclerosis – the principal cause of heart attack, stroke and gangrene of the extremities (Brownlee 2001). Microangiopathy, atherosclerosis, and abnormalities in small vessel function significantly contribute to the development of diabetes-induced morbidity (Jensen et al 1989). During the development of atherosclerosis, pathological proliferation and migration of vascular smooth muscle cells are necessary to form atherosclerotic plaques. C-peptide has been shown to prevent vascular dysfunction in diabetic rats (Ido et al 1997), and to reduce proliferation of smooth muscle cells exposed to high glucose (Kobayashi et al 2005; Cifarelli et al 2008), a reduction that may suppress diabetes-induced atherosclerosis. It should be noted that under normoglycemic conditions C-peptide stimulates cultured smooth muscle cell proliferation (Walcher et al 2006).

Effects on nitric oxide and vasodilation

More than thirty years ago, impaired circulation, relative tissue hypoxia, and an impaired maximal oxygen uptake were reported in insulinopenic diabetes (Ditzel and Standl 1975). Shortly after induction of diabetes in lambs, coronary vascular resistance is elevated (Lee and Downing 1979), and diabetic animals are prone to develop increased total peripheral resistance and hypertension (Bell et al 2006). Administration of C-peptide restores diabetes-impaired skeletal muscle perfusion (Johansson et al 1992a), improves capillary skin red blood cell velocity (Forst et al 1998), and myocardial blood perfusion (Hansen et al 2002) as well as pancreatic islet arteriole diameter (Nordquist et al 2008b). In type 2 diabetes, C-peptide concentrations correlate with the

presence of coronary artery disease and peripheral vascular disease (Sari and Balci 2005). The C-peptide-induced beneficial effects are, however, not restricted to the diabetic state. Surprisingly, in myocardial ischemia-reperfusion, C-peptide has been shown to exert cardioprotective effects through nitric oxide (NO) release (Young et al 2000).

Effects on erythrocyte deformability

When erythrocytes are deformed by the narrow space in the capillaries, they release adenosine triphosphate (ATP) (Sprague et al 1996). The ATP released from the erythrocyte stimulates NO synthesis and release from vascular endothelial cells, an event that will regulate vascular resistance and improve oxygenation (Sprague et al 2003). In diabetes, erythrocyte deformability is impaired (Brown et al 2005) and erythrocyte aggregation increases. The impaired deformability results in increased blood viscosity and decreases capillary blood flow and oxygen availability in the tissue. C-peptide levels have been suggested to positively correlate with erythrocyte deformability (De La Tour et al 1998). Decreased membrane sodium, potassium adenosine triphosphatase (Na^+/K^+ -ATPase) activity in erythrocytes is associated with diabetic complications (Raccach et al 1996; De La Tour et al 1998), and there are indications that C-peptide concentrations correlate with erythrocyte Na^+/K^+ -ATPase activity in type 1 as well as type 2 diabetic subjects (De La Tour et al 1998). C-peptide is known to ameliorate the impaired deformability of erythrocytes in blood drawn from insulinopenic diabetic patients, and ouabain-inhibition of this effect suggests that the C-peptide-induced effects on erythrocyte deformability are mediated through restoration of the diabetes-impaired Na^+/K^+ -ATPase activity (Kunt et al 1999). Recently, it was shown by Meyer and colleagues (2008) that in the presence of metal ions, C-peptide promotes the release of ATP from erythrocytes, and that this release is mediated via activation of the GLUT1 transporter.

Renoprotection

Early in the development of diabetic nephropathy, the glomerular filtration rate (GFR) increases. Diabetes-induced glomerular hyperfiltration has been proposed as an independent risk factor for the development of renal complications, and it is well known that C-peptide prevents hyperfiltration as well as renal damage in experimental diabetes. C-peptide reduces glomerular hyperfiltration in diabetic patients, and long-term substitution therapy improves renal function (Johansson et al 1992b, 2000). In animal models of diabetes mellitus, exogenous C-peptide has been shown to reduce

diabetes-induced glomerular hyperfiltration and decrease albuminuria (Samnegard et al 2001, 2005; Huang et al 2002; Rebsomen and Tsimaratos 2005; Maezawa et al 2006). Structurally, renal glomerular hypertrophy and mesangial matrix expansion is prevented by C-peptide treatment, and it has been shown by Maezawa and colleagues (2006) that podocyte collagen gene expression is normalized by C-peptide treatment in a mouse model of type 1 diabetes (Samnegard et al 2001, 2005).

The rat C-peptide carboxy-terminal penta-fragment EVARQ has been shown to abolish diabetes-induced glomerular hyperfiltration. C-peptide and the penta-fragment both reduce diabetic hyperfiltration (Nordquist et al 2007). Hence, it is possible that the five amino acid sequence of the carboxy-terminal of C-peptide is the sequence, or one of the sequences, mediating the normalizing effect on GFR in these diabetic rats. It is common that proteins have defined functional sites. A fragment of gonadotropin-releasing peptide displays increased activity compared to the intact peptide (Sandow and Konig 1979; Ramasharma et al 1988). Similar to C-peptide gastrin, cholecystokinin and osteogenic growth peptide, have active sites in their C-terminal penta-peptides (Portelli and Renzi 1973; Yajima et al 1977; Bab et al 1999; He et al 2004).

Afferent arteriolar constriction

The glomerular microcirculation is regulated to a great extent through alterations in the vascular resistance of the afferent and efferent arterioles. In insulinopenic diabetes, afferent diameter is increased (Ikenaga et al 2000). C-peptide constricts isolated renal afferent arterioles from diabetic mice, but not from normoglycemic animals (Nordquist et al 2008a). Constriction of the afferent arteriole lowers glomerular filtration pressure and thus decreases GFR. However, C-peptide does not appear to diminish renal blood flow (Huang et al 2002, Samnegard et al 2004). Taken together, these results suggest that C-peptide reduces diabetes-induced glomerular hyperfiltration at least partly via constriction of the afferent glomerular arteriole, but with a simultaneous dilation of the efferent arteriole that counterbalances the effect on renal blood flow. It should be noted that efferent dilation and increased afferent vascular tone have also been reported for insulin (Juncos and Itos 1993).

Tubuloglomerular feedback

It has previously been postulated that diabetic hyperfiltration occurs due to alterations in tubuloglomerular feedback (TGF) (Vallon et al 1995; Thomson et al 2001). TGF is an

intrarenal mechanism that stabilizes GFR, and thus the tubular Na^+ -load to match the tubular Na^+ handling capacity. The anatomical prerequisite for TGF is the return of the tubule to its own glomerulus. These, together with the macula densa (MD) make up the juxtaglomerular apparatus. The MD consists of specialized epithelial cells localized where the returning tubule passes the glomerulus, and constitutes a sensor mechanism for Na^+ by sensing Cl^- , which relates to Na^+ levels. Increased tubular flow rate will increase the tubular NaCl load, which is sensed by the MD, and results in a constriction of the afferent arteriole. However, the afferent constriction caused by C-peptide was demonstrated in isolated arterioles, where the arteriole is not set up with an intact tubulus and thus is independent of TGF. In addition, it was recently shown by Sallstrom et al that TGF does not mediate diabetes-induced hyperfiltration, since diabetes-induced glomerular hyperfiltration occurs in adenosine A1-receptor-deficient mice known to lack a functional TGF mechanism (Sallstrom et al 2007). If TGF is not the mediator of diabetic hyperfiltration, it is unlikely that C-peptide would exert its effect on filtration via a TGF-dependent mechanism. Therefore, a possible conclusion is that C-peptide exerts its effect directly on the glomerular afferent arterioles.

Many aspects remain to be investigated

Blood flow is differentially affected in the kidney vasculature versus peripheral vasculature in the diabetic state. Multiple studies show normalizing effects of C-peptide on diabetes-impaired blood perfusion and blood cell velocity (Forst et al 1998; Johansson et al 2003). However, in these studies, C-peptide reverses diabetes-induced decreases in peripheral blood perfusion, whereas in renal vessels C-peptide reverses diabetes-induced increases in blood flow (Bank and Aynedjian 1993; Wang et al 1993; Komers et al 1994). It remains to be determined whether these beneficial, but seemingly opposing effects of C-peptide reflect tissue-specific mechanisms.

Another aspect of C-peptide as a vasoactive substance is that the vasoconstrictive effect of C-peptide takes considerably longer time to develop compared to well known constrictors such as angiotensin II or norepinephrine (Nordquist et al 2008a). An explanation of this could be a sequential cascade of intracellular events leading to vasoconstriction. Although C-peptide clearly possesses a reducing effect on diabetic hyperfiltration, there is still no effector, no receptor, and no downstream signalling cascade reported for this phenomenon. So what are possible causes of the effect of

C-peptide on hyperfiltration? In the next section we review likely candidates mediating C-peptide's physiological properties.

C-peptide cellular signaling

A putative G protein-coupled receptor

The first report of a possible receptor was published in 1986 when rat C-peptide I was found to bind specifically to cultured rat islet tumor B-cells (Flatt et al 1986). Early studies suggested that C-peptide mimics insulin action, but that its action on the renal Na^+/K^+ -ATPase is completely abolished by pretreatment with pertussis toxin (PTX) (Ohtomo et al 1996). PTX is an inhibitor of $\text{G}\alpha_{i/o}$ family of heterotrimeric G proteins (Katada and Ui 1982; West et al 1985), and has been shown to eliminate nearly all C-peptide signaling activities (Rigler et al 1999; Kitamura et al 2001; Zhong et al 2005; Al-Rasheed et al 2006; Lindahl et al 2007), something that strongly suggests that the putative C-peptide receptor is a seven transmembrane G protein-coupled receptor (GPCR).

Identification of GPCRs is not an easy task, and to date the cell surface receptor(s) for C-peptide remain unknown. However, multiple studies have been conducted via manipulating the ligand–C-peptide (Ido et al 1997; Ohtomo et al 1998; Rigler et al 1999). Initially, rat and human C-peptides, but not pig C-peptide, were shown to act like insulin and return elevated glucose-mediated blood flow to normal in two models (Ido et al 1997). The structure of C-peptide was examined by circular dichroism revealing that human and rat C-peptide begin and end with alpha helical domain linked by a flexible glycine rich domain containing a distinctive proline kink in the middle of the sequence; sadly pig C-peptide was not examined (Ido et al 1997). Additionally, Ido and colleagues indicated that the reverse sequence, all D amino acids, and the middle glycine rich section had similar effects as full length rat and human C-peptide. It should be noted that if there is a receptor involved, then the D amino acids should be inert. The unexpected data lead the authors to conclude that, contrary to previous studies (Ohtomo et al 1996), the effects are not receptor dependent and that C-peptide enters cells similar to some bacterial proteins (Ido et al 1997). Shortly thereafter, activity of the glycine rich region was confirmed when examining renal Na^+/K^+ -ATPase activity (Ohtomo 1998). The glycine rich region was 20% less effective than full length C-peptide, but interestingly, the last 4 amino acids were only 8% less active than full length C-peptide, and the terminal penta-peptide had slightly greater activity than the full length peptide. Moreover, randomly scrambled C-peptide and the D-amino

acid penta-peptide had no effect (Ohtomo et al 1998). These data indicate that indeed sequence specificity and chirality, an essential component of ligand-receptor interactions, are essential for C-peptide activity.

Binding studies demonstrated that C-peptide specifically binds to human renal tubular cells, fibroblasts from the upper arm, and saphenous vein endothelial cells, but not umbilical cord endothelial cells (Rigler et al 1999). Rigler and colleagues found that the half maximal binding (B_{50}) was 0.3 nM and $B_{\text{max}} = 0.9$ nM. Importantly, C-peptide binding was not displaced by all D-amino acid C-peptide, insulin, proinsulin, or insulin like growth factor (IGF) I and II; however, the terminal penta-peptide was as efficient in displacement as full length C-peptide (Rigler et al 1999). These data support the theory that the last 5 amino acids mediate the binding of C-peptide to its receptor and that it has a unique receptor independent of the insulin family of receptors. Lastly, Rigler and colleagues (1999) confirmed earlier studies that the majority of the receptor is a $\text{G}\alpha_{i/o}$ coupled receptor via use of PTX. However, there appears to be a small pool of receptors that are PTX insensitive, suggesting that C-peptide either has two receptors or a single GPCR that couples to $\text{G}\alpha_{i/o}$ and another $\text{G}\alpha$ subunit (Rigler et al 1999). More recently, multiple studies have confirmed that the secondary structure of C-peptide is essential for proper signaling (Kitamura et al 2002; Li et al 2003; Walcher et al 2006), and that the penta-peptide mimics C-peptides effects (Zhong et al 2005). Therefore, the earlier studies by Ido and colleagues (1997) have not been repeated, and it appears that C-peptide does bind to yet unidentified receptors.

Recently, the coupling of the C-peptide receptor to $\text{G}\alpha_i$ has been proven examining C-peptide-mediated activation of $\text{G}\alpha_i$ (Al-Rasheed et al 2006). C-peptide, but not scrambled C-peptide, caused the nonhydrolyzable GTP analogue ^{35}S -GTP γ S to bind to $\text{G}\alpha_i$, indicating that C-peptide activates $\text{G}\alpha_i$. Unfortunately no other G-protein was examined in this assay, so it is unknown if the PTX insensitive receptor pool is acting through a different G-protein or a different class of receptors. However, these data prove that stimulation of cells with C-peptide activates $\text{G}\alpha_i$.

In contrast to the studies suggesting that C-peptide acts through a GPCR, studies using isolated L6 rat myoblast membranes demonstrated that insulin and C-peptide, but not scrambled C-peptide, increased membrane associated tyrosine kinase activity (Grunberger et al 2001). Moreover, C-peptide enhances insulin receptor tyrosine kinase activity, although with very low level of activation. These data allowed for theories to be generated about C-peptide binding

to, or merely activating, the insulin receptor. In response to the re-emergent theory that C-peptide binds to the insulin receptor, surface plasma resonance (SPR), a technique that detects protein-protein interactions, was used to show little to no binding of C-peptide to insulin or IGF-I receptors (Henriksson et al 2006). Moreover, Henriksson and colleagues demonstrated that insulin, but not C-peptide, activates the glucokinase and insulin promoter. Therefore, although the receptors signal similarly in physiological conditions, the molecular mechanisms are not identical and C-peptide binds to a unique receptor.

In support of the GPCR theory, rhodamine-labeled C-peptide binds to Swiss-3T3 and HEK-293 cells and internalizes in a temperature and PTX dependent mechanism (Lindahl et al 2007). Additionally, pre-incubation of C-peptide with a C-peptide antibody or unlabeled C-peptide inhibits rhodamine-labeled C-peptide binding. In order to identify a C-peptide receptor, Lindahl and colleagues (2007) utilized Biacore sensor chips, SPR, and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) to identify C-peptide binding partners from HEK-293 cell extracts. Intracellular proteins were found to bind to C-peptide, but although the rhodamine-labeled C-peptide was found to internalize, receptor mediated endocytosis does not expose the ligand to the cytosol. Thus, these data need to be examined further to exclude false positive associations, since the ligand may never be exposed to those proteins unless C-peptide is entering the cells in a nonclassical manner as proposed by Ido and colleagues (1997).

In conclusion, it is clear that C-peptide binds to receptor(s) distinctive of the insulin family of receptors. Additionally, one of the receptors stimulates $G\alpha_i$. Collectively, the data strongly suggest that the receptor is a GPCR, in fact this amount of evidence was sufficient for classifying receptors before the advent of cloning. However, since the receptor is still unknown this is not a definitive conclusion. To further complicate the potential identity of the C-peptide receptor, activators of G-protein signaling (AGS) family of proteins are known to activate $G\alpha_i$ independently of GPCRs (Blumer et al 2005). Therefore the precise nature of the C-peptide receptor cannot be determined from the literature and requires further investigation.

Receptor-mediated signal transduction

As mentioned previously, the physiological properties mirror, for the most part, insulin-mediated effects. In fact studies conducted in L6 rat myoblasts indicate that C-peptide signals nearly identically to insulin, except in regard to

phosphorylation of Akt (Grunberger et al 2001). These data along with the physiological effects of C-peptide were strong contributing factors to the proposal that C-peptide signals through the insulin receptor. In this section we will review the general signaling of C-peptide. However, it must be noted that it has already become evident that there are cell-specific signaling mechanisms. For example, in rat medullary thick ascending limb (mTAL) cells C-peptide causes translocation of protein kinase C (PKC) α , but not PKC δ , ϵ , or ζ , to the plasma membrane (Tsimaratos et al 2003), and in human renal tubular cells C-peptide causes translocation of PKC δ and ϵ , but not PKC α , γ , ζ , and θ (Zhong et al 2005). Similar discrepancies have been shown for Akt (Grunberger et al 2001; Zhong et al 2005) and the NOS isoforms (Kitamura et al 2003; Wallerath et al 2003; Li et al 2004). Therefore, we will include the cell types when describing the signaling pathways, and synthesize a generic signaling paradigm for C-peptide.

Na⁺/K⁺-ATPase

Initial studies demonstrated that C-peptide activates the renal Na⁺/K⁺-ATPase through a PTX and FK-506-dependent mechanism (Ohtomo et al 1996). FK-506 is a specific inhibitor of calcineurin (also known as protein phosphatase 3 and formerly called protein phosphatase 2B), which has been shown to be involved in activation of Na⁺/K⁺-ATPase (Lea et al 1994). Importantly, calcineurin is activated by binding to calmodulin after increased intracellular Ca²⁺ levels (Klee et al 1979) indicating that C-peptide must increase intracellular Ca²⁺ levels. This was confirmed in bovine aortic endothelial cells (Wallerath et al 2003). Additionally, extensive studies in human renal tubular cells indicate that the L-type Ca²⁺ channel blockers nifedipine and verapamil, as well as the phospholipase C (PLC) inhibitor U73122 inhibit C-peptide-mediated signaling, thus indicating that the Ca²⁺ entry is through L-type Ca²⁺ channels (Zhong et al 2005). The activation of PLC and Ca²⁺ influx provides a mechanism for C-peptide-mediated activation of classical PKC family members, such as PKC α . In rat mTAL cells C-peptide-mediated Na⁺/K⁺-ATPase activity and phosphorylation of the α -subunit of Na⁺/K⁺-ATPase is blocked by the general PKC inhibitor GF109203X (Tsimaratos et al 2003). Further studies in human renal tubular cells confirm the role of PTX and GF109203X, but also show that phosphorylation of the α -subunit of Na⁺/K⁺-ATPase is blocked by PD98059, a MEK inhibitor (Zhong et al 2004). Furthermore, the C-peptide-mediated phosphorylation of the α -subunit of Na⁺/K⁺-ATPase has been shown to be a threonine residue residing within an ERK consensus

site. This suggests that ERK, not PKC, is phosphorylating the Na⁺/K⁺-ATPase (Zhong et al 2004) and that PKC is involved in activating ERK, as described in the next section.

These data collectively suggest that C-peptide activates PLCβ2 through Gβγ subunits associated with Gα_i (Katz et al 1992; Wu et al 1993), and that C-peptide activates L-type Ca²⁺ channels through Gβγ subunits (Viard et al 1999). The activation of the L-type Ca²⁺ channel by Gβγ alone is rather minimal and requires phosphatidylinositol 3-kinase (PI3K), which is activated by C-peptide (Grunberger et al 2001; Kitamura et al 2001; Li et al 2003; Al-Rasheed et al 2006; Walcher et al 2006), to allow for a full Ca²⁺ transient (Viard et al 1999). Additionally there is a role for PKC in the Gβγ-mediated activation of L-type Ca²⁺ channels (Viard et al 1999). Since PKCδ and ε do not require Ca²⁺ for activation they may play a role in enhancing the Ca²⁺ transient since they translocate to the membrane after stimulation with C-peptide (Zhong et al 2005). However, the level of C-peptide-mediated activation of the L-type Ca²⁺ channel was not compared to a positive control, and no experiments examining the mechanism of C-peptide-mediated Ca²⁺ entry into cells has been conducted. Thus it is unknown if PKC and PI3K are needed for C-peptide-mediated activation of L-type Ca²⁺ channels and subsequent PKCα activation. As described in the next section, PI3K is most likely needed

for activation of ERK, which in humans is a necessary component of C-peptide-mediated phosphorylation of the α-subunit of Na⁺/K⁺-ATPase (Zhong et al 2004). Further experiments are required to definitively describe the signaling cascade from the C-peptide receptor(s) to Na⁺/K⁺ ATPase; the current model is shown in Figure 1.

MAPKs

Activation of mitogen-activated protein kinases (MAPKs) is common to many signaling molecules; there are three major MAPK proteins: ERK, p38, and JNK. However, the mechanism of receptor-mediated, especially GPCR-mediated, activation of MAPKs is not uniform and is still contested. C-peptide activates ERK, p38, and JNK, but as detailed below only ERK is consistently shown to be activated by C-peptide.

C-peptide signaling to ERK was first reported in 2001 (Grunberger et al 2001; Kitamura et al 2001). In Swiss 3T3 fibroblasts Kitamura and colleagues (2001) demonstrated that C-peptide-mediated phosphorylation of ERK has an EC₅₀ = 0.25 ± 0.05 nM, which is in accordance with the B₅₀ = 0.3 nM (Rigler et al 1999), and ERK is phosphorylated within 10 min. Additionally, signaling to ERK is completely inhibited by PTX, nearly abolished by GF109203X, and inhibited by 50% by the PI3K inhibitor

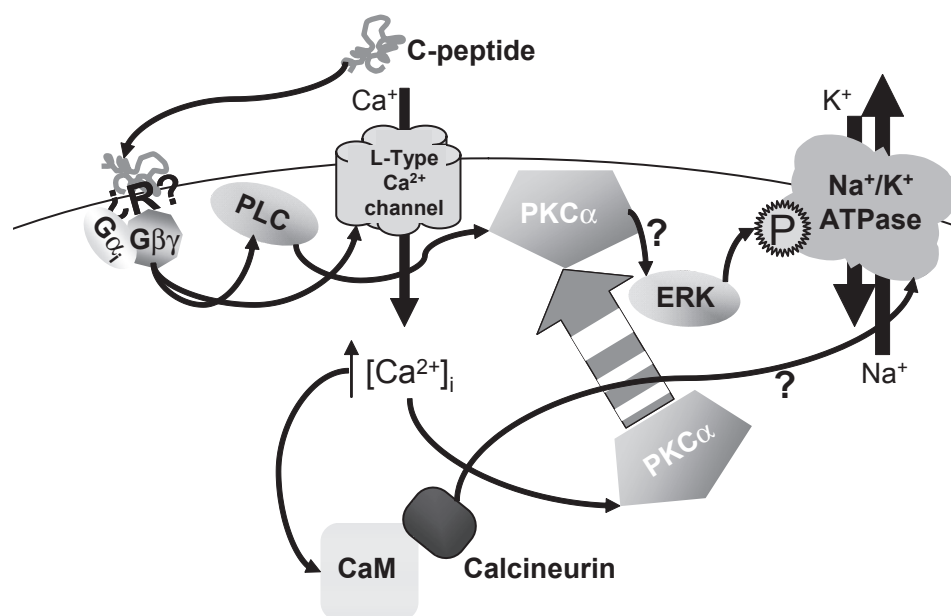


Figure 1 Molecular mechanisms of C-peptide-mediated activation of Na⁺/K⁺ ATPase. This cartoon simplifies the signaling cascade resulting in activation of the Na⁺/K⁺ ATPase. Arrows represented signaling cascades. The activation of ERK is not completely understood. For simplicity it is drawn with a single arrow from PKCα; however, it is known not to be a direct mechanism. Similarly, the precise role of calcineurin in regulation of the Na⁺/K⁺-ATPase is unknown, and is represented here by a simple arrow. The question mark (?) indicates simplified or unknown pathways.

Abbreviations: PLC, phospholipase C; PKCα, protein kinase Cα; ERK, extracellular regulated kinase; CAM, calmodulin.

wortmannin. Thus, indicating that $G\alpha_i$ and PKC mediate C-peptide-dependent phosphorylation of ERK, and that PI3K also has a significant role. Grunberger and colleagues (2001) demonstrated in L6 rat myoblasts a similar concentration response curve that is bell-shaped and showed no response at 10 nM and higher concentrations of C-peptide. This bell-shaped concentration-response curve is unusual; however, the bell-shaped curve is reported throughout the C-peptide literature. Not all studies with C-peptide result in a bell-shaped curve. For instance, C-peptide stimulation of mouse lung capillary endothelial cells results in a standard sigmoidal activation curve for ERK (Kitamura et al 2002). The potential physiological consequences of the bell-shaped curve in regards to diabetes treatment will be discussed in the conclusion.

The studies by Kitamura and colleagues (2002, 2003) indicate that C-peptide increased the phosphorylation of ERK and p38, but not JNK. These studies were reinforced via kinase assays, and thus are the first conclusive proof that C-peptide activates ERK and p38 (Kitamura et al 2002). C-peptide-mediated activation of ERK and p38 was confirmed in rat aortic endothelial cells (Kitamura et al 2003). In contrast, in human renal tubular cells C-peptide increases phosphorylation of ERK and JNK, but not p38 (Zhong et al 2005). These and other data suggest that ERK is the primary MAPK activated by C-peptide. Via use of specific inhibitors, Kitamura and colleagues demonstrated that ERK leads to phosphorylation of RSK1, but not MSK1. C-peptide-mediated phosphorylation and activation of RSK1 also occurs in L6 rat myoblasts (Grunberger et al 2001). Importantly, RSK1 is downstream of ERK and PDK1, and is involved in multiple signaling cascades including inactivation of glycogen synthase kinase (Hauge and Frodin 2006). Thus C-peptide-mediated activation of ERK may be important in processing glucose.

The most complete C-peptide-mediated signaling to ERK has been worked out in vascular smooth muscle cells. C-peptide-mediated ERK and JNK phosphorylation absolutely requires PLC, L-type Ca^{2+} channels, PKC, and MEK activity (Walcher et al 2006). Furthermore, C-peptide signaling to ERK is significantly reduced by treatment with the src family kinase inhibitor PP2 and the PI3K inhibitor LY294002 (Walcher et al 2006). PP2 is a dirty drug (Bain et al 2007), however Walcher and colleagues also used siRNA demonstrating that c-src is involved in C-peptide-mediated phosphorylation of ERK. Additionally, PP2 significantly, but not completely, blocks C-peptide mediated generation of PIP3, indicating that src is involved in the activation of PI3K. Activation of ERK in vascular smooth muscle cells leads

to cellular proliferation and increased cyclin D1 expression (Walcher et al 2006).

Collectively the data indicate that C-peptide is a potent stimulant of MAPKs, specifically ERK. Most of the studies have used insulin or PDGF as a positive control and found C-peptide to activate ERK to a much lesser extent; however, how C-peptide acts in comparison to ligands with similarly expressed receptors has not been examined. It is known that the C-peptide receptor(s) are less dense than the insulin receptor (Rigler et al 1999). Although there appears to be tissue specificity as to which MAPKs are activated, in all cases ERK is activated. ERK phosphorylation is a common occurrence for $G\alpha_i$ -coupled receptors (L'Allemain et al 1991), and can occur through multiple pathways including through $G\alpha_i$ (Hedin et al 1999) and through $G\beta\gamma$ (Crespo et al 1994; Koch et al 1994) activation of PI3K (Stoyanov et al 1995; Hawes et al 1996; Lopez-Illasaca et al 1997). Hedin and colleagues (1999) demonstrate that the $G\alpha_i$ -dependent mechanism is independent of Ras and PI3K, but requires PKC, which may explain the 50% inhibition seen with wortmannin (Kitamura et al 2001) and allow for more traditional roles for PKC (Schonwasser et al 1998) and c-src (Luttrell et al 1996) as is eluded to by Walcher and colleagues (2006). These accepted pathways partially unite the disparate C-peptide signaling pathways into one mechanism. However, the precise molecular mechanisms of how ERK is activated remain unknown.

In conclusion, the use of PTX, PI3K, and MEK inhibitors suggest that the mechanism involves the classical Ras-Raf/MEK/ERK cascade that is activated by $G\beta\gamma$ -mediated activation of PI3K. However, this does not account for how PKC and c-src are involved in activating this cascade. Thus, although C-peptide clearly activates ERK through a $G\alpha_i$ -mediated mechanism that involves PI3K, the multiple cell types used, lack of standardized experiments across cell types, and lack of a known receptor make it impossible to clearly unite all of the data into a single pathway. Our experience indicates that signaling to ERK is cell type-dependent (Escano et al 2008). Additionally, the disparities between the phosphorylation of p38 and JNK support the idea that cell type dependent mechanisms are involved in the activation of the MAPKs. Thus, we are hesitant to combine all of the data into one pathway.

NO synthase

There are three isoforms of nitric oxide synthase (NOS): iNOS, nNOS, and eNOS (Mashimo and Goyal 1999). C-peptide has been shown to activate eNOS in rat and bovine

aortic endothelial cells (Kitamura et al 2003; Wallerath et al 2003). Wallerath and colleagues demonstrated that C-peptide rapidly generates cGMP in a concentration-dependent manner. cGMP levels peak within 5 min and by 10 min the levels are similar to baseline levels, which can be attributed to phosphodiesterases degrading the cGMP. Furthermore, Wallerath and colleagues (2003) indicate that C-peptide-mediated increase in cGMP levels were abrogated by the NOS inhibitor L-NNA and by Ca^{2+} free media. This suggests that eNOS is involved in C-peptide-mediated NO production. C-peptide did not increase eNOS mRNA. In contrast, Kitamura and colleagues (2003) demonstrated that C-peptide transiently increases eNOS, but not iNOS, and that mRNA levels and eNOS protein levels are increased for up to 6 hours. eNOS expression was blocked by inhibiting gene transcription with actinomycin D and inhibition of MEK. Thus, Kitamura and colleagues (2003) indicate that the ERK MAPK pathway is important in C-peptide-mediated generation of NO. Additionally, Kitamura and colleagues (2003) observe NO production utilizing the NO sensitive dye DAF-2. The procedure followed, 3 hours of treatment followed by washing and then exposure to DAF-2 for 1 hour, cannot discriminate between the increased levels of eNOS generating increased basal NO and C-peptide-mediated release of NO. Given that Wallerath and colleagues (2003) show that C-peptide-mediated production of cGMP occurs within 10 min, the DAF-2 results from Kitamura and colleagues are most likely due to the enhanced eNOS protein levels. Supporting, in part, the study by Kitamura and colleagues (2003), Li and colleagues (2004) indicate that 24 hour exposure of human smooth muscle cells to C-peptide increases iNOS and eNOS mRNA and protein levels. Though generally reported to be a stimulator of NOS, C-peptide counteracts diabetes-induced increases in renal eNOS levels (Kamikawa et al 2008). The seeming contradictions in the data may be due to altered mechanisms in the diabetic state, cell type, or methodology, which is not standard between the studies. In summary, C-peptide, like insulin, is involved in generation of NO through activation of eNOS, but the mechanism is largely unknown.

NF- κ B activation and protection of diabetic nephropathy

There are a few reports indicating that C-peptide activates nuclear factor κ B (NF- κ B) (Al-Rasheed et al 2006; Kitazawa et al 2006). In Swiss 3T3 cells inhibition of PKC inhibits NF- κ B activation (Kitazawa et al 2006). In OK cells C-peptide protects against tumor necrosis factor α

(TNF- α)-mediated apoptosis through an NF- κ B mediated mechanism (Al-Rasheed et al 2006). C-peptide-mediated NF- κ B activity was ablated by PTX and wortmannin, but not PD98059. Al-Rasheed and colleagues (2006) also demonstrate that C-peptide increases TRAF2, an NF- κ B driven survival gene, and blocks high concentration TNF- α -mediated decreased expression of TRAF2. Together the data indicate that C-peptide may activate NF- κ B through a PKC- and PI3K-dependent phosphorylation of I κ B that then leads to increased translation of NF- κ B driven genes that can inhibit TNF- α -mediated signaling. It should be noted that recent data suggest C-peptide rather reduces NF- κ B activation (Cifarelli et al 2008).

Importantly, TNF- α has been suggested in the pathogenesis of diabetic nephropathy (Moriwaki et al 2007). Additionally, TNF- α is increased in diabetic nephropathy (Kalantarinia et al 2003) and correlates with urinary protein excretion (Moriwaki et al 2007). Injection of TNF- α decreases renal function (Schmidt et al 2007), and Moriwaki and colleagues (2007) demonstrated that inhibiting TNF- α reduces albuminuria in experimental diabetic rats. Therefore, C-peptide-mediated inhibition of TNF- α -mediated effects in renal cells may have clinical benefits.

Activation of RhoA and physiological consequences

C-peptide activates the small guanosine triphosphatase (GTPase) RhoA through an unknown mechanism in human renal tubular cells (Zhong et al 2005). Physiologically, Rho-kinase, an effector of RhoA, is known to be responsible for mediating “ Ca^{2+} -sensitization”, a condition where smooth muscle constriction is enhanced in similar or lower levels of Ca^{2+} influx (Kimura et al 1996). Rho-kinase is of particular interest because it plays an important role in mediating vasoconstriction in the kidney (Roos et al 2006). Additionally, RhoA/Rho-kinase regulates blood flow, GFR, and alters the function and structure of renal tubular epithelial cells and mesangial cells (Wakino et al 2005).

We have previously shown that the Rho-kinase inhibition with Y-27632 prevents the vasoconstrictive effects of C-peptide in isolated afferent arterioles, thus suggesting that C-peptide activates RhoA and consequently Rho-kinase in the renal vasculature (Nordquist et al 2008a). However, reduction of C-peptide-induced vasoconstriction by inhibition of Rho-kinase does not necessarily imply that C-peptide activates Rho-kinase (Figure 2). C-peptide is known to increase PKC and MAPKs, both of which are capable of mediating contraction (via CPI-17 and caldesmon, respectively

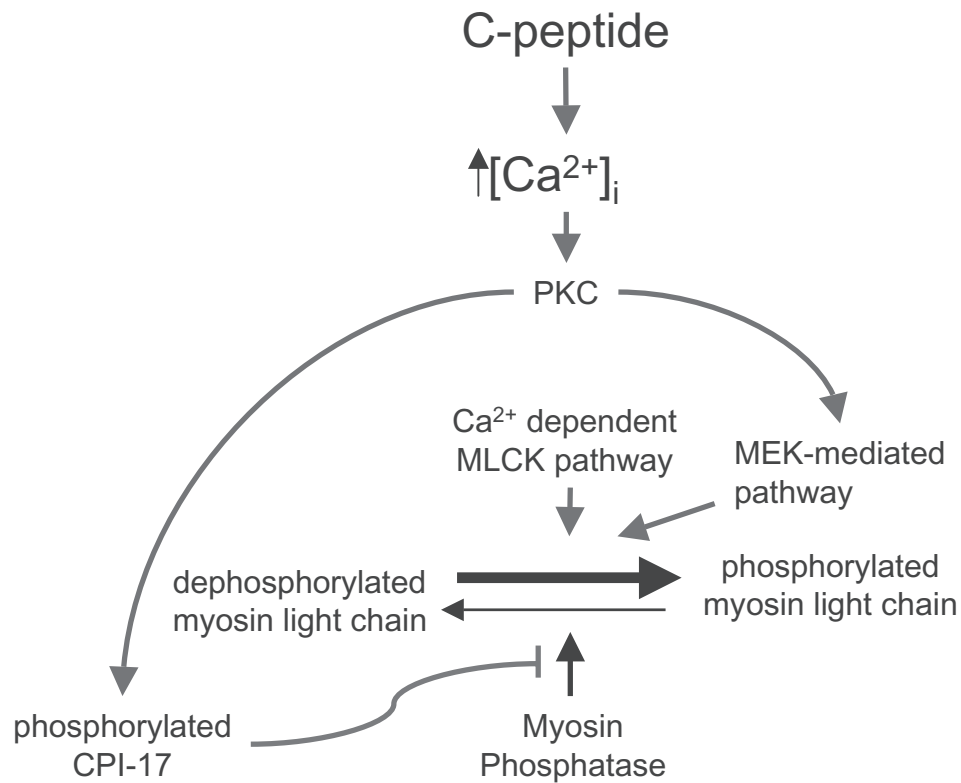


Figure 2 Schematic illustrations of sequences of events leading to constriction. This schematic drawing illustrates possible downstream effects of C-peptide.

(Gerthoffer et al 1996; Walsh et al 2007; Sakai et al 2007). Additionally, MAPK has been linked to phosphorylation of the regulatory subunit of the myosin light chain in the same location as myosin light chain kinase (Roberts 2004). Moreover, G α_i -coupled receptors can activate integrin-linked kinase (ILK) through a PI3K-dependent pathway that results in phosphorylation of CPI-17 and the myosin light chain at the same site as myosin light chain kinase (Huang et al 2006). Since inhibition of Rho-kinase allows for greater myosin phosphatase activity, which in turn will decrease the phosphorylation status of the regulatory subunit of the myosin light chain, it is possible that C-peptide induces phosphorylation of the myosin light chain through one of the aforementioned pathways, and that Y-27632 is only increasing the basal level of myosin phosphatase. Thus, more investigation is warranted into the link between C-peptide and RhoA/Rho-kinase activation in the renal vasculature.

Concluding remarks

Thus far, the results from C-peptide studies display several unexplored research directions and opportunities for novel therapeutic interventions. It is clear that C-peptide possesses beneficial effects on diabetes-induced complications. This is supported by pancreas and islet of Langerhans transplantation

in diabetic patients, restoring not only the patients' insulin production, but also production of C-peptide. Thus, large scale clinical trials examining the benefits of C-peptide should be initiated in diabetic patients. In type 1 and stage 2 type 2 diabetes restoring circulating C-peptide is most likely going to be beneficial to the patient. However, the aforementioned bell-shaped concentration response curve may, paradoxically, help potentiate insulin resistance. In many cases mid to high nanomolar concentrations of C-peptide signalled less than low nanomolar concentrations, which could reduce the physiological effect of C-peptide and may explain why the increased levels of C-peptide in phase 1 type 2 diabetics is not beneficial. This apparent paradox must be more fully examined before C-peptide is administered to hyperinsulinemic type 2 diabetic patients. Additionally, if this paradox is physiologically relevant then the level of C-peptide administration would have to be carefully monitored, such as is done with insulin.

Finally, there are multiple basic and clinically relevant questions that remain unanswered. The identity of the receptor is clinically important, and the downstream signalling cascade, as currently known, does not fully explain amelioration of diabetes-induced complications. Further studies should be undertaken, investigating the intracellular

effects of C-peptide. If the C-peptide receptor is a GPCR, then an agonist to the receptor can be generated. This is important since C-peptide may not be easily administered to patients, whereas a stable agonist may be able to be supplied as a once a day pill. Similarly, more fully understanding the cellular signaling of C-peptide may lead to novel interventions in diabetes associated diseases, such as nephropathy.

In conclusion, the field of C-peptide biology has accelerated greatly in the last 15 years. It is clear that C-peptide is biologically active, binds to cell surface receptors, and signals in a manner similar but unique from insulin. Physiologically, the effects are in some aspects similar to that of giving insulin to type 1 diabetic patients; however, C-peptide appears to restore insulin-treated type 1 diabetic patients to normal physiology. Given that C-peptide is a hormone co-secreted with insulin, and thus is absent in type 1 diabetes, the beneficial effects of C-peptide are not surprising.

Disclosure

The authors report no conflicts of interest.

References

- Al-Rasheed NM, Willars GB, Brunskill NJ. 2006. C-peptide signals via Galpha i to protect against TNF-alpha-mediated apoptosis of opossum kidney proximal tubular cells. *J Am Soc Nephrol*, 17:986–95.
- Bab I, Gavish H, Namdar-Attar M, et al. 1999. Isolation of mitogenically active C-terminal truncated pentapeptide of osteogenic growth peptide from human plasma and culture medium of murine osteoblastic cells. *J Pept Res*, 54:408–14.
- Bain J, Plater L, Elliott M, et al. 2007. The selectivity of protein kinase inhibitors: a further update. *Biochem J*, 408:297–315.
- Bank N, Aynedjian HS. 1993. Role of EDRF nitric oxide in diabetic renal hyperfiltration. *Kidney Int*, 43:1306–12.
- Bell TD, DiBona GF, Wang Y, et al. 2006. Mechanisms for renal blood flow control early in diabetes as revealed by chronic flow measurement and transfer function analysis. *J Am Soc Nephrol*, 17:2184–92.
- Blumer JB, Cismowski MJ, Sato M, et al. 2005. AGS proteins: receptor-independent activators of G-protein signaling. *Trends Pharmacol Sci*, 26:470–6.
- Brown CD, Ghali HS, Zhao Z, et al. 2005. Association of reduced red blood cell deformability and diabetic nephropathy. *Kidney Int*, 67:295–300.
- Brownlee M. 2001. Biochemistry and molecular cell biology of diabetic complications. *Nature*, 414:813–20.
- Chakrabarti S, Khan ZA, Cukiernik M, et al. 2004. C-peptide and retinal microangiopathy in diabetes. *Exp Diabetes Res*, 5:91–6.
- Cifarelli V, Luppi P, Tse HM, et al. 2008. Human proinsulin C-peptide reduces high glucose-induced proliferation and NF-KB activation in vascular smooth muscle cells. *Atherosclerosis*, online first.
- Crespo P, Xu N, Simonds WF, et al. 1994. Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. *Nature*, 369:418–20.
- [DCCT] The Diabetes Control and Complications Trial Research Group. 1993. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. *N Engl J Med*, 329:977–86.
- De La Tour DD, Raccach D, Jannot MF, et al. 1998. Erythrocyte Na/K ATPase activity and diabetes: relationship with C-peptide level. *Diabetologia*, 41:1080–4.
- Ditzel J, Standl E. 1975. The problem of tissue oxygenation in diabetes mellitus. *Acta Med Scand Suppl*, 578:59–68.
- Escano CS, Keever LB, Gutweiler AA, et al. 2008. Angiotensin II activates extracellular signal-regulated kinase independently of receptor tyrosine kinases in renal smooth muscle cells: implications for blood pressure regulation. *J Pharmacol Exp Ther*, 324:34–42.
- Fiorina P, Folli F, Zerbini G, et al. 2003a. Islet transplantation is associated with improvement of renal function among uremic patients with type 1 diabetes mellitus and kidney transplants. *J Am Soc Nephrol*, 14:2150–8.
- Fiorina P, Folli F, Maffi P. 2003b. Islet transplantation improves vascular diabetic complications in patients with diabetes who underwent kidney transplantation: a comparison between kidney-pancreas and kidney-alone transplantation. *Transplantation*, 75:1296–301.
- Fiorina P, Gremizzi C, Maffi P, et al. 2005a. Islet transplantation is associated with an improvement of cardiovascular function in type 1 diabetic kidney transplant patients. *Diabetes Care*, 28:1358–65.
- Fiorina P, Venturini M, Folli F, et al. 2005b. Natural history of kidney graft survival, hypertrophy, and vascular function in end-stage renal disease type 1 diabetic kidney-transplanted patients: beneficial impact of pancreas and successful islet cotransplantation. *Diabetes Care*, 28:1303–10.
- Flatt PR., Swanson-Flatt SK, Hampton SM, et al. 1986. Specific binding of the C-peptide of proinsulin to cultured B-cells from a transplantable rat islet cell tumor. *Biosci Rep*, 6:193–9.
- Forst T, Kunt T, Pohlmann T, et al. 1998. Biological activity of C-peptide on the skin microcirculation in patients with insulin-dependent diabetes mellitus. *J Clin Invest*, 101:2036–41.
- Gerthoffer WT, Yamboliev IA, Shearer M, et al. 1996. Activation of MAP kinases and phosphorylation of caldesmon in canine colonic smooth muscle. *J Physiol*, 495(Pt 3):597–609.
- Grunberger G, Qiang X, Li Z, et al. 2001. Molecular basis for the insulinomimetic effects of C-peptide. *Diabetologia*, 44:1247–57.
- Hansen A, Johansson BL, Wahren J, et al. 2002. C-peptide exerts beneficial effects on myocardial blood flow and function in patients with type 1 diabetes. *Diabetes*, 51:3077–82.
- Hauge C, Frodin M. 2006. RSK and MSK in MAP kinase signalling. *J Cell Sci*, 119(Pt 15):3021–3.
- Hawes BE, Luttrell LM, van Biesen T, et al. 1996. Phosphatidylinositol 3-kinase is an early intermediate in the G beta gamma-mediated mitogen-activated protein kinase signaling pathway. *J Biol Chem*, 271:12133–6.
- He H, Shehan BP, Barnham KJ, et al. 2004. Biological activity and ferric ion binding of fragments of glycine-extended gastrin. *Biochemistry*, 43:11853–61.
- Hedin KE, Bell MP, Huntton CJ, et al. 1999. Gi proteins use a novel beta gamma- and Ras-independent pathway to activate extracellular signal-regulated kinase and mobilize AP-1 transcription factors in Jurkat T lymphocytes. *J Biol Chem*, 274:19992–20001.
- Henriksson M, Johansson J, Moede T, et al. 2006. Proinsulin C-peptide and insulin: Limited pattern similarities of interest in inter-peptide interactions but no C-peptide effect on insulin and IGF-1 receptor signaling. *Cell Mol Life Sci*, 63:3055–60.
- Huang DY, Richter K, Breidenbach A, et al. 2002. Human C-peptide acutely lowers glomerular hyperfiltration and proteinuria in diabetic rats: a dose-response study. *Naunyn Schmiedebergs Arch Pharmacol*, 365:67–73.
- Huang J, Mahavadi S, Sriwai W, et al. 2006. Gi-coupled receptors mediate phosphorylation of CPI-17 and MLC20 via preferential activation of the PI3K/ILK pathway. *Biochem J*, 396:193–200.
- Ido Y, Vindigni A, Chang K, et al. 1997. Prevention of vascular and neural dysfunction in diabetic rats by C-peptide. *Science*, 277:563–6.
- Ikenaga H, Bast JP, Fallet RW, et al. 2000. Exaggerated impact of ATP-sensitive K+ channels on afferent arteriolar diameter in diabetes mellitus. *J Am Soc Nephrol*, 11:1199–207.

- Jensen T, Bjerre-Knudsen J, Feldt-Rasmussen B, et al. 1989. Features of endothelial dysfunction in early diabetic nephropathy. *Lancet*, 18636:461–3.
- Johansson BL, Linde B, Wahren J. 1992a. Effects of C-peptide on blood flow, capillary diffusion capacity and glucose utilization in the exercising forearm of type 1 insulin-dependent diabetic patients. *Diabetologia*, 3512:1151–8.
- Johansson BL, Sjöberg S, Wahren J. 1992b. The influence of human C-peptide on renal function and glucose utilization in type 1 insulin-dependent diabetic patients. *Diabetologia*, 352:121–8.
- Johansson BL, Borg K, Fernqvist-Forbes E, et al. 2000. Beneficial effects of C-peptide on incipient nephropathy and neuropathy in patients with Type 1 diabetes mellitus. *Diabet Med*, 173:181–9.
- Johansson BL, Wahren J, Pernow J. 2003. C-peptide increases forearm blood flow in patients with type 1 diabetes via a nitric oxide-dependent mechanism. *Am J Physiol Endocrinol Metab*, 2854:E864–70.
- Juncos L A, Ito S. 1993. Disparate effects of insulin on isolated rabbit afferent and efferent arterioles. *J Clin Invest*, 924:1981–5.
- Kalantarinia K, Awad AS, Siragy HM. 2003. Urinary and renal interstitial concentrations of TNF-alpha increase prior to the rise in albuminuria in diabetic rats. *Kidney Int*, 644:1208–13.
- Kamikawa A, Ishii T, Shimada K, et al. 2008. Proinsulin C-peptide abrogates type-1 diabetes-induced increase of renal endothelial nitric oxide synthase in rats. *Diabetes Metab Res Rev*, 24:331–8.
- Katada T, Ui M. 1982. ADP ribosylation of the specific membrane protein of C6 cells by islet-activating protein associated with modification of adenylate cyclase activity. *J Biol Chem*, 25712:7210–16.
- Katz A, Wu D, Simon MI. 1992. Subunits beta gamma of heterotrimeric G protein activate beta 2 isoform of phospholipase C. *Nature*, 3606405:686–9.
- Kimura K, Ito M, Amano M, et al. 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase Rho-kinase. *Science*, 2735272:245–8.
- Kitamura T, Kimura K, Jung BD, et al. 2001. Proinsulin C-peptide rapidly stimulates mitogen-activated protein kinases in Swiss 3T3 fibroblasts: requirement of protein kinase C, phosphoinositide 3-kinase and pertussis toxin-sensitive G-protein. *Biochem J*, 355(Pt 1):123–9.
- Kitamura T, Kimura K, Jung BD, et al. 2002. Proinsulin C-peptide activates cAMP response element-binding proteins through the p38 mitogen-activated protein kinase pathway in mouse lung capillary endothelial cells. *Biochem J*, 366(Pt 3):737–44.
- Kitamura T, Kimura K, Makondo K, et al. 2003. Proinsulin C-peptide increases nitric oxide production by enhancing mitogen-activated protein-kinase-dependent transcription of endothelial nitric oxide synthase in aortic endothelial cells of Wistar rats. *Diabetologia*, 4612:1698–705.
- Kitazawa M, Shibata Y, Hashimoto S, et al. 2006. Proinsulin C-peptide stimulates a PKC/IkappaB/NF-kappaB signaling pathway to activate COX-2 gene transcription in Swiss 3T3 fibroblasts. *J Biochem*, 1396:1083–8.
- Klee CB, Crouch TH, Krinks MH. 1979. Calcineurin: a calcium- and calmodulin-binding protein of the nervous system. *Proc Natl Acad Sci USA*, 7612:6270–3.
- Kobayashi Y, Naruse K, Hamada Y, et al. 2005. Human proinsulin C-peptide prevents proliferation of rat aortic smooth muscle cells cultured in high-glucose conditions. *Diabetologia*, 4811:2396–401.
- Koch WJ, Hawes BE, Allen LF, et al. 1994. Direct evidence that Gi-coupled receptor stimulation of mitogen-activated protein kinase is mediated by G beta gamma activation of p21ras. *Proc Natl Acad Sci USA*, 9126:12706–10.
- Komers R, Allen TJ, Cooper ME. 1994. Role of endothelium-derived nitric oxide in the pathogenesis of the renal hemodynamic changes of experimental diabetes. *Diabetes*, 4310:1190–7.
- Kunt T, Schneider S, Pflutzner A, et al. 1999. The effect of human proinsulin C-peptide on erythrocyte deformability in patients with Type I diabetes mellitus. *Diabetologia*, 424:465–71.
- L'Allemain G, Pouyssegur J, Weber MJ. 1991. p42/mitogen-activated protein kinase as a converging target for different growth factor signaling pathways: use of pertussis toxin as a discrimination factor. *Cell Regul*, 28:675–84.
- Lea JP, Sands JM, McMahon SJ. 1994. Evidence that the inhibition of Na⁺/K⁺-ATPase activity by FK506 involves calcineurin. *Kidney Int*, 463:647–52.
- Lee JC, Downing SE. 1979. Coronary dynamics and myocardial metabolism in the diabetic newborn lamb. *Am J Physiol*, 2372:H118–24.
- Lee TC, Barshes NR, Agee EE, et al. 2006. The effect of whole organ pancreas transplantation and PIT on diabetic complications. *Curr Diab Rep*, 64:323–7.
- Li H, Xu L, Dunbar JC, et al. 2004. Effects of C-peptide on expression of eNOS and iNOS in human cavernosal smooth muscle cells. *Urology*, 643:622–7.
- Li ZG, Zhang L, Sima AA. 2003. C-peptide enhances insulin-mediated cell growth and protection against high glucose-induced apoptosis in SH-SY5Y cells. *Diabetes Metab Res Rev*, 195:375–85.
- Lindahl E, Nyman U, Melles E, et al. 2007. Cellular internalization of proinsulin C-peptide. *Cell Mol Life Sci*, 644:479–86.
- Lindstrom K, Johansson C, Johnsson E, et al. 1996. Acute effects of C-peptide on the microvasculature of isolated perfused skeletal muscles and kidneys in rat. *Acta Physiol Scand*, 1561:19–25.
- Lopez-Illasaca M, Crespo P, Pellici PG, et al. 1997. Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase gamma. *Science*, 2755298:394–7.
- Luttrell LM, Hawes BE, van Biesen BE, et al. 1996. Role of c-Src tyrosine kinase in G protein-coupled receptor- and Gbetagamma subunit-mediated activation of mitogen-activated protein kinases. *J Biol Chem*, 27132:19443–50.
- Maezawa Y, Yokote K, Sonezaki K, et al. 2006. Influence of C-peptide on early glomerular changes in diabetic mice. *Diabetes Metab Res Rev*, 224:313–22.
- Mashimo H, Goyal RK. 1999. Lessons from genetically engineered animal models. IV. Nitric oxide synthase gene knockout mice. *Am J Physiol*, 2774(Pt 1):G745–50.
- Meyer JA, Froelich JM, Reid GE, et al. 2008. Metal-activated C-peptide facilitates glucose clearance and the release of a nitric oxide stimulus via the GLUT1 transporter. *Diabetologia*, 51:175–82.
- Moriwaki Y, Inokuchi T, Yamamoto A, et al. 2007. Effect of TNF-alpha inhibition on urinary albumin excretion in experimental diabetic rats. *Acta Diabetol*, 444:215–18.
- Nordquist L, Moe E, Sjöquist M. 2007. The C-peptide fragment EVARQ reduces glomerular hyperfiltration in streptozotocin-induced diabetic rats. *Diabetes Metab Res Rev*, 23:400–405.
- Nordquist L, Lai EY, Sjöquist M, et al. 2008a. Proinsulin C-peptide constricts glomerular afferent arterioles in diabetic mice. A potential renoprotective mechanism. *Am J Physiol Regul Integr Comp Physiol*, 294:R836–41.
- Nordquist L, Lai EY, Sjöquist M, et al. 2008b. C-peptide constricts pancreatic islet arterioles in diabetic, but not normoglycaemic mice. *Diabetes Metab Res Rev*, 24:165–8.
- Ohtomo Y, Aperia A, Sahlgren B, et al. 1996. C-peptide stimulates rat renal tubular Na⁺, K⁺-ATPase activity in synergism with neuropeptide Y. *Diabetologia*, 392:199–205.
- Ohtomo Y, Bergman T, Johansson BL, et al. 1998. Differential effects of proinsulin C-peptide fragments on Na⁺, K⁺-ATPase activity of renal tubule segments. *Diabetologia*, 413:287–91.
- Portelli M, Renzi G. 1973. Pentapeptide derivatives of the C-terminal tetrapeptide of gastrin. *Farmaco Sci*, 284:316–22.
- Raccach D, Fabreguetts C, Azulay JP, et al. 1996. Erythrocyte Na⁺-K⁺-ATPase activity, metabolic control, and neuropathy in IDDM patients. *Diabetes Care*, 196:564–8.
- Ramasharma K, Yamashiro D, Li CH. 1988. Human follicular gonadotropin releasing peptide analogs. Evaluation of biological in vitro and immunological activity. *Int J Pept Protein Res*, 326:419–24.
- Rebsomen L, Tsimaratos M. 2005. Association of reduced red blood cell deformability and diabetic nephropathy. *Kidney Int*, 675:2066; author reply 2066–7.
- Rigler R, Pramanik A, Jonasson P, et al. 1999. Specific binding of proinsulin C-peptide to human cell membranes. *Proc Natl Acad Sci USA*, 9623:13318–23.

- Roberts RE. 2004. The role of Rho kinase and extracellular regulated kinase-mitogen-activated protein kinase in alpha2-adrenoceptor-mediated vasoconstriction in the porcine palmar lateral vein. *J Pharmacol Exp Ther*, 311:742–7.
- Roos MH, van Rodijnen WF, van Lambalgen AA, et al. 2006. Renal microvascular constriction to membrane depolarization and other stimuli: pivotal role for rho-kinase. *Pflugers Arch*, 452:471–7.
- Sakai H, Chiba Y, Misawa M. 2007. Role of Rho kinase in endothelin-1-induced phosphorylation of CPI-17 in rat bronchial smooth muscle. *Pulm Pharmacol Ther*, 20:734–9.
- Sallstrom J, Carlsson PO, Fredholm BB, et al. 2007. Diabetes-induced hyperfiltration in adenosine A1-receptor deficient mice lacking the tubuloglomerular feedback mechanism. *Acta Physiol Oxf*, 190:253–9.
- Samnegard B, Brundin T. 2001. Renal extraction of insulin and C-peptide in man before and after a glucose meal. *Clin Physiol*, 21:164–71.
- Samnegard B, Jacobson SH, Jaremko G, et al. 2001. Effects of C-peptide on glomerular and renal size and renal function in diabetic rats. *Kidney Int*, 60:1258–65.
- Samnegard B, Jacobson SH, Johansson BL, et al. 2004. C-peptide and captopril are equally effective in lowering glomerular hyperfiltration in diabetic rats. *Nephrol Dial Transplant*, 19:1385–91.
- Samnegard B, Jacobson SH, Jaremko G, et al. 2005. C-peptide prevents glomerular hypertrophy and mesangial matrix expansion in diabetic rats. *Nephrol Dial Transplant*, 20:532–8.
- Sandow J, Konig W. 1979. Studies with fragments of a highly active analogue of luteinizing hormone releasing hormone. *J Endocrinol*, 81:175–82.
- Sari R, Balci MK. 2005. Relationship between C peptide and chronic complications in type-2 diabetes mellitus. *J Natl Med Assoc*, 97:1113–18.
- Schmidt C, Hoehrl K, Schweda F, et al. 2007. Regulation of renal glucose transporters during severe inflammation. *Am J Physiol Renal Physiol*, 292:F804–11.
- Schonwasser DC, Marais RM, Marshall CJ, et al. 1998. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. *Mol Cell Biol*, 18:790–8.
- Sima AA, Li ZG. 2005. The effect of C-peptide on cognitive dysfunction and hippocampal apoptosis in type 1 diabetic rats. *Diabetes*, 54:1497–505.
- Sprague RS, Ellsworth ML, Stephenson LH, et al. 1996. ATP: the red blood cell link to NO and local control of the pulmonary circulation. *Am J Physiol*, 271(6Pt 2):H2717–22.
- Sprague RS, Olearczyk JJ, Spence DM, et al. 2003. Extracellular ATP signaling in the rabbit lung: erythrocytes as determinants of vascular resistance. *Am J Physiol Heart Circ Physiol*, 285:H693–700.
- Stoyanov B, Volinia S, Hanch T, et al. 1995. Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. *Science*, 269:690–3.
- Sutherland C, Leighton IA, Cohen P. 1993. Inactivation of glycogen synthase kinase-3 beta by phosphorylation: new kinase connections in insulin and growth-factor signalling. *Biochem J*, 296(Pt 1):15–19.
- Thomson SC, Deng A, Bao D, et al. 2001. Ornithine decarboxylase, kidney size, and the tubular hypothesis of glomerular hyperfiltration in experimental diabetes. *J Clin Invest*, 107:217–24.
- Tsimaratos M, Roger F, Chabardes D, et al. 2003. C-peptide stimulates Na⁺/K⁺-ATPase activity via PKC alpha in rat medullary thick ascending limb. *Diabetologia*, 46:124–31.
- Wakino S, Kanda T, Hayashi K. 2005. Rho/Rho kinase as a potential target for the treatment of renal disease. *Drug News Perspect*, 18:639–43.
- Walcher D, Babiak C, Poletsek P, et al. 2006. C-Peptide induces vascular smooth muscle cell proliferation: involvement of SRC-kinase, phosphatidylinositol 3-kinase, and extracellular signal-regulated kinase 1/2. *Circ Res*, 99:1181–7.
- Wallerath T, Kunt T, Forst T, et al. 2003. Stimulation of endothelial nitric oxide synthase by proinsulin C-peptide. *Nitric Oxide*, 9:95–102.
- Vallon V, Blantz RC, Thomson S. 1995. Homeostatic efficiency of tubuloglomerular feedback is reduced in established diabetes mellitus in rats. *Am J Physiol*, 269(6Pt 2):F876–83.
- Walsh MP, Susnjak M, Deng J, et al. 2007. Phosphorylation of the protein phosphatase type 1 inhibitor protein CPI-17 by protein kinase C. *Methods Mol Biol*, 365:209–23.
- Wang YX, Brooks DP, Edwards RM. 1993. Attenuated glomerular cGMP production and renal vasodilation in streptozotocin-induced diabetic rats. *Am J Physiol*, 264(5Pt 2):R952–6.
- Venturini M, Fiorina P, Maffi P, et al. 2006. Early increase of retinal arterial and venous blood flow velocities at color doppler imaging in brittle type 1 diabetes after islet transplant alone. *Transplantation*, 81:1274–7.
- West RE Jr, Moss J, Vaughan M, et al. 1985. Pertussis toxin-catalyzed ADP-ribosylation of transducin. Cysteine 347 is the ADP-ribose acceptor site. *J Biol Chem*, 260:14428–30.
- Viard P, Exner T, Maier U, et al. 1999. Gbetagamma dimers stimulate vascular L-type Ca²⁺ channels via phosphoinositide 3-kinase. *FASEB J*, 13:685–94.
- Wu D, Katz A, Simon MI. 1993. Activation of phospholipase C beta 2 by the alpha and beta gamma subunits of trimeric GTP-binding protein. *Proc Natl Acad Sci U S A*, 90:5297–301.
- Yajima H, Kai Y, Ogawa H, et al. 1977. Structure-activity relationships of gastrointestinal hormones: motilin, GIP, and [27-TYR]CCK-PZ. *Gastroenterology*, 72(4Pt 2):793–6.
- Young LH, Ikeda Y, Scalia R, et al. 2000. C-peptide exerts cardioprotective effects in myocardial ischemia-reperfusion. *Am J Physiol Heart Circ Physiol*, 279:H1453–9.
- Zhong Z, Kotova O, Davidescu A, et al. 2004. C-peptide stimulates Na⁺/K⁺-ATPase via activation of ERK1/2 MAP kinases in human renal tubular cells. *Cell Mol Life Sci*, 61:2782–90.
- Zhong Z, Davidescu A, Ehren I, et al. 2005. C-peptide stimulates ERK1/2 and JNK MAP kinases via activation of protein kinase C in human renal tubular cells. *Diabetologia*, 48:187–97.