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The Physiology of Proinsulin C-Peptide: Unanswered Questions and a Proposed Model

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C-peptide is produced, processed, and secreted with insulin, and appears to exert separate but intimately related effects. In this review, we address the existence of the C-peptide receptor, the interaction between C-peptide and insulin, and the potential physiological significance of proinsulin C-peptide.

The human insulin prohormone consists of a 110-amino acid precursor protein, including a signal peptide, the A and B chains of insulin, and the connecting peptide, or C-peptide. Within the endoplasmic reticulum of the pancreatic β-cells, the signal peptide is cleaved, and the cysteine residues of the insulin A and B chains are oxidized to form disulfide bonds between the two chains (7, 21). The prohormone is then exported to the Golgi apparatus where it is packaged into secretory vesicles. Within these secretory vesicles, the prohormone is proteolytically cleaved by prohormone convertase 2 at site 65/66 and by prohormone convertase 1/3 at site 32/33, forming distinct insulin and C-peptide molecules (FIGURE 1) (54). Lastly, the B chain of insulin and C-peptide are further processed by carboxypeptide E to remove terminal basic residues and form the mature insulin and C-peptide peptides (54). Insulin and C-peptide are stored in the secretory vesicles until stimulation of the pancreatic β-cells by elevations in extracellular glucose concentration. Increased uptake of glucose by the β-cells leads to downstream closure of ATP-sensitive potassium channels, depolarization, and influx of calcium into the cells, ultimately resulting in the fusion of secretory vesicles and simultaneous release of insulin and C-peptide into the extracellular space.

In plasma, insulin and C-peptide exhibit remarkably different kinetics, since insulin has a serum half-life of ~2–3 min, and C-peptide, which escapes first-pass metabolism by the liver, has a half-life of ~30 min (10, 46, 52). For many years, C-peptide was thought to be a biological by-product of insulin processing, but because of its stability in plasma, it was and still is used clinically as a marker of β-cell function. It is now known that C-peptide is a biologically active peptide, although its physiological significance in normal physiology has not been fully elucidated. Although the role of C-peptide in mitigating diabetes-related complications (20, 33, 36), its potential clinical relevance in diabetes (20, 33, 47, 52), and proposed signaling mechanisms underlying these effects (18, 19) have been reviewed elsewhere, multiple questions remain. First, are the effects of C-peptide receptor mediated, and, if so, what is the C-peptide receptor(s)? Second, what is the relationship between the actions of C-peptide and insulin? Last, and most importantly, what is the potential physiological significance of C-peptide? In this review, we will address these questions and specifically evaluate the role of C-peptide in normal physiology.

Are the Effects of C-Peptide Receptor Mediated?

The existence of a specific C-peptide receptor remains a controversial topic. Multiple studies have shown that C-peptide binds specifically to a plethora of cell types, including human skin fibroblasts, kidney tubule cells, and endothelial cells (11, 16, 37, 42). C-peptide binding was not displaced by insulin, indicating that C-peptide does not interact directly with the insulin receptor (42). C-peptide also has been shown to initiate multiple intracellular signaling cascades, including protein kinase A (PKA), protein kinase C (PKC), and activation of MAK kinase, all of which are consistent with the interaction of C-peptide with a G-protein-coupled receptor (GPCR). In agreement with this assertion, the actions of C-peptide in several cell systems are pertussis toxin sensitive (26, 48, 52), indicating that C-peptide may signal via a GPCR coupled to $G\alpha_{i/o}$. Using a unique deductive ligand-receptor matching strategy (53), our group identified the orphan GPCR GPR146 as an essential part of the C-peptide signalosome and a potential receptor for C-peptide (51). Knockdown of GPR146 completely inhibited C-peptide-induced cFos mRNA expression in a human gastric cell line (KATOIII), and C-peptide exhibited significant co-localization with GPR146 on KATOIII cell membranes (51). Furthermore, incubation with an antibody directed against the second extracellular domain of GPR146 completely blocked the effect of C-peptide, but not insulin, on ATP release from human erythrocytes (41). These data suggest that the actions of C-peptide indeed are receptor mediated and that GPR146 is part of

the C-peptide signaling complex, if not the endogenous receptor of this peptide hormone.

Despite these data, alternative, nonreceptor-mediated mechanisms have been proposed for C-peptide's biological effects, including non-chiral interactions with cells (22). For example, several groups have suggested that C-peptide is internalized and localizes to intracellular compartments, including the cytosol of Swiss 3T3 and HEK293 cells (34) and in the nucleus of mesangial cells (29). Likewise, C-peptide has been reported to directly interact with protein tyrosine phosphatase 1B, a distinctly intracellular protein (24). Recently C-peptide was shown to interact with and alter the activity of alpha-enolase (23), a multi-functional enzyme that plays a role in multiple cellular processes, including glycolysis and cell growth, and localizes to the cell membrane. These data suggest that C-peptide may exert its cellular actions via nonreceptor-mediated signaling events in addition to or as opposed to interaction with a GPCR.

To answer the question of whether C-peptide interacts with a cell surface receptor, several experiments are needed. First, co-immunoprecipitation and pulldown assays would demonstrate a physical interaction between C-peptide and its putative receptor GPR146, or other binding partners. Those studies then could be confirmed with radioligand or fluorophore-based binding experiments using a cell line overexpressing the potential C-peptide receptor. However, the binding kinetics of C-peptide to human cell membranes indicates that C-peptide interacts with multiple membrane-bound proteins (37, 42), and C-peptide likely signals via a receptor complex, which may include GPR146.

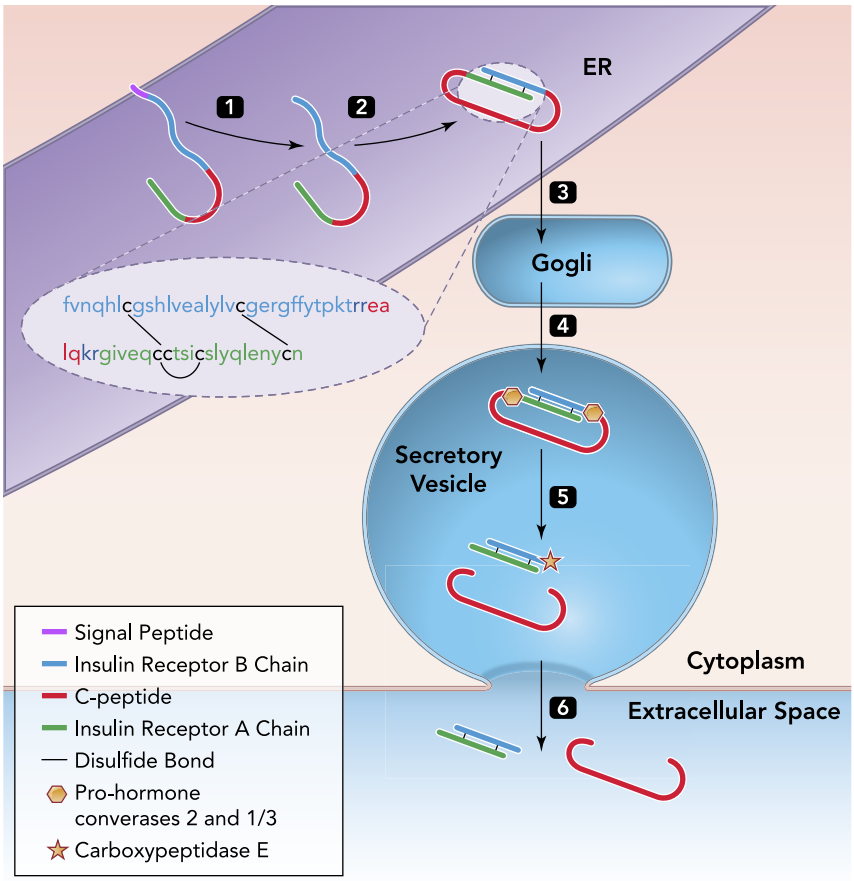

FIGURE 1. **Processing of the proinsulin prohormone**
Initially the preprohormone of insulin is translated in the ER, and the signal peptidase cleaves the signal peptide (1) followed by folding of the prohormone and formation of disulfide bonds (2) between and within the A and B chains of insulin. The C-peptide plays an essential role in orienting the two chains of insulin during this step. The entire prohormone is then secreted into the golgi (3) and ultimately packaged into secretory vesicles (4), where prohormone convertases 2 and 1/3 create separate and distinct insulin and C-peptide molecules (5), and carboxypeptidase E removes terminal amino acids from both molecules. Both insulin and C-peptide remain in the secretory vesicles of the β-cell until high-glucose conditions stimulate their release (6). Purple line, signal peptide; blue line, insulin B chain; green line, insulin A chain; red line, C-peptide; orange hexagons, prohormone convertase cleavage sites; orange star, carboxypeptidase E cleavage site.

What is the Relationship Between C-Peptide and Insulin?

Insulin and C-peptide are released simultaneously from the same secretory vesicles, and thus the physiological stimuli for the release of C-peptide is, like insulin, elevations in plasma glucose levels. Despite this fact, C-peptide does not appear to directly alter glucose metabolism (12, 25, 47, 52). However, it does appear that C-peptide- and insulin-initiated signaling cascades interact (FIGURE 2) and that this interaction is important for the normal functioning of both peptides, particularly in erythrocytes (39–41).

In addition to regulating GLUT4 translocation and glucose uptake, activation of the insulin receptor triggers signaling events related to gene expression, cell growth, cell proliferation, and cell viability (4). C-peptide may interact with insulin-induced signaling cascades to alter these actions at multiple points. First, C-peptide has been shown to enhance the tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) (14), an adapter protein that, when phosphorylated, facilitates the induction of multiple signaling pathways, including the MAP kinase pathway and the PI3 kinase pathway. Likewise, C-peptide was shown to modulate ERK1/2 phosphorylation (26, 31) and Akt activity (14, 31), as well as AMPK phosphorylation (2), all downstream targets of insulin receptor activation. However, C-peptide regulates insulin-independent signaling molecules as well, including PKC (26, 39, 45), which has been shown to dampen insulin signaling via serine/threonine phosphorylation of IRS-1 (28, 38). These data present an interesting paradox: How and why would a peptide that enhances the activation of insulin-regulated signaling molecules also activate second messengers that dampen insulin signaling? Furthermore, what are the functional implications of these findings?

C-peptide exhibits a bell-shaped dose-response curve in most cell systems, and it has been hypothesized that C-peptide function is dependent on the molar ratio of C-peptide to insulin (14), as has been observed in human erythrocytes (40, 41).

Human erythrocytes release adenosine triphosphate (ATP) in response to low oxygen tension, which can act directly as a vasodilator and cause the release of endothelium-derived vasodilatory substances, such as nitric oxide, thus increasing blood flow to areas of high oxygen demands (9). Exposure to physiological levels of insulin in the absence of C-peptide inhibits low oxygen-induced ATP release from healthy and diabetic human erythrocytes (39–41), and thus may contribute to deficits in blood flow observed in diabetes (9). Interestingly, exposure to C-peptide in the absence of insulin similarly inhibits low oxygen-induced ATP release from erythrocytes (40). However, when erythrocytes are exposed to both insulin and C-peptide in ratios observed in the plasma of healthy humans (1:1 to 1:5.5, C-peptide:insulin), low oxygen-induced ATP release is restored (39, 41). Incubation with ratios of C-peptide to insulin above or below this range results in deficient ATP release from both healthy and diabetic human erythrocytes (39, 41). Importantly, C-peptide-mediated rescue of insulin-induced inhibition of low oxygen-induced ATP release is blocked by PKC-α inhibitors (39). These data indicate that, not only are C-peptide and insulin produced, packaged, and released together, but they share similar cellular targets and act together to modulate intracellular signaling of those target cells.

What is the Physiological Significance of C-Peptide?

No physiological or pathophysiological situation exists in which cells in vivo are exposed to C-peptide in the absence of insulin, and thus the precise role of C-peptide in normal physiology has been difficult to ascertain. Because C-peptide is essential for proper proinsulin processing (7, 21), experimental knockout models of C-peptide deficiency are not available. However, Type 1 diabetic patients offer a natural experiment that provides essential clues as to the physiological significance of C-peptide. Type 1 diabetes is characterized by a loss of β-cell mass and function, usually due to autoimmune destruction of the pancreatic β-cells (46, 52). Although both insulin and C-peptide secretion are lost in Type 1 diabetics, patients receive insulin therapy without C-peptide replacement. In these patients, loss of C-peptide does not appear to produce any immediate deleterious effects. However, most Type 1 diabetics develop microvascular dysfunction, such as retinopathy, neuropathy, nephropathy, or impairments in wound healing (31, 46, 52) at some point in their lives, usually a decade or more following disease onset, suggesting that C-peptide may play a role in the long-term maintenance of endothelial function. Type 1 diabetes certainly is not a perfect C-peptide "knockout" model, since insulin replacement therapy does not model perfectly endogenous insulin secretion and since many Type 1 diabetic patients retain some residual β-cell activity for years following diagnosis and consequently exhibit varying plasma levels of C-peptide. Interestingly, higher levels of residual plasma C-peptide correlate positively and significantly with a decrease in the incidence of microvascular disease and endothelial dysfunction in Type 1 diabetic humans (3, 27). Furthermore, C-peptide replacement in Type 1 diabetics reverses microvascular dysfunction leading to neuropathy (8), providing further evidence of C-peptide's role in normal endothelial function.

The precise role of C-peptide in endothelial biology has been difficult to dissect, since many in vitro studies exclude insulin from the treatment media, and the functional interaction between

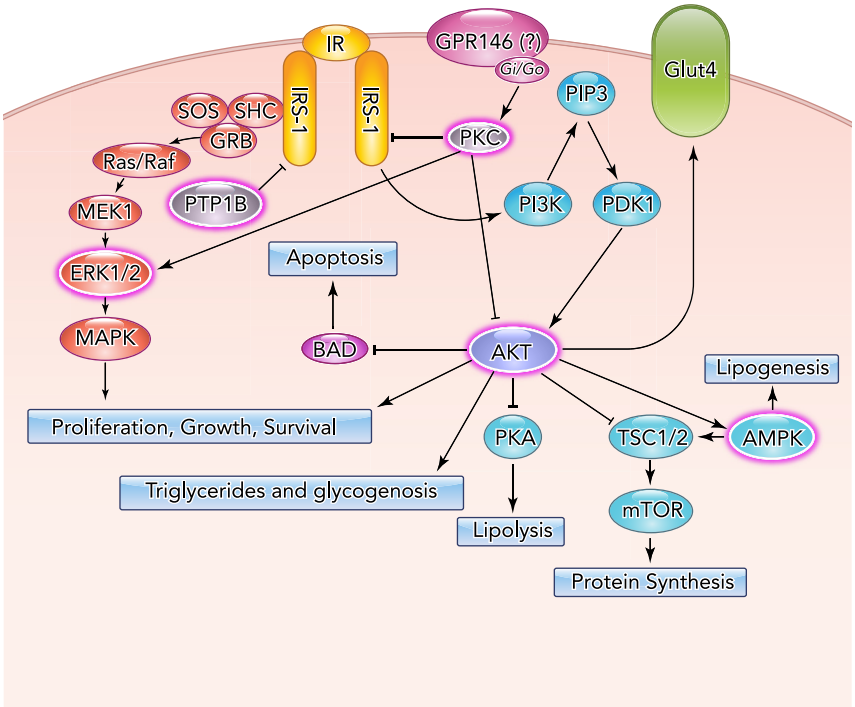

FIGURE 2. **Potential interactions between C-peptide- and insulin-initiated signaling cascades**
Insulin binds to the insulin receptor and exhibits downstream effects through the PI3K stimulation of the AKT and AMPK pathways as well as the ERK1/2 pathway. All three of these pathways responsible for multiple metabolic events, cell proliferation, growth, and survival are also reportedly targets of C-peptide actions either directly or through PKC. Direct interaction with PTP1B has also been proposed, which itself is a negative regulator of IRS-1. Points of interaction between C-peptide- and insulin-initiated signaling cascades are highlighted in pink.

C-peptide and insulin may be important for the normal activity of both hormones. However, C-peptide appears to exert a number of actions directly and indirectly on the endothelium through modulation of red and white blood cell activity (FIGURE 3). In the endothelium, C-peptide has been shown to prevent the formation of reactive oxygen species (ROS) (1, 2, 6, 30) via modulation of AMPK activity (2). C-peptide also enhances endothelial production of the gaseous vasodilator nitric oxide (NO) (31, 49), likely through activation of Akt (31). C-peptide was shown to inhibit leukocyte adhesion to the endothelium (43) through a decrease in MCP-1 secretion and VCAM-1 expression in endothelial cells, which attract and bind leukocytes to the endothelium, respectively (32). In addition, C-peptide exerts anti-apoptotic effects in endothelial cells by inhibiting caspase3 activation and stimulation of the anti-apoptotic protein, BCL-2 (6).

In addition to direct actions in the endothelial cells, C-peptide modulates endothelial biology indirectly through actions in the erythrocytes and the white blood cells as well. In the erythrocytes, C-peptide interacts with insulin to normalize ATP release via a PKCα-dependent mechanism (39). Erythrocyte-derived ATP stimulates NO production from endothelial cells, enhancing blood flow to regions of high oxygen demand (13). Although some groups have reported that C-peptide enhances monocyte chemotaxis and infiltration (35, 48), most studies have shown that C-peptide inhibits leukocyte adhesion to the endothelium (15, 43), potentially through modulation of NFkB signaling (15). Additionally, C-peptide reduces inflammatory cytokine release from LPS-stimulated monocytes (15), which complements the anti-inflammatory effects of C-peptide on the endothelium.

Proposed Model

In general, C-peptide appears to exert anti-inflammatory, anti-apoptotic, vasodilatory, and anti-oxidant effects directly and indirectly on the vascular endothelium, and, intriguingly, these effects are dependent on activation of signaling mechanisms that are usually associated with insulin receptor activity. Together with the observation that the combination of C-peptide with insulin results in a

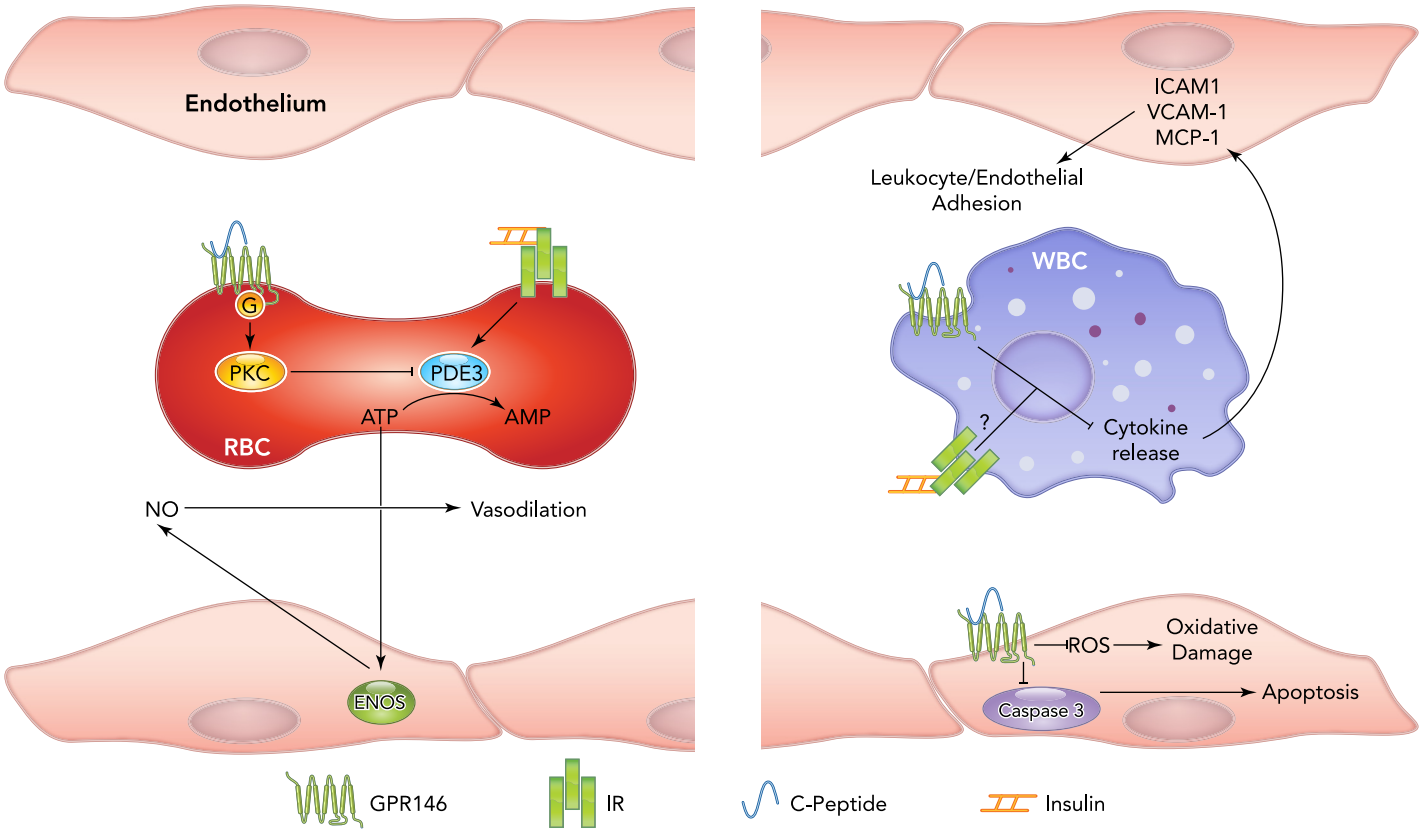

FIGURE 3. **Roles of C-peptide in endothelial physiology**
C-peptide and insulin are responsible for competing as well as complementary activities in the vascular system. C-peptide and insulin have opposing actions on the activity of PDE3 in the red blood cell (RBC), leading with the appropriate molar ratio to increased ATP release, which has both direct and indirect effects on vasodilation. Inhibitory actions of both C-peptide and insulin decrease cytokine release from leukocytes (WBC) that result in decreases in endothelial expression of cell adhesion molecules needed for WBC margination, rolling, and migration out of blood vessels. In addition, C-peptide exerts direct effects on endothelial cells that result in decreased formation of reactive oxygen species (ROS) and activity of caspase 3, which results in an anti-apoptotic effect.

biological output that is different from that of either peptide alone (39–41), these findings suggest that C-peptide plays a physiologically relevant role in the modulation or tuning of insulin signaling. While some studies have shown that certain actions of C-peptide are pertussis toxin sensitive, and therefore dependent on coupling of the C-peptide receptor to $G\alpha_{i/o}$, exposure to C-peptide also activates signaling molecules that are not usually associated with $G\alpha_{i/o}$-coupled GPCRs, such as PKC, which is usually associated with GPCRs coupled to $G\alpha_q$ (50). This discrepancy could be explained by G-protein promiscuity. In other words, some GPCRs, including the oxytocin receptor, have been reported to switch G-protein coupling based on ligand concentration or changes in the external or internal cellular environment (5, 17, 44). We propose that, in the setting of low insulin, the C-peptide receptor, presumably GPR146, couples to $G\alpha_{i/o}$ and that activation of GPR146 by C-peptide leads to enhanced MAPK and subsequent Akt signaling through a Gβγ-dependent signaling cascade, thus mimicking insulin signaling mechanisms. However, in the setting of high insulin, we propose that GPR146 switches G-protein affinity to $G\alpha_q$, which can lead to the downstream activation of PKC and inhibition of insulin signaling via serine/threonine phosphorylation of IRS-1 (FIGURE 4). G-protein coupling to the C-peptide receptor would exist in a continuum based on the ratio of insulin and C-peptide in the extracellular space. Loss of adequate C-peptide levels, as in Type 1 diabetes, would eliminate this tuning of insulin signaling, which, presumably, would not result in immediate deleterious effects but over time could lead to an accumulation of cellular defects (due to the unopposed actions of insulin) and ultimately to endothelial and subsequent microvascular dysfunction.

Summary

In summary, C-peptide likely interacts with a GPCR, perhaps GPR146, to exert anti-inflammatory, anti-apoptotic, vasodilatory, and anti-oxidant effects in the vascular endothelium through both direct actions on the endothelial cells and indirectly through interaction with erythrocytes and immune cells. We propose that C-peptide plays a physiologically relevant role in the tuning of insulin signaling and that the C-peptide receptor switches G-protein affinity based on relative concentrations of insulin and C-peptide in the extracellular space. Thus loss of C-peptide could have important implications for endothelial physiology and could account for the microvascular dysfunction observed in diabetes. ■

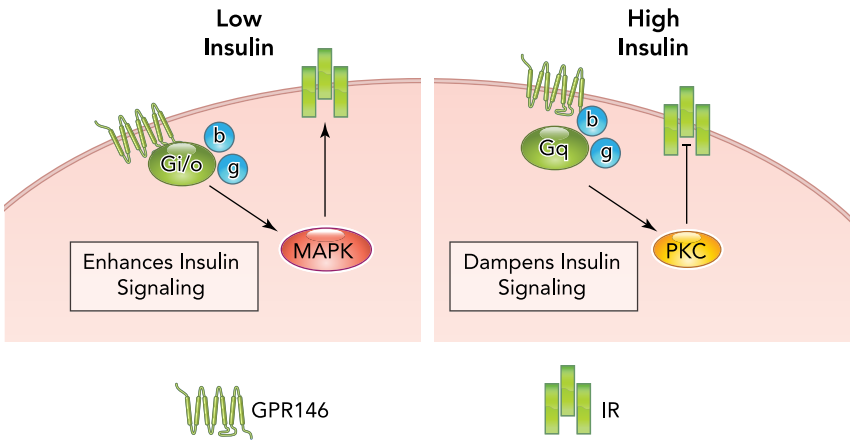

FIGURE 4. **Proposed model for C-peptide-mediated tuning of insulin signaling**
In low insulin conditions (*left*), the C-peptide receptor couples to Gαi/o, and C-peptide binding leads to enhanced activation of insulin-related MAPK signaling pathways (including downstream Akt activation). In high insulin conditions (*right*), the C-peptide receptor couples to Gαq, and C-peptide binding leads to the downstream activation of PKC, followed by subsequent dampening of insulin-related signaling cascades through serine/threonine phosphorylation of IRS-1.

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