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Insulin-like actions of glucagon-like peptide-1: a dual receptor hypothesis

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GLP-1 (9-36)amide is the cleavage product of GLP-1(7-36) amide, formed by the action of diaminopeptidyl peptidase-4 (Dpp4), and is the major circulating form in plasma. Whereas GLP-1(7-36)amide stimulates glucose-dependent insulin secretion, GLP-1(9-36)amide has only weak partial insulinotropic agonist activities on the GLP-1 receptor, but suppresses hepatic glucose production, exerts antioxidant cardioprotective actions and reduces oxidative stress in vasculature tissues. These insulin-like activities suggest a role for GLP-1 (9-36)amide in the modulation of mitochondrial functions by mechanisms independent of the GLP-1 receptor. In this paper, we discuss the current literature suggesting that GLP-1(9-36)amide is an active peptide with important insulin-like actions. These findings have implications in nutrient assimilation, energy homeostasis, obesity, and the use of Dpp4 inhibitors for the treatment of diabetes.

Multiple glucagon-like peptides modulate glucose metabolism

Glucagon-like peptide-1 (GLP-1) is a glucoincretin hormone produced in the enteroendocrine cells of the intestines, and is secreted in response to feeding. It is one of several peptide hormones cleaved from the precursor protein, proglucagon. Other cleavage products include glucagon and the glucagon-like peptides 1 and 2 (Figure 1). Glucagon is the major hormone produced by the α -cells of the pancreatic islets during fasting conditions, whereas the GLPs are produced by the intestine in response to feeding [1]. GLP-2 promotes growth of the intestinal mucosa [2], whereas glucagon promotes hepatic glucose production (gluconeogenesis) during the postabsorptive period [3]. GLP-1 is best known as a potent insulinotropic hormone that stimulates glucose-dependent insulin secretion [1,4]. Both GLP-1 agonists and inhibitors of the enzyme diaminopeptidyl peptidase-4 (Dpp4) (which inactivates the insulinotropic actions of GLP-1) are approved and in use for the treatment of type 2 diabetes [4]. It is now becoming understood, however, that GLP-1 also has insulin-like (insulinomimetic) actions independent of its insulinotropic actions, which is the focus of this article (see also an informative recent review of the extra-pancreatic actions of GLP-1 [5]).

The GLP-1 hormones consist of a complex family of peptides of 28–37 amino acids in length, formed by multiple post-translational enzymatically mediated cleavages by diaminopeptidyl peptidases and endopeptidases, and C-terminal amidation by peptide amidating monooxygenases (Figure 2). Much of the current literature in the field considers that these cleavages represent degradation pathways for inactivation and disposal of GLP-1 [6,7]. Alternatively, the temporal, successive enzymatic cleavages of GLP-1 might importantly modify its biological activities, such as changing its function from an insulin-releasing hormone to an insulinomimetic hormone that acts on insulin-sensitive target tissues to facilitate nutrient assimilation and metabolism.

In this paper we propose a dual receptor hypothesis of GLP-1 actions, whereby the initial GLP-1 isopeptide acts on cell surface receptors on pancreatic β -cells to augment glucose-dependent insulin secretion. The initial insulinotropic peptide is rapidly modified by removal of two amino acids, allowing access of the modified GLP-1 to a translocation receptor that transports GLP-1 across the plasma membrane into the cell, where additional cleavages by endopeptidases release small peptides of 5–9 amino acids from the C-terminus of GLP-1. These small peptides target the mitochondria to modulate oxidative phosphorylation, involving fatty acid and glucose metabolism, energy expenditure, and programmed cell death (apoptosis). By this mechanism, GLP-1 has a distinct dual role in nutrient metabolism: first, to stimulate pancreatic insulin secretion and second, to enhance nutrient uptake and metabolism by peripheral insulin-sensitive tissues.

Insulinotropic actions of GLP-1

The insulinotropic actions of GLP-1 were discovered 2 decades ago and have been thoroughly described in numerous review articles [1,8]. In brief, the amino-terminally extended insulinotropic hormones, GLP-1(7-37) and GLP-1(7-36)amide, rapidly stimulate glucose-dependent insulin secretion within 1 minute after their systemic administration into animals or humans, working via G-protein-coupled receptors on pancreatic insulin-producing β -cells. GLP-1 receptors (GLP-1Rs) are present in several organs including the pancreatic islets, heart, lungs, skin (hair follicle), stomach, hypothalamus and brain stem. In addition to stimulating insulin secretion, GLP-1R activation enhances growth and survival of β -cells, inhibits

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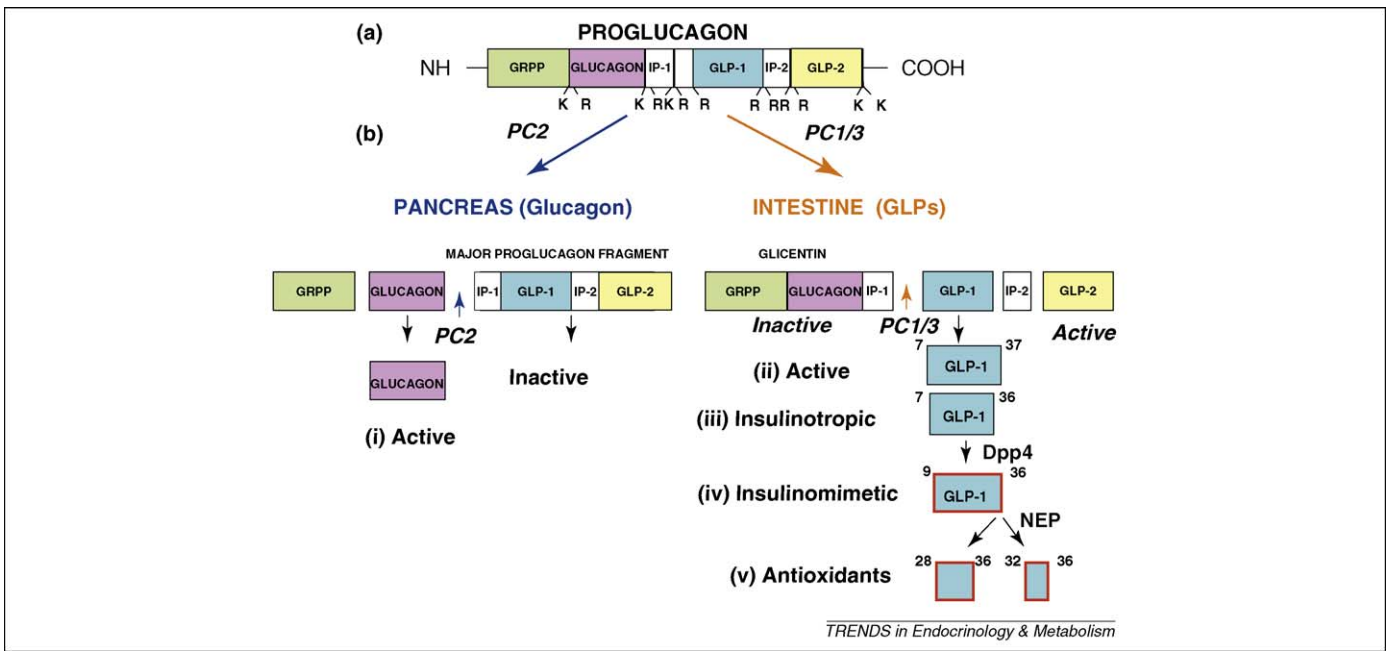


Figure 1. Proglucagon is a protein precursor of glucagon and GLPs. (a) The multifunctional proglucagon is cleaved by (b) site-selective proteases (PC2, PC1/3) in the pancreas to liberate glucagon as the active peptide and GLP-1 in the intestine. The prohormone convertases PC2 and PC1/3 selectively cleave proglucagon to produce (i) glucagon and (ii) GLPs, respectively. (iii) The insulinotropic GLP-1 peptides are GLP-1(7-36)amide and a glycine-extended form GLP-1(7-37). They are released from the intestine in response to feeding and stimulate glucose-dependent insulin secretion. These insulinotropic GLP-1s are rapidly converted after their secretion to (iv) insulinomimetic hormones GLP-1(9-36)amide and GLP-1(9-37) by removal of the N-terminal two amino acids by Dpp4. These might be further cleaved by NEP to produce (v) small C-terminal peptides; a nonapeptide and pentapeptide, GLP-1(28-36)amide and GLP-1(32-36)amide, and the corresponding decapeptide and hexapeptide GLP-1(28-37) and GLP-1(32-37). It is proposed that these small peptides might target mitochondria and modulate oxidative phosphorylation, glucose and fatty acid metabolism, and energy expenditure, resulting in attenuation of oxidative stress (ROS formation) and promotion of cell survival.

gastric emptying and glucagon secretion, has a cardioprotective function and promotes satiety [8]. The GLP-1R is coupled to G_s and activates signal transduction pathways that include cAMP/protein kinase A (PKA), phosphoinosi-

tol 3 kinase (PI3K)/Akt and mitogen-activated protein kinase kinase (MEK)/extracellular regulated kinase (ERK).

Several GLP-1R agonist-based therapies are currently approved and in use for the treatment of type 2 diabetes [4,9]. Activation of cAMP in β -cells by GLP-1 appears to act synergistically with glucose metabolism in increasing insulin secretion and renders β -cells competent to respond to glucose [10]. Glucose is required for GLP-1-mediated stimulation of insulin secretion, the so-called 'glucose competence concept' [10].

Selective enzymatic cleavages of GLP-1 into peptides with novel bioactivities

Of considerable importance is the finding that the insulinotropic GLP-1s have a very short existence in the circulation. They are rapidly ($t_{1/2} = 1-2$ min) cleaved after their secretion into the circulation by Dpp4, resulting in the production of the amino-terminally shortened peptides, GLP-1(9-37) and GLP-1(9-36)amide, which have weak, if any, insulin-releasing activities [11]. GLP-1(9-37) and GLP-1(9-36)amide are the major circulating forms of GLP-1, comprising about 80–90% of the total circulating GLP-1s [12], with a half-life of 8–10 minutes. In studies of GLP-1 secretion from the pig intestine, approximately 60% of GLP-1 released exists in the form of GLP-1(9-36)amide as a result of rapid cleavage of GLP-1(7-36)amide by Dpp4 located on vascular endothelial cells at the site of secretion from L-cells [13]. Because levels of total GLP-1 in the circulation are only in the range of 100 pmol/L, the active insulinotropic hormones GLP-1(7-37) and GLP-1(7-36)amide are remarkably low (10 pmol/L), indicative of a relatively high affinity of the GLP-1 receptor for

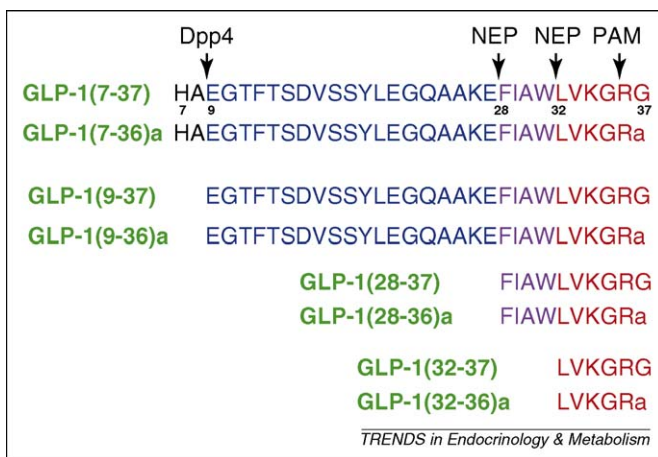


Figure 2. Amino acid sequences of GLP-1 and subpeptides generated by selective modifications by specific proteases. The parent insulinotropic peptide is cleaved out from the proglucagon precursor by the actions of prohormone convertase type 1/3. The carboxyterminus of GLP-1 is modified at the time of synthesis by the actions of peptide amidating monooxygenase (PAM), which removes the carboxyterminal glycine (G) and amidates the penultimate arginine (R). Within minutes after their secretion into the circulation, the GLP-1(7-36)amide and GLP-1(7-37) are modified by removal of the aminoterminal amino acids, histidine (H) and alanine (A) (black font) by Dpp4. Cleavage by Dpp4 yields the insulinomimetic peptides, GLP-1(9-36)amide and GLP-1(9-37). It is proposed that additional cleavages within the carboxyterminal domains of the insulinotropic peptides by NEP, such as the selective cleavages by NEP 24.11, generates small penta and hexa- (red font) to nona and deca-peptides (purple + red font) that target mitochondria and thereby modulate oxidative phosphorylation, energy expenditure and apoptosis.

interactions with the insulinotropic GLP-1s. That GLP-1(9-37) and GLP-1(9-36)amide peptides make up the majority of circulating GLP-1 hormone and are relatively stable suggests that they have biological activities apart from stimulating insulin secretion.

The neutral endopeptidase NEP 24.11 (also known as neprilysin, CD10 and CALA antigen [14]) is another endopeptidase, distinct from Dpp4, that is implicated in GLP-1 degradation. NEP 24.11 cleaves GLP-1 at several internal sites amino-proximal to bulky hydrophobic amino acids such as phenylalanine and leucine [15]. NEP 24.11 is predominantly a membrane-associated enzyme that is expressed in the central nervous system, at high levels in hepatocytes on the surfaces of bile caniculi and in other organs [16]. Therefore, an intracellular form of NEP 24.11, or an endopeptidase with similar specificity, might be responsible for internal cleavage of GLP-1. Similar to Dpp4, NEP 24.11 is currently considered to function as a degrading enzyme for the destruction and disposal of GLP-1 [6].

Insulin-like and cytoprotective actions of GLP-1(7-36)amide and GLP-1(7-37)

Although it is difficult to detect insulin-like effects of the insulinotropic GLPs, GLP-1(7-37) and GLP-1(7-36)amide, because of their insulin-releasing activities, several studies have suggested that GLP-1 has insulin-like actions on peripheral glucose uptake and metabolism independent of its effects on insulin secretion and changes in plasma insulin levels. Egan and co-workers [17] showed that hepatic glucose uptake (Rd) was greater in response to GLP-1(7-37)-induced insulin release than to insulin given by infusion in obese insulin-resistant human subjects. Notably, a similar study in lean insulin-sensitive subjects showed no effects of GLP-1(7-37) on Rd [18] compared with insulin infusion. Subsequent studies in obese insulin-resistant *ob/ob* mice using GLP-1(7-37) or the long-acting GLP-1 agonist exendin-4 revealed inhibition of hepatic glucose production (gluconeogenesis) [19] and reduction in hepatic steatosis [20], respectively. A single injection of an adenovirus exendin-4 expression vector to diet-induced obese mice decreased weight gain without detectable changes in food intake, increased energy expenditure and enhanced insulin action as shown by reductions in hepatic fat and enzymes involved in gluconeogenesis and fatty acid synthesis [21]. These findings of Sampson and co-workers are similar to those reported by Ding *et al.*, showing that administration of exendin-4 to *ob/ob* mice reduced steatosis and increased the expression of hepatic enzymes involved in fatty acid oxidation [20].

In isolated rat hepatocytes *ex vivo*, GLP-1(7-36)amide activates the activity of glycogen synthase-a, proposed to be via PI3 kinase and Akt signaling because the activation by GLP-1 was inhibited by wortmannin, a fungal metabolite that inhibits PI3 kinase [22]. This study suggests that GLP-1 acts on the liver by receptor mechanisms that are distinct from those that mediate the insulinotropic actions on pancreatic β -cells, but cannot definitively exclude the possibility that these peripheral extrapancreatic actions of GLP-1 are attributable to

small changes in plasma insulin levels stimulated by insulinotropic GLP-1s.

Evidence for direct GLP-1(9-36)amide actions on the heart

One of the first observations of direct effects of GLP-1(9-36)amide on the heart was made by Nikolaidis and co-workers [23], who infused GLP-1(9-36)amide into dogs with severe heart failure caused by experimentally induced rapid pacing that resulted in dilated cardiomyopathy. These dogs were severely stressed and hyperinsulinemic. Direct infusion of GLP-1(9-36)amide rapidly reversed the heart failure, improved end diastolic pressure, and markedly stimulated myocardial glucose uptake without changing plasma insulin levels. These findings are indicative of enhanced glycolysis (glucose oxidation) [23]. Cardioprotective effects of GLP-1(9-36)amide were also demonstrated in mice with experimentally induced ischemic injury and postischemic-reperfusion injury [24]. GLP-1(9-36)amide perfused in isolated mouse hearts with ischemic myocardial damage improved cardiac function, increased vasodilatation and coronary blood flow and reduced the extent of ischemia-induced injury to the myocardium. These beneficial effects of GLP-1(9-36)amide in the heart were accompanied by demonstrable increases in myocardial cGMP, inducible nitric oxide synthase activity and nitric oxide generation, consistent with an antioxidant action and reduction in reactive oxygen species (ROS) [24]. Remarkably, many of the improvements in the murine myocardium produced by GLP-1(9-36)amide were seen in conditions of complete receptor blockade, for example, in the hearts of *GLP-1R* null mice, and in mice co-infused with GLP-1(7-36)amide and the GLP-1R antagonist exendin(9-39) [24,25]. Similarly, Sonne *et al.* [26] tested the effects of both the GLP-1 agonist, exendin-4 and GLP-1(9-36)amide on postischemic-reperfusion injury in the isolated rat heart and found that both peptides augmented left ventricular performance and were only partially inhibited by the GLP-1R antagonist exendin(9-39).

These findings in ischemic hearts under conditions of increased oxidative stress lead to the conclusion that GLP-1 exerts important biologic actions on the ischemic heart, independent of those mediated by GLP-1R, suggesting the existence of novel mechanisms of actions of GLP-1(9-36)amide in this tissue. These antioxidant cardiac actions of GLP-1(9-36)amide are reminiscent of those of SS (Szeto-Schiller) peptides derived by modifications of carboxyterminal sequences of cholecystokinin with opioid agonist activities [27]. The SS peptides consist of a family of tetrapeptides of the general structure H-B-H-B, where H is a bulky hydrophobic amino acid and B is a basic amino acid, either arginine or lysine. SS peptides readily enter cells and target mitochondria to inhibit ROS formation and apoptosis, which can protect against ischemia-reperfusion-induced myocardial stunning in guinea pigs [28] and rats [29]. It is tempting to speculate that if GLP-1 can enter target cells by endocytosis or other mechanisms, the C-terminal peptides LVKGR and LVKGRG, potentially derived by cleavages by an intracellular form of the neutral endopeptidase NEP24.11 [15], or an enzyme of similar specificity, might target mitochondria and serve as antioxidant peptides.

Evidence for direct actions of GLP-1(9-36)amide on vasculature

As has been found for the heart, GLP-1(9-36)amide also exerts potent antioxidant actions on the vasculature. Studies by Green and co-workers [30] demonstrate that the insulinotropically inactive peptides GLP-1(9-36)amide and exendin(9-39) (the latter a GLP-1R antagonist) and the agonists, GLP-1(7-36)amide and exendin-4 all have significant vasorelaxant properties on the isolated rat aorta. These relaxant actions of the GLP-1s appear to be mediated, at least in part, via cAMP and opening of ATP-sensitive potassium channels [30]. Remarkably, in studies carried out in rat femoral arterial rings (conduit arteries) *ex vivo*, both GLP-1(7-36)amide and GLP-1(9-36)amide produced a marked vasorelaxation response, whereas exendin-4 exerted no observable vasorelaxation activity [31]. The reason for these observed differences between the GLP-1 peptides and the long-acting GLP-1 agonist exendin-4 in promoting vasorelaxation remains unexplained [30]. Both exendin-4 and GLP-1(7-36)amide are full agonists and GLP-1(9-36)amide is a weak partial agonist on the GLP-1 receptor [11]. One speculation derived from these studies is that GLP-1(9-36)amide mediates vasorelaxation by a GLP-1 receptor-independent mechanism, and that the vasorelaxation activities observed with GLP-1(7-36)amide is a consequence of its cleavage to GLP-1(9-36)amide by ambient Dpp4 activity present in the arterial ring preparations. Exendin-4 is resistant to cleavage by Dpp4, an important property that enhances its insulinotropic actions. Furthermore, these actions of GLP-1 peptides on rat conduit arteries do not require insulinotropic activity because GLP-1(9-36)amide is devoid of insulinotropic activities and exendin-4 is a potent insulinotropic peptide [30].

Collectively, these studies in vasculature tissues clearly demonstrate actions of GLP-1(9-36)amide on vascular cell metabolism independent of the GLP-1 receptor, suggesting that novel GLP-1R-independent mechanisms mediate these actions.

Evidence for direct GLP-1(9-36)amide actions on liver

As described above, both GLP-1(7-37) and GLP-1(7-36)amide suppress hepatic glucose production (HGP) in obese, insulin-resistant mice and humans. However, it is assumed that these actions are attributable to GLP-1(9-36)amide, based on the known actions of Dpp4 to rapidly modify GLP-1(7-36)amide to the GLP-1(9-36)amide metabolite. Recently, studies have shown that infusion of GLP-1(9-36)amide in obese, insulin-resistant human subjects under fasting and glucose clamp conditions dramatically lowers hepatic glucose production by up to 50%, beginning 15–20 minutes after the start of the infusions [32]. This remarkable inhibition of hepatic glucose production was observed in the obese, insulin-resistant subjects, but not in the lean, insulin-sensitive subjects [32]. It is worth noting that an earlier study of the infusion of GLP-1(9-36)amide into healthy non-obese, presumably insulin-sensitive subjects also produced no effects on insulin release or on glucose metabolism [33]. The delay of 15–20 minutes before the onset of the suppression of HGP by GLP-1(9-36)amide in obese, insulin-resistant subjects in the post-absorptive state

strongly suggests that GLP-1(9-36)amide exerts actions on intermediary metabolism. It is well known that plasma glucose levels are maintained by hepatic gluconeogenesis in the post-absorptive state, after glycogen stores are depleted. Because conditions of fasting and insulin resistance limit the availability of glucose for oxidation as an energy source, energy production occurs predominantly from fatty acid oxidation. The glucose metabolism and fatty acid metabolism cycles are known to be reciprocally coupled [34]. Thus, a reduction of gluconeogenesis induced by GLP-1(9-36)amide is possibly a consequence of inhibition of fatty acid oxidation. The major factors that fuel gluconeogenesis in the liver are acetylcoenzyme A (coA) (derived from fatty acid oxidation) and glycerol (derived from lipolysis). Therefore, it seems reasonable to speculate that a target action of GLP-1(9-36)amide on the suppression of gluconeogenesis might occur at the level of the inhibition of fatty acid oxidation (beta oxidation) that takes place in the matrix of mitochondria.

The suppression of hepatic glucose production by infusion of GLP-1(9-36)amide might not be due entirely to a direct effect of the peptide on hepatocytes, but rather to effects mediated by actions of the peptide on the autonomic nervous system. Recent evidence suggests that GLP-1 receptors exist on vagal afferent nerve terminals in the portal vein. Studies in rats [35] and dogs [36] indicate that GLP-1 infusions into the portal vein enhance hepatic glucose uptake and production; these effects are reversed by the administration of exendin9-39, a specific GLP-1 receptor antagonist. Moreover, the direct administration of GLP-1 centrally into the arcuate nucleus of the hypothalamus in rats augmented glucose-stimulated insulin secretion and reduced hepatic glucose production, and administration of the antagonist exendin9-39 caused relative hyperglycemia [37]. These studies, however, do not address actions of the insulinomimetic GLP-1(9-36)amide, which is only a weak partial agonist of the GLP-1 receptor [11] and unlikely to act on the GLP-1 receptors in the portal vein and hypothalamus. If the GLP-1(9-36)amide used in the studies reported by Elahi *et al.* [32] reduced hepatic glucose output via a central effect, it probably does not involve GLP-1 receptors. Indeed, there is evidence that the intraportal GLP-1 actions on glucose uptake and metabolism through the hepatic vagal nerve are mediated by GLP-1 receptors, although the evidence is conflicting. Intraportal injections of the GLP-1 agonist exendin-4 had no effect, and the GLP-1 receptor antagonist exendin(9-39) failed to modify GLP-1-stimulated afferent vagal nerve activity in rats [38]. Moreover, GLP-1 administered either via the hepatic artery or portal vein in dogs increased hepatic glucose uptake equivalently, suggesting that the increased hepatic glucose uptake does not involve GLP-1 receptors located in the portal vein [39]. Based on the sum of the current evidence, it seems reasonable to hypothesize that at least some of the actions of GLP-1 on glucose uptake and metabolism in the liver occurs via mechanisms that are independent of the GLP-1 receptor. An alternative, as yet unidentified, receptor-independent mechanism might be involved in mediating the actions of GLP-1 on hepatic glucose metabolism.

Enigma of GLP-1 receptor expression in hepatocytes

Whether or not the GLP-1R is expressed in hepatocytes remains an enigma, with some studies reporting GLP-1R expression in rat [20,40] and human [41,42] hepatocytes, whereas others report no GLP-1R expression in rat [43,44], mouse [45], or human [42,46] hepatocytes. Of interest, not only did Flock and co-workers [45] not detect GLP-1R mRNA transcripts by RT-PCR analysis of RNA isolated from cultured murine hepatocytes, but they also were unable to detect any stimulation of cAMP formation after incubation of murine hepatocytes with GLP-1 or exendin-4. Notably, both glucagon and forskolin significantly increased levels of hepatocyte cAMP in the same experiments [45].

A recent paper by Aviv and co-workers [42] describes the activation of cAMP formation, CREB, PKB and ERK1/2 with burst-decay kinetics within 5–15 minutes after the addition of exendin-4 to isolated human hepatocytes. These remarkable findings of the activation of cAMP formation by exendin-4 are reminiscent of those reported by Ding *et al.* [20] in isolated rat hepatocytes. Notably, however, Aviv *et al.* failed to detect GLP-1 receptor expression in human hepatocytes before their trans-differentiation into pancreatic β -cells [42]. Such an observed burst-decay response of cells to a peptide hormone is a representative signature response of a G_s -coupled receptor, and suggests that the actions of exendin-4 on hepatocytes are mediated by a G-protein-coupled receptor other than GLP-1R. Candidate alternative receptors could be members of the family of structurally related receptors for the glucagon superfamily of peptide hormones. It is tempting to speculate that such a receptor might even be the glucagon receptor itself. Because the glucagon receptor is structurally similar to the GLP-1 receptor and is abundantly expressed in hepatocytes, these findings raise the intriguing possibility that exendin-4, a GLP-1 receptor agonist, activates the glucagon receptor in liver. This theoretical activation of glucagon receptors by exendin-4 might in some way depend on the metabolic state of the hepatocytes. A further speculation is that the amino-terminally truncated GLP-1 peptide, GLP-1(9-36)amide, might act as an antagonist, or even as an inverse agonist, of the glucagon receptor in the liver. If so, this circumstance could offer an explanation for the suppression of hepatic glucose production (gluconeogenesis) in response to infusions of GLP-1(9-36)amide in human subjects reported by Elahi and co-workers [32]. Of note are the findings that the actions of GLP-1(9-36) in suppressing hepatic glucose production in human subjects were manifested in obese, insulin-resistant subjects but not in lean, insulin-sensitive subjects [32] suggesting that some unknown alteration of the hepatocytes (e.g. steatosis) in obese people might be a prerequisite for the inhibitory actions of GLP-1(9-36)amide on the glucagon receptor and the consequent inhibition of gluconeogenesis.

In summary, there appears to be a clear effect of GLP-1 on peripheral glucose metabolism, independent of its insulinotropic actions. Some of these actions on liver appear to be mediated through the vagal nerve terminals in the portal vein, centrally in the hypothalamus or directly in liver. Notably, actions of GLP-1 on vagal nerve terminals in the portal vein seem to be mediated by GLP-1 receptor-

independent mechanisms. It is important to note that all of these mechanisms of GLP-1 actions on glucose metabolism, on hepatocytes directly and on vagal nerve terminals in the portal vein, as well as central actions in the hypothalamus, are not necessarily mutually exclusive, and could also be shared by GLP-1 (9-36)amide.

Mechanisms of insulin-like actions of GLP-1 on liver, heart and vasculature: A hypothetical model

Most evidence regarding the insulinomimetic actions of GLP-1 on the heart, liver and vasculature indicates that both GLP-1R dependent and independent mechanisms are involved. Moreover, it seems that insulin-like actions of GLP-1 on liver, heart and vasculature could be mediated at the level of mitochondrial functions such as regulation of oxidative phosphorylation, ROS formation, gluconeogenesis and fatty acid oxidation. Mitochondria are complex intracellular organelles with their own genome whose primary roles are to regulate energy production and utilization and modulate apoptosis [47]. Two hypothetical mechanisms are proposed, based on the cumulative evidence supporting insulin-like actions of GLP-1 on insulin-sensitive tissues, independent of its insulinotropic actions (Figure 3). One mechanism is mediated by GLP-1(7-36)amide binding to GLP-1R, activating downstream signaling pathways such as cAMP/PKA and PI3K/Akt. Both PKA and Akt target mitochondria and regulate apoptosis

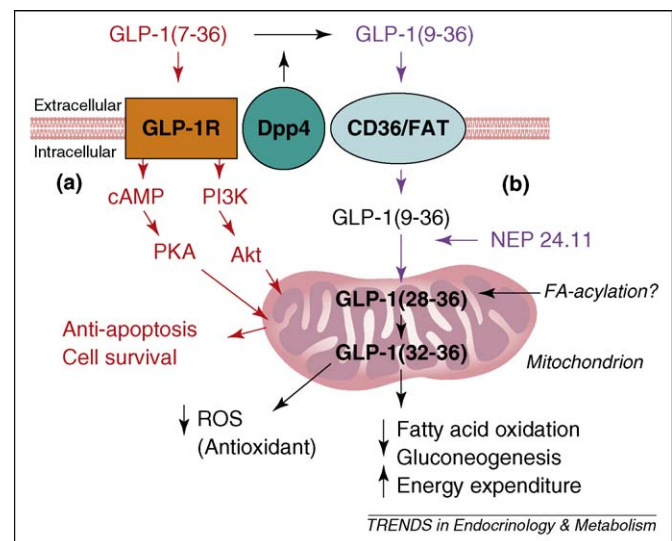


Figure 3. Model depicting two hypothetical cell signaling pathways by which GLP-1 exerts insulinomimetic actions on insulin-sensitive target tissues. (a) In one mechanism, GLP-1(7-36)amide acts on its GPCR receptor, GLP-1R, to activate cAMP-dependent PKA and PI3K-dependent Akt, the pro-survival kinase. Both PKA and Akt are known to target mitochondria and modulate cytochrome and caspase-dependent apoptosis. In the absence of the expression of GLP-1R, it is hypothesized that some other G-protein-coupled receptor related to GLP-1R, such as the glucagon receptor, might convey signaling in response to GLP-1 agonists. (b) In the absence of GLP-1R (e.g. in the liver), GLP-1(9-36) binds to a novel translocator receptor, such as the pattern-recognition scavenger receptor CD36/FAT, which transports the peptide into the cell, where it is cleaved internally by selective endopeptidases (e.g. NEP 24.11), to liberate a small nonapeptide GLP-1(28-36)amide and a pentapeptide GLP-1(32-36)amide that target to mitochondria. The epsilon amino group on lysine-34 might be fatty-acylated by the fatty-acylCoA ligase. These small peptides are proposed to contain consensus mitochondrial targeting sequences, LVKGRamide or LVKGRG, and to have antioxidant actions by suppression of fatty acid oxidation and gluconeogenesis in the liver. Transport of these peptides into the matrix of the mitochondria might be facilitated by fatty acylation of the epsilon amino group on the lysine-34 by fatty-acylCoA ligase/synthase.

[47]. Activated PKA targets the cytoplasmic surface of mitochondria, resulting in phospho-inhibition of the pro-apoptotic protein BAD, enhancing cell survival [48]. Akt enhances cell survival by targeting mitochondrial membranes, and to some extent the matrix, where it inhibits GSK3, an important enzyme that enhances apoptosis by inhibiting mitochondrial pyruvate dehydrogenase, an enzyme important for glucose oxidation [49]. A caveat to the GLP-1R activation hypothesis is that the expression of the GLP-1R in hepatocytes remains controversial. In the absence of hepatic GLP-1R expression, it could be speculated that some other G-protein receptor expressed in hepatocytes is activated by GLP-1 agonists, such as the glucagon receptor or another receptor in the glucagon superfamily of peptides related to the GLP-1R.

The second mechanism proposed involves the binding of GLP-1(9-36) to a novel receptor such as, for a theoretical example, CD36/FAT, a pattern-recognition receptor that binds and transports peptides and oxidized low-density lipoproteins into cells [50–52] (Figure 3). In this mechanism, GLP-1(9-36)amide is transported into the cell, where it is hypothetically cleaved internally in the C-terminal region by an intracellular form of an endopeptidase, such as neutral endopeptidase (NEP) 24.11 (also known as neprilysin, CALLA, CD10 [14]) or an enzyme with similar specificity. Cleavage of GLP-1 by NEP 24.11 occurs between amino acids glutamate-27 and phenylalanine-28, yielding the nona-peptide FIAWLVKGRamide [GLP-1(28–36)] and the pentapeptide LVKGRamide [GLP-1(32–36)] [15]. The sequence LVKGRamide, or LVKGRG in the example of cleavage of the glycine-extended GLP-1(9-37), is highly reminiscent of several known mitochondrial targeting motifs. These motifs include RXXRRLRG determined by random screening [53]; the mitochondrial targeting sequence, LKTRV of the protein PB1-F2, produced by the influenza B virus upon infection of cells, where it modulates fatty acid oxidation and apoptosis [54]; the opioid agonist/antioxidant tetra-peptide, YRFR [27]; and the internal latent mitochondrial targeting sequence in NPY, KGLK, which targets internally translated, truncated NPY protein into mitochondria rather than into the endoplasmic reticulum and subsequent secretory pathway [55]. A second theoretical modification of the C-terminal nona- and penta-peptides derived from GLP-1(9-36)amide is that of fatty acylation of the epsilon amino group of lysine-34 by the mitochondrial enzyme fatty-acyl-CoA ligase/synthase that acylates lysines in histones [56]. Palmitoylation of peptides controls Golgi versus mitochondrial subcellular targeting [57].

In this hypothetical model, these small C-terminal peptides derived by directed proteolytic actions of NEP 24.11 might be transported across the outer and inner mitochondrial membranes into the matrix where the tri-functional protein and associated fatty-acylCoA dehydrogenases cleave fatty acids into the two carbon acetate fragments in the form of Acetyl-CoA (beta-oxidation). Perhaps the presence of a peptide at the site of beta-oxidation in the mitochondria would be inhibitory, because the enzymatic machinery is designed to cleave alkane bonds in fatty acids and not peptide bonds.

Based on these proposed mechanisms, the insulin-like actions of GLP-1(9-36)amide operate independently of the

insulin receptor kinase signaling network. In the liver, GLP-1(9-36)amide and/or the smaller peptides GLP-1(28-36)amide and GLP-1(32-36)amide might inhibit hepatic fatty acid oxidation and thereby inhibit gluconeogenesis. Uncontrolled fatty acid oxidation and corresponding gluconeogenesis in the liver promote oxidative stress, a condition believed to contribute to steatohepatitis and eventually to the development of hepatic cirrhosis [58,59]. The attenuation of fatty acid oxidation rates in the liver would benefit obese patients with metabolic syndrome, which consists of insulin resistance, hepatic steatosis, high rates of uncontrolled fatty acid oxidation and lipid turnover, and accompanying elevated hepatic glucose production. In the heart and vasculature, GLP-1(9-36)amide inhibits mitochondrial ROS formation and thereby acts as an antioxidant to promote the survival and functions of myocardium and vascular endothelium. Therefore, GLP-1(9-36)amide, and C-terminal peptides derived from it, attenuate oxidative stress and might have beneficial effects on the prevention or amelioration of lipotoxic cardiomyopathy [60].

Summary

GLP-1 is a multifunctional hormone secreted from the intestine into the circulation in response to feeding, initially as a potent insulinotropic hormone that stimulates glucose-dependent insulin secretion from the pancreas and then as an insulinomimetic hormone that helps insulin in the stimulation of nutrient uptake and utilization by peripheral organs. The insulinotropic hormones GLP-1(7-36)amide and GLP-1(7-37) are rapidly modified by enzymatic cleavage to form the insulinomimetic hormones GLP-1(9-36)amide and GLP-1(9-37) with insulin-like actions on insulin-sensitive tissues. In this manner, GLP-1 first helps deliver insulin from the pancreas into the circulation and then helps insulin in the assimilation and utilization of nutrients by peripheral organs such as the liver, heart, and vasculature. The insulinotropic actions of GLP-1(7-36)amide and GLP-1(9-37) on the β -cells of the endocrine pancreas are mediated by a G-protein coupled receptor (GLP-1R) that activates cAMP-dependent PKA and PI3 kinase-dependent Akt signal transduction pathways. However, the insulin-like actions of GLP-1(9-36)amide and GLP-1(9-37) appear to occur by way of GLP-1R-independent mechanisms, suggesting the existence of a novel pathway for the actions of insulinomimetic GLP-1s.

That GLP-1 modulates ROS formation in heart and vasculature suggests that its actions are manifested at the level of mitochondrial functions involving oxidative phosphorylation, such as fatty acid oxidation, glycolysis, and energy formation and expenditure. The discovery of insulin-like actions of GLP-1 raises interesting possibilities for the development of novel GLP-1-based therapies for treating obesity and accompanying manifestations of metabolic syndrome, including insulin resistance, excessive oxidative stress, hepatic steatosis, accelerated cardiovascular disease, and type 2 diabetes.

Future directions

The evidence supporting direct actions of GLP-1(9-36)amide on heart and vasculature appear convincing.

Box 1. Outstanding questions

Is the suppression of hepatic glucose production by GLP-1(9-36)amide a result of a direct effect of the peptide on hepatocytes or is it centrally mediated, for example, by receptors in the hypothalamus or nerve terminals in the portal vein? Are GLP-1(9-36)amide actions mediated through GLP-1 receptor, or by an alternative mechanism?

Could the expression of GLP-1 receptors be modulated by changes in the physiological state of the liver that facilitate the binding of GLP-1(9-36)amide?

Could some other G-protein coupled receptor, such as the glucagon receptor, serve as the hepatic GLP-1 receptor, via biochemical modification of the agonist and/or changes in the conformation of the receptor in liver?

Does GLP-1(9-36)amide suppress hepatic glucose production by modulating mitochondrial functions in hepatocytes as is implied for its actions on heart and vasculature?

Does GLP-1(9-36)amide, or peptides derived from it, mediate its mitochondrial actions of anti-oxidation and oxidative phosphorylation by signal transduction pathways conveyed by cell surface receptors or by its internalization into cells and direct targeting to mitochondria?

Does the endopeptidase NEP24.11, or similar endopeptidases, generate new bioactive peptides from GLP-1 in the target cells of its actions, or do endopeptidases simply degrade GLP-1 to inactive peptides?

Does the use of Dpp4 inhibitors for the treatment of type 2 diabetes impair important insulin-like actions of GLP-1 mediated by the cleavage product GLP-1(9-36)amide?

Based on the existence of insulinomimetic actions and absence of insulinotropic actions of GLP-1(9-36)amide, would it be a suitable therapy for the treatment of obesity-related diabetes when insulin resistance and hyperinsulinemia prevail? Would GLP-1(9-36)amide serve as an "insulin sensitizer" in pharmacologic doses, providing insulin-like actions in the presence of severe insulin resistance?

Studies have been carried out in isolated perfused heart and vascular tissues *ex vivo*. Such experimental models exclude the participation of centrally mediated pathways of hormone action. Furthermore, the studies suggest that the actions of GLP-1(9-36)amide are conveyed by GLP-1 receptor-independent mechanisms and involve the modulation of mitochondrial functions such as oxidative phosphorylation. However, additional studies are needed with the use of both *in vitro* myocardial cell and vascular endothelium systems to explore the cellular biochemistry and pathways by which GLP-1(9-36)amide exerts anti-oxidant actions and modulates oxidative phosphorylation. Studies of GLP-1(9-36)amide actions in isolated mitochondria would be most informative. The information currently available on potential actions of GLP-1(9-36)amide on liver are somewhat limited and as yet undefined. The demonstration of suppression of hepatic glucose production by infusion of GLP-1(9-36)amide in obese, insulin-resistant human subjects indicates that the peptide has biological activities in the liver and is not a circulating inactive degradation product of GLP-1 metabolism. However, these *in vivo* studies leave open the question of whether the actions of GLP-1(9-36) on the liver are direct actions on receptors in hepatocytes, and whether they are centrally mediated via hypothalamic receptors or neurally mediated by receptors on vagal afferent nerve fibers in the portal vein. It is possible that all of the aforementioned mechanisms are at play. Future studies of isolated hepatocyte systems are needed to address the question of whether

GLP-1(9-36)amide has direct effects on oxidative phosphorylation or intermediary metabolism, or any biochemical reactions for that matter (Box 1).

An additional question that remains unanswered at present is whether GLP-1 receptors are expressed in the liver. The evidence on this matter is controversial, in that GLP-1(9-36)amide binds only poorly to the GLP-1 receptor. This suggests that if receptors are involved in sensing GLP-1(9-36)amide actions on the liver, they must be novel receptors distinct from the known GLP-1 receptor. Moreover, it remains to be determined whether GLP-1(9-36) acts on putative novel receptors to convey signals to mitochondria or whether GLP-1(9-36)amide and/or peptides derived from it by further endopeptidase cleavages, enter hepatocytes and target the mitochondria. Likewise, the physiological state of the liver such as inflammation or lipid accumulation might condition the expression of GLP-1 receptors, novel or otherwise, and thereby activate downstream signaling mechanism that convey GLP-1 actions.

The discovery of the insulinomimetic actions of the GLP-1 metabolite, GLP-1(9-36)amide in heart, vasculature and possibly liver, raises implications regarding the use of Dpp4 inhibitors for the treatment of diabetes. Prevention of the formation of GLP-1(9-36)amide might ultimately augment oxidative stress in heart, vasculature, and liver, and promote hepatic glucose production – untoward effects that would be undesirable in the treatment of obesity-related diabetes. Potent, short-acting Dpp4 inhibitors taken at the time of meals might provide brief stimulation of insulin secretion by increasing levels of insulinotropic GLP-1(7-36)amide, allowing escape from Dpp4 inhibition and the intermittent production of insulinomimetic GLP-1(9-36)amide during interprandial periods. Discoveries in the area of insulin-like actions of GLP-1 derived peptides on insulin-sensitive tissues have occurred only recently and much is yet to be learned about the multiple actions of GLP-1-related peptide hormones.

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