Development of Glucagon-Like Peptide-1-Based Pharmaceuticals as Therapeutic Agents for the Treatment of Diabetes

Daniel J. Drucker*

Department of Medicine, Banting and Best Diabetes Centre, Toronto General Hospital, University of Toronto, Toronto, Canada

Abstract: Glucagon-like peptide-1 (GLP-1) is released from gut endocrine cells following nutrient ingestion and acts to regulate nutrient assimilation via effects on gastrointestinal motility, islet hormone secretion, and islet cell proliferation. Exogenous administration of GLP-1 lowers blood glucose in normal rodents and in multiple experimental models of diabetes mellitus. Similarly, GLP-1 lowers blood glucose in normal subjects and in patients with type 2 diabetes. The therapeutic utility of the native GLP-1 molecule is limited by its rapid enzymatic degradation by the serine protease dipeptidyl peptidase IV. This review highlights recent advances in our understanding of GLP-1 physiology and GLP-1 receptor signaling, and summarizes current pharmaceutical strategies directed at sustained activation of GLP-1 receptor-dependent actions for glucoregulation *in vivo*. Given the nutrient-dependent control of GLP-1 release, neutraceuticals or modified diets that enhance GLP-1 release from the enteroendocrine cell may exhibit glucose-lowering properties in human subjects. The utility of GLP-1 derivatives engineered for sustained action and/or DP IV-resistance, and the biological activity of naturally occurring GLP-1-related molecules such as exendin-4 is reviewed. Circumventing DP IV-mediated incretin degradation via inhibitors that target the DP IV enzyme represents a complementary strategy for enhancing GLP-1-mediated actions *in vivo*. Finally, the current status of alternative GLP-1-delivery systems via the buccal and enteral mucosa is briefly summarized. The findings that the potent glucose-lowering properties of GLP-1 are preserved in diabetic subjects, taken together with the potential for GLP-1 therapy to preserve or augment β cell mass, provides a powerful impetus for development of GLP-1-based human pharmaceuticals.

INTRODUCTION

Glucagon-like peptide-1 is a posttranslational product of the proglucagon gene liberated from gut endocrine cells in response to nutrient ingestion. GLP-1 exerts multiple actions that converge on the lowering of blood glucose in rodents and human subjects. The pleiotropic actions of GLP-1 (Figure 1) are preserved in human subjects and GLP-1 administration lowers blood glucose in patients with both type 1 and type 2 diabetes [1-8]. These findings suggest that strategies for enhancing GLP-1 action (Figure 2), either via stimulating GLP-1 release, reducing GLP-1 degradation, delivery of more potent GLP-1 peptide analogues, or derivation of small molecules that activate GLP-1 receptor signaling, warrant vigorous and rigorous scientific assessment. The aim of this review is to highlight our current understanding of GLP-1 action with a focus on reviewing the efficacy, and theoretical advantages and pitfalls of different pharmaceutical approaches that converge on increasing signaling through the GLP-1 receptor. The reader is referred to several comprehensive recent reviews on GLP-1 action for an introduction to GLP-1 physiology [9-12].

GLP-1 SYNTHESIS AND SECRETION

Nutrients are the primary physiological regulators of GLP-1 secretion from gut endocrine cells. Both fats and carbohydrates stimulate GLP-1 secretion in rodent and human studies. The precise mechanisms underlying the detection and

*Address correspondence to this author at the Toronto General Hospital, 101 College Street CCRW3-845, Toronto Ontario Canada, M5G 2C4; Ph.:+416-340-4125; Fax: 416-978-4108; e-mail: d.drucker@utoronto.ca

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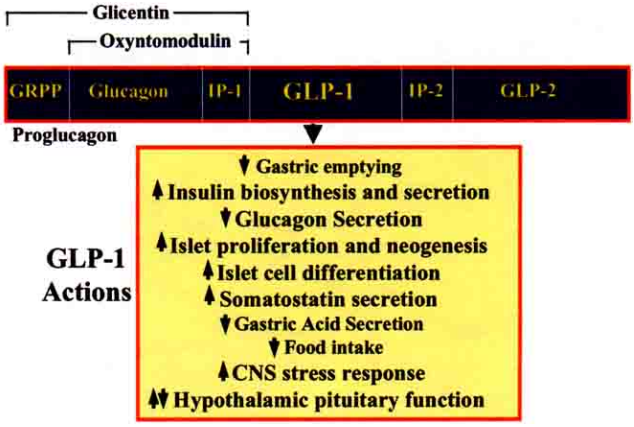

Fig. (1). The biological actions of GLP-1.

Glucagon-like peptide-1 is derived from proglucagon by tissue-specific posttranslational processing. The actions of GLP-1 are shown below the proglucagon molecule.

transmission of the nutrient signal to the secretory apparatus of the gut L cell remain unclear [13]. Although the majority of gut endocrine cells are located in the distal ileum and colon, GLP-1 secretion occurs within minutes of nutrient ingestion, implying the existence of a proximal-distal intestinal loop for rapid transmission of nutrient-induced secretory signals from the duodenum and jejunum to the distal ileum and colon. GLP-1 release appears biphasic, with a rapid early response (mediated by humoral or neural mechanisms) followed by a more delayed response (direct nutrient contact with the distal L cells). Various mediators of this proximal-distal axis have been proposed based on rodent studies, including gastrin-releasing peptide and gastrointestinal inhibitory peptide (GIP) [14-16]. Taken together, these findings suggest that identification of nutrient components that function as potent GLP-1 secretagogues represents a useful strategy for enhancing GLP-1 activity *in vivo*.

Among various nutrients examined, fatty acids and dietary fiber up regulate both proglucagon mRNA transcripts and GLP-1 secretion in the rodent gastrointestinal tract [17-22]. Luminal glucose, peptones, and fatty acids increase GLP-1 secretion from the isolated rat ileum [23]. The rapid rise of circulating levels of GLP-1 within minutes of food ingestion has stimulated inquiry into the endocrine and neural mediators, activated in the proximal gut, that signal the distal ileum and colon to release GLP-1.

Neuromedin C [24], calcitonin gene-related peptide [15, 25] acetylcholine and muscarinic agonists [26, 27], GIP [14, 28-30] and gastrin-releasing peptide [16, 31] stimulate GLP-1 secretion; the latter two peptides have been identified as putative peptide mediators of the proximal to distal signal in rodents [14, 16, 24]. The stimulatory effects of GIP on GLP-1 release from canine ileal cells was inhibited by somatostatin and the protein kinase A inhibitor H-89 [28]. Consistent with a physiological role for gastrin-releasing peptide in the regulation of GLP-1 release, mice with inactivation of the GRP gene exhibit defective glucose-stimulated insulin release in association with reduced glucose-stimulated GLP-1 and insulin secretion [32].

Adrenaline, acting through the β2-adrenergic receptor stimulates GLP-1 release from the perfused rat ileum [33]. The importance of the vagus nerve for transmission of the proximal-distal secretory signal has been demonstrated in studies examining the effect of ganglionic blockade or vagal transection, maneuvers which significantly diminish GLP-1 secretion in rodents [30]. Pharmacological or surgical selective hepatic branch vagotomy significantly attenuates GIP-stimulated increases in GLP-1 secretion [30]. Furthermore, bilateral subdiaphragmatic vagotomy abolishes fat-stimulated intestinal PGDP secretion in the rat [30]. Somatostatin-28 exerts inhibitory effects on GLP-1 secretion, as somatostatin immunoneutralization increases GLP-1 release in

the perfused porcine ileum preparation [24]. Similarly, galanin also inhibits intestinal GLP-1 release [34, 35]. In contrast, although insulin inhibits pancreatic glucagon secretion and biosynthesis, a direct role for insulin in the regulation of gut GLP-1 secretion remains unclear.

PHYSIOLOGY OF GLP-1 ACTION

Original concepts of GLP-1 action focused primarily on its role as a meal-stimulated incretin that functioned by potentiation of glucose-stimulated insulin release from the islet β cell following nutrient ingestion [36-39]. Accordingly, administration of exogenous GLP-1 immediately prior to a meal would be predicted to mimic the incretin-like actions of endogenous GLP-1 and control postprandial glycemic excursion. A large body of evidence from animal and human studies has shown that GLP-1 exerts multiple effects that serve to lower blood glucose independent of its actions on the islet β cell. It is now clear that GLP-1 potently inhibits gastric emptying [40-45] and glucagon secretion [46-50], additional actions that lower glucose in rodent and human studies. Indeed, the potent inhibition of gastric emptying might be predicted to reduce the rate of nutrient absorption and decrease the requirement for insulin secretion from the islet β cell [51].

Continuous intravenous or subcutaneous GLP-1 infusion is effective in controlling blood glucose around the clock, and not just following meal ingestion [4, 6, 52]. Continuous subcutaneous infusion of GLP-1 for 48 hours in human subjects with type 2 diabetes lowered fasting and meal-related plasma glucose and reduced appetite [53]. Furthermore, injection of subcutaneous GLP-1 three times daily immediately before meals increased insulin, lowered glucagon, and decreased blood glucose in patients with early type 2 diabetes over a 3 week study period [54]. Encouragingly, GLP-1 also improved postprandial glycemic control in a similar experimental design over a 3 week period in 5 patients with poorly controlled diabetes [1].

The results of short-term studies have shown that GLP-1 retains its glucose-lowering potency in human subjects after 7 days of continuous infusion. Nevertheless, infusion studies with the native molecule have shown a reduction of glucose-lowering effectiveness with increasing duration of GLP-1 infusion, suggesting that degradation of the intact peptide to GLP-$1^{9\text{-}36amide}$ may potentially limit its sustained activity in this setting [4, 55, 56]. Hence it seems clear that long acting GLP-1 analogues or more stable formulations would exhibit considerable advantages over native GLP-1 for achieving prolonged reduction of blood glucose over long periods of time.

The physiological importance of GLP-1 for glucoregulation has been defined in experiments employing receptor antagonists, immunoneutralizing antisera, and knockout mice. In human subjects, GLP-1 administration reduces gastric emptying which may paradoxically reduce meal-stimulated insulin secretion [51]. Both rodent and human studies employing GLP-1 antagonists reveal an essential role for GLP-1 in the control of postprandial nutrient disposal and insulin secretion. Infusion of GLP-1 immunoneutralizing antisera or the GLP-1 receptor antagonist exendin (9-39) increased glycemic excursion and decreased insulin secretion in baboons, rats and human subjects [57-60]. Surprisingly, GLP-1 action is also essential for control of fasting glycemia and glucose clearance following non-enteral glucose challenge in mice [61, 62]. These latter observations are likely attributable to the importance of GLP-1 for both basal β cell function and for glucagon secretion. The comparatively modest degree of glucose intolerance observed in GLP-1R-/- mice is accounted for in part by compensatory up-regulation of GIP secretion and enhanced sensitivity to GIP action [63]. In contrast to the role of GLP-1 for glucose homeostasis following both enteral and non-enteral glucose challenge, the role of GIP appears more restricted as GIP regulates glucose absorption and glycemic excursion only following enteral glucose challenge [62, 64].

DIPEPTIDYL PEPTIDASE IV

As GLP-1 degradation represents a significant obstacle to the use of the native peptide for the chronic treatment of diabetic patients, the rapid enzymatic inactivation of the two naturally ocurring forms of GLP-1 has been carefully studied. Both full length bioactive GLP-$1^{7\text{-}36amide}$ and GLP-$1^{7\text{-}37}$ (Fig. **2**) are degraded within seconds of their release by the gut endocrine cell [65]. As

GLP-1 contains an alanine at position 2, it is an excellent substrate for the enzyme dipeptidyl peptidase IV (also known as the transmembrane protein CD26), leading to the generation of GLP-$1^{9\text{-}36amide}$ and GLP-$1^{9\text{-}37}$ [56, 66-68]. Although these N-terminally truncated peptides have been shown to be weak antagonists of GLP-1 action *in vitro* [69], the physiological significance of circulating GLP-$1^{9\text{-}36amide}$ and GLP-$1^{9\text{-}37}$ remains unclear.

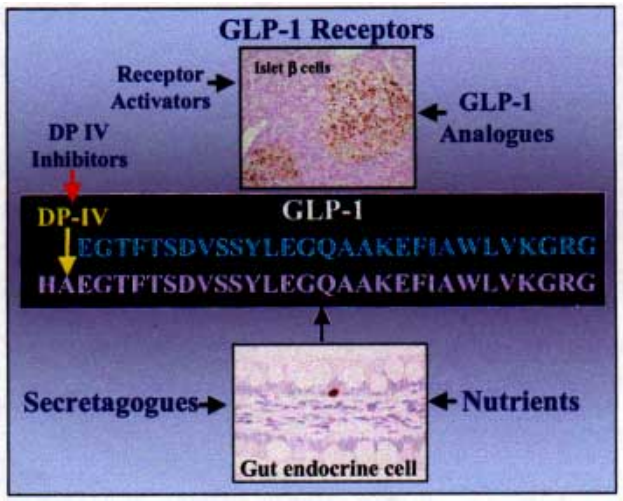

Fig. (2). Strategies for enhancing GLP-1 action in diabetic patients.

GLP-1 is synthesized in and secreted from gut endocrine cells and acts on distant target tissues, including islet β cells. DP IV=dipeptidyl peptidase IV.

In the absence of plasma, GLP-1 is a fairly stable peptide, as incubations at up to 55°C for 72 h did not result in significant peptide degradation [70]. Furthermore, storage of the peptide at 4°C for 11 months did not result in significant peptide degradation as assessed by high pressure liquid chromatography [70]. In contrast, intravenous or subcutaneous infusion of GLP-1 is associated with rapid degradation of the full-length bioactive peptide in both normal and diabetic subjects, and the *in vivo* elimination half life of GLP-1 in human subjects is estimated to be approximately 90 seconds [56].

Inhibition of the serine protease dipeptidyl peptidase IV (DP IV) appears to represent a useful strategy for enhancing the bioactivity of GLP-1 *in vivo* [71]. DP IV is a widely expressed soluble and membrane-associated enzyme present in many tissues including the kidney, lung, liver, pancreas and intestine, and is highly expressed on both lymphocytes and endothelial cells [72]. The expression of DP IV on vascular endothelial cells that surround the GLP-1-producing gut endocrine cell, taken together with the expression of DP IV in gut epithelium [73], provides an explanation for the finding that over 50% of GLP-1 leaving the intestinal venous circulation has already been degraded at the N-terminus [65]. Unlike other endocrine systems such as the parathyroid cell that degrades intracellular parathyroid hormone, GLP-1 is stored within gut endocrine cells as primarily intact biologically active GLP-$1^{7\text{-}36amide}$ and GLP-$1^{7\text{-}37}$.

DP IV inhibitors represent effective glucose-lowering compounds *in vivo*. The DP IV inhibitor valine-pyrrolidide prevented degradation of intact GLP-1 and potentiated the action of exogenously administered GLP-1, leading to enhanced glucose clearance and increased insulin secretion in the non-diabetic pig [74]. Valine-pyrrolidide also increased levels of glucose-stimulated GLP-1 and improved insulin secretion and glucose tolerance in glucose intolerant C57BL/6J mice [75]. Similar results were obtained in Zucker fatty rats using the inhibitors Ile-thiazolidide [76] and NVP-DPP728 [77]. Hence, the available data from short term studies clearly demonstrates that inhibition of DP IV activity is an effective method for improving glucose tolerance via potentiation of incretin action. Whether long-term inhibition of DP IV activity will result in sustained improvement in glycemic control is a subject of ongoing current investigation.

The glucose lowering properties of DP IV enzyme inhibitors are clearly not attributable solely to reduced degradation of GLP-1. Gastric inhibitory polypeptide, secreted from duodenal K cells in a nutrient-dependent manner, is a potent stimulator of glucose-dependent insulin release that is also inactivated by DP IV cleavage [66, 67]. Furthermore, DP IV inhibitors potently lower blood glucose in mice with complete inactivation of GLP-1 receptor signaling, likely due to potentiation of the bioactivity of insulinotropic DP IV substrates such as GIP [78].

The consequences of inactivating mutations of the DP IV gene has been reported in two different animal models, the Fischer 344 DP IV mutant rat [79] and the CD26-/- mouse [78]. The Fischer 344 DP IV deficient rat expresses a DP IV mRNA

transcript that encodes a mutation at position 633 of the enzyme in the catalytic site, leading to defective processing and activity of the enzyme [79-81]. Remarkably, the levels of circulating GLP-1 and GLP-1 action are not perturbed in the Fischer 344 mutant rat, whereas glucose-stimulated GIP and GIP action are diminished, for reasons that remain unclear [82]. In contrast, inactivation of the murine DP IV gene by homologous recombination results in apparently normal mice with normal fasting glucose but enhanced glucose clearance following oral glucose challenge [78]. Consistent with the central importance of DP IV for incretin action, the levels of bioactive GLP-1 and GIP are increased following glucose administration in DP IV-/- mice [78].

DP IV is also known as the lymphocyte cell surface membrane-associated peptidase CD 26, a molecule that regulates chemokine cleavage and T cell responses to antigen stimulation. CD26 was originally identified as an adenosine deaminase binding protein [83]. CD26, herein referred to as DP IV, cleaves peptides with an alanine or proline at position 2. Numerous chemokines are substrates for DP IV. In some instances, DP IV cleavage appears to have no effect on chemokine activity [84]. In other studies, DP IV may act as a costimulatory molecule for T cell activation, and DP IV processing may yield N-terminally modified chemokines with novel biological activities [85-87]. Inhibition of DP IV activity with relatively specific inhibitors may reduce T cell activation and hence DP IV is thought to exert immunomodulatory properties *in vitro* and *in vivo* [84].

A large number of CNS and gut regulatory peptides including NPY, GHRH, GIP, PYY, PACAP and GLP-2 are also substrates for DP IV activity [84]. Accordingly, the use of DP IV inhibitors for potentiation of GLP-1 activity is likely to be associated with reduced degradation of numerous bioactive peptides and chemokines. These considerations suggest that inhibition of DP IV activity for the treatment of diabetes may be associated with additional biological consequences beyond simple potentiation of incretin (GLP-1 and GIP) action. Hence it seems prudent to assess immune function and additional physiological endpoints in both short and long term studies of DP IV inhibitors *in vivo*.

Several lines of evidence support a role for one or more DP IV-related enzymes in the cleavage of substrates exhibiting alanine or proline at position 2. For example, the levels of incretin hormones are actually lower than normal in the DP IV mutant rat [82]. Furthermore, residual DP IV-like activity has been detected in plasma from DP IV-mutant rats [88], and on DP IV-negative human T lymphoblastoid cells [89]. These findings may be explained in part by evidence that DP IV appears to be a member of an expanding enzyme family, whose related members that share overlapping substrate specificity include attractin [90], fibroblast activation protein [91], quiescent peptidyl peptidase [92], N-acetylated alpha-linked acidic dipeptidase II [93], DPP6 [94], and DPP8 [95]. The contribution if any of these related enzymes to incretin degradation, and their comprehensive substrate specificity profiles remains unclear and requires further investigation.

GLP-1 DEGRADATION AND CLEARANCE

Although gut L cells store GLP-1 predominantly as intact GLP-1$^{7\text{-}36amide}$, a substantial proportion of degraded GLP-1$^{9\text{-}36amide}$ is detected even in the intestinal venous circulation [65]. GLP-1 also undergoes endoproteolysis and is a substrate for membrane-associated neutral endopeptidase (NEP) 24.11 [96, 97]. Despite the importance of enzymatic degradation for termination of GLP-1 activity, additional mechanisms such as renal metabolism of GLP-1 account for substantial clearance of the peptide from the circulation [98-100]. The liver and lung also contribute to removal of GLP-1 from the circulation [99]. A small amount (14%) of glycated GLP-1 has been detected in mouse intestinal extracts and glycation of GLP-1 *in vitro* at position 7 impaired GLP-1-stimulated insulin release from BRIN-BD11 rat insulinoma cells *in vitro* [101]. Nevertheless, the biological significance of GLP-1 glycation for development of a GLP-1-based therapeutic remains uncertain.

THE GLUCAGON-LIKE PEPTIDE 1 RECEPTOR

The rat GLP-1 receptor was identified through expression cloning in 1992 and is a 463 amino acid member of the G protein coupled receptor

superfamily [102]. Transfection of the receptor cDNA into COS cells yields high affinity binding sites for GLP-1 with a Kd of 0.6 nM, comparable to the Kd observed for GLP-1 binding to rat insulinoma INS-1 cells [103]. Glucagon displaces GLP-1 binding only at concentrations of ~ 1 uM. The GLP-1 receptor exhibits amino acid identity not only with the glucagon and GLP-2 receptors [104, 105], but also with receptors for secretin (40% identity), parathyroid hormone (32.4%), and calcitonin (27.5%) [102]. The human GLP-1 receptor exhibits 90% identity with the rat receptor, and binds GLP-1 with comparable affinity, KD=0.5 nM [106, 107]. Although the ligand binding pocket of the GLP-1R has not been defined, the purified N-terminal extracellular domain competes for GLP-1 receptor binding *in vitro* [108]. The human GLP-1 receptor has been localized to chromosome 6p21 [109], however linkage studies have not demonstrated significant association between type 2 diabetes or obesity and inheritance of specific GLP-1 receptor polymorphisms [110-112].

The GLP-1 receptor is expressed in the lung, stomach, pancreas, heart, and brain [102, 113, 114]. *In situ* hybridization studies localized GLP-1R transcripts to the gastric pits, duodenal crypts and pancreatic islets [113]. GLP-1R mRNA transcripts have also been detected in the jejunum, ileum and colon of rats and mice [114, 115]. Despite suggestions that additional GLP-1 receptors may exist that mediate one or more 'non-classical' actions of GLP-1, RNA hybridization and RT-PCR studies have not detected evidence for a second GLP-1 receptor [113-115]. Furthermore, cloning of human GLP-1 receptors from lung, brain, heart and pancreas has revealed that all GLP-1 receptor cDNAs isolated to date exhibit identical amino acid sequences [102, 107, 116, 117]. Similarly, GLP-1 binding and GLP-1 actions are completely eliminated in GLP-1R-/- mice [61].

Although the majority of islet GLP-1 receptor expression is localized to β cells, GLP-1 receptor expression was detected in isolated rat islet α cells by RT-PCR, and 20% of dispersed islet alpha cells and 76% of islet delta cells exhibited GLP-1 receptor immunopositivity [118]. In contrast, other studies failed to detect evidence for GLP-1R expression in isolated rat islet α cells using Western blot analysis [119]. It is not clear whether transcriptional regulation represents a major locus for control of GLP-1R expression. A small but significant increases in the levels of GLP-1R mRNA transcripts was observed when rat islets were cultured under high glucose (20 mM) conditions. In contrast to the glucagon receptor, GLP-1R transcripts were not regulated by activators of the cAMP-dependent pathway, however both glucagon and GLP-1R mRNA transcripts were negatively regulated by 10 nM dexamethasone [120].

The rat and human GLP-1 receptors are coupled to adenylate cyclase and cAMP formation, with the human receptor exhibiting an EC_{50} for cAMP formation of 93 pM [107]. The GLP-1 receptor also mediates a cAMP-dependent increase in free cytosolic calcium in studies of islet cells and transfected COS-7 cells [106, 121-123]. Although increased cAMP formation is a common feature of GLP-1 receptor activation, several studies demonstrate downstream GLP-1 actions are mediated by PKA-independent signaling pathways [124]. For example, in some cell lines and in *Xenopus* oocytes, GLP-1R activation increases inositol triphosphate-dependent intracellular Ca^{2+} mobilization in a PKA-independent manner [125, 126]. Similarly, GLP-1 effects on immediate early gene expression were markedly attenuated by the calcium channel blocker nifedipine in islet cell lines [127]. Furthermore, the effects of GLP-1 on DNA synthesis, and induction of (PDX-1) DNA binding activity in beta (INS-1)-cells appears to be mediated through the phosphatidylinositol 3-kinase-dependent pathway [128]. Moreover, disruption of GLP-1R signaling in mouse β cells results in abnormal glucose-stimulated calcium oscillations that are not corrected by addition of cAMP agonists *in vitro* [129].

Understanding the conditions and mechanisms underlying GLP-1 receptor desensitization represents an important question with clinical relevance. Exposure of cells expressing a transfected or endogenous GLP-1R to GLP-1 results in decreased expression of plasma membrane associated GLP-1R and receptor internalization [130, 131]. In contrast, the GLP-1 receptor antagonist exendin (9-39) did not affect cell surface expression of the GLP-1R or receptor endocytosis [130]. In normal islet cells, rodents, and human subjects, GLP-1 and GIP exhibit

modest but detectable additive effects on insulin secretion [132-134]. Supraphysiological concentrations of GLP-1 (100 nM) induce reversible homologous desensitization on hamster insulinoma cells. In contrast, pretreatment with comparable concentrations of GIP or glucagon did not affect the insulin secretory response to GLP-1 [135]. Similarly, pre-exposure of rat and mouse islet cells to 10 nM GLP-1 or the phorbol ester PMA induced desensitization of subsequent GLP-1 stimulated cAMP accumulation [136, 137]. The protein kinase C inhibitor RO-318220 inhibited desensitization induced by PMA but not by GLP-1 [136]. The importance of phosphorylation of specific amino acids in the carboxyterminal region of the GLP-1R was illustrated by studies demonstrating marked attenuation of both homologous and PMA-induced GLP-1R desensitization following mutation of specific serine doublets in the cytoplasmic tail [138, 139].

STRUCTURAL DETERMINANTS OF GLP-1 ACTION

The amino acid sequences of glucagon, GLP-1, exendin-4, and GLP-2 are 100% conserved at positions 1, 4, 6, 22, 26, and 27, and GLP-1 and exendin-4 exhibit amino acid identity at 16 of 30 positions in the native GLP-$1^{7\text{-}36amide}$ molecule. The structure of GLP-1, as characterized in studies using 2D ^{1}H NMR, is similar to glucagon, as GLP-1 exhibits an N-terminal random coil (residues 1-7), and two helical segments (7-14 and 18-29) separated by a linker region (15-17) [140]. Despite only 53% identity with GLP-1, the lizard peptide exendin-4 is a highly potent agonist at the GLP-1 receptor both *in vitro* and *in vivo* [107, 141, 142]. In contrast, although glucagon and GLP-1 exhibit identity at 14 amino acids, glucagon and GLP-1 do not exhibit cross-reactivity at their respective receptors at physiologically relevant concentrations [102]. However at higher concentrations, 3×10^{-7} M glucagon is a weak agonist at the GLP-1 receptor [70]. Furthermore, the GLP-1 receptor antagonist exendin (9-39) inhibited glucagon-stimulated cAMP formation in purified rat β cells at glucagon concentrations of 10^{-8} M [143]. The N-terminus of GLP-1 is unlikely to be responsible for differential recognition of the GLP-1 compared to the glucagon receptor given the strong sequence conservation in this region, with identity at 10/14 N-terminal amino acids [144]. The carboxy-terminus of GLP-1 is essential for ligand binding, as deletion of these sequences results in peptides that do not recognize the GLP-1 receptor [70, 145] and are partially or completely inactive in assays measuring stimulation of cAMP formation and insulin secretion [146]. Similarly, removal of the N-terminal histidine at position 1 significantly attenuates the insulinotropic action of GLP-1 [70, 146].

Initial studies of the structural determinants of GLP-1 action included analyses of specific amino acids substitutions via site-directed mutagenesis. The results of studies using alanine-substitutions demonstrated that side chains in positions 7, 10, 12, 13, 15, 28, and 29 are critically important for ligand-receptor interaction as alanine substitutions lead to a significant loss in receptor affinity and in some instances changes in peptide conformation as assessed by circular dichroism spectroscopy [144, 147]. The positive charge of the imidazole side chain at position 7 accounts for the importance of the position histidine for receptor binding [148]. Swapping selective residues from growth hormone releasing hormone into the GLP-1 backbone identified amino acid positions 1, 10, 15, and 17 as essential for receptor binding and cAMP activation in a RINm5F cell assay [149].

The functional domains of GLP-1 have also been studied using domain swap experiments and chimeric peptides. Substitutions of glucagon for GLP-1 amino acid sequences at the carboxyterminus produced significant decreases in affinity for the GLP-1 receptor [150]. Conversely, transfer of GLP-1 carboxyterminal sequences to the glucagon molecule improved affinity for the GLP-1 receptor. Substitutions at the N-terminus (positions 2, 3, 10, and 12) were generally well tolerated, and produced only modest decreases in receptor affinity [150]. Substitution of GIP sequences for GLP-1 sequences in the N-terminus retained binding specificity for the GLP-1 receptor but led to a significant diminution of GLP-1 receptor binding [151]. Carboxyterminal substitutions also diminished GLP-1 receptor binding, but were better tolerated compared to substitutions at the N-terminus. Consistent with the results described above, substitutions at positions 13 (tyrosine) and 15 (glutamic acid) were poorly tolerated, leading to decreased GLP-1 receptor affinity [151].

GLP-1 ANALOGUES

GLP-1 analogues engineered for DP IV resistance *in vitro* generally exhibit longer plasma half lives and enhanced bioactivity *in vivo*. Clearly preferred analogues are those which exhibit preserved to enhanced receptor binding concomitant with increased resistance to DP IV-mediated degradation. GLP-1 derivatives with a glycine or α-aminoisobutyric acid at position 8 exhibit both DP IV-resistance and retained binding affinity for the GLP-1 receptor [152]. A considerable number of GLP-1 derivatives have been developed that exhibit enhanced stability and sustained bioactivity *in vivo*. Derivativization of GLP-1 by addition of fatty acid moieties at the carboxyterminus promotes albumin bindingleading to molecules with prolonged duration of action. In contrast, fatty acid derivatization at the N-terminus is less well tolerated, leading to reduced receptor binding and loss of potency [153]. GLP-1 peptides acylated with simple fatty acids alone, or with a L-glutamoyl spacer or diacids exhibit an enhanced negative charge, leading to greater albumin binding and improved solubility at physiologically relevant pH [153]. In contrast to native GLP-1, such derivatives exhibit half-lives of at least 9 hours and potently lower blood glucose *in vivo*.

GLP-1 analogues with position 1 substitutions may also exhibit enhanced resistance to DP IV-mediated degradation. N-methylated, desamidated, and imidazole-lactic acid-substituted GLP-1 molecules were resistant to DP IV degradation and both N-methylated and and imidazole-lactic acid-substituted GLP-1 exhibited preserved affinity and cAMP stimulatory activity in studies using RINm5F cells [154]. Similarly, GLP-1 analogues engineered for resistance to DP IV with substitutions at position 2 alone, or positions 2 and 8 exhibited DP IV resistance but preserved insulinotropic properties in isolated rat islets [155, 156]. Similarly, GLP-1 derivatives with N-terminal substitutions at positions 8 with either alpha-aminoisobutyric acid, threonine, glycine or serine exhibited DP IV resistance, with the alpha-aminoisobutyric acid and glycine-substituted peptides retaining similar affinities for the GLP-1 receptor compared to native GLP-1 [152].

EXENDIN-4

The GLP-1-related peptide exendin-4 was originally isolated as a salivary gland peptide that stimulated cAMP formation in guinea pig pancreatic acinar cells [141, 157-159]. Subsequent experiments demonstrated that the truncated peptide, exendin (9-39) functions as a GLP-1 receptor antagonist [141]. As the amino acid sequence of exendin-4 does not contain a position 2 alanine recognized by DP IV, exendin-4 exhibits a longer half life and is more potent than native GLP-1 in normal and diabetic rodents [141, 142, 160-162]. The exendin-4 sequence is encoded by a lizard gene distinct from that encoding GLP-1 and is primarily expressed in lizard salivary gland [163]. The available evidence suggests that the glucose-lowering and islet growth-promoting actions of exendin-4 are transduced by the known islet GLP-1 receptor [9, 164]. The enhanced potency of exendin-4 in multiple mammalian species suggests that human studies examining the efficacy of exendin-4 treatment of diabetic subjects appear warranted.

GLP-1 DELIVERY SYSTEMS

The majority of studies examining GLP-1 administration have employed intravenous or subcutaneous injection for delivery of the peptide. Nevertheless, alternative non-injecatble GLP-1 delivery systems have also been explored and appear promising. Administration of GLP-1 in a tablet formulation in the buccal mucosa stimulated insulin secretion and lowered blood glucose in normal human volunteers [165]. Buccal delivery of GLP-1 was also effective in lowering blood glucose in human subjects with Type 2 diabetes [166]. GLP-1 analogues also lowered blood glucose in db/db mice following acute oral administration of a GLP-1 analogue in a modified polymer formulation [167]. Encapsulated engineered cells that secrete GLP-1 have also been proposed for the treatment of diabetes [168]. Evidence that gene-based delivery of GLP-1 analogues may be feasible derives from studies of exendin-4 transgenic mice that exhibit preserved responsivity to exendin-4 even after several months of continuous exendin-4 expression [169].

SUMMARY

Since the isolation of the cDNAs and genes encoding proglucagon in the early 1980s, considerable progress has been made in understanding the multiple actions of GLP-1 *in vivo*. The findings from multiple clinical studies that the glucose-lowering actions of GLP-1 are preserved in patients with Type 2 diabetes has provided a strong impetus for GLP-1-based pharmaceutical development programs. Furthermore, recent findings that suggest GLP-1 receptor signaling may promote islet and β cell regeneration in rodents [128, 164, 170, 171] has engendered considerable enthusiasm for examining whether the trophic effects of GLP-1 are preserved in patients with type 2 diabetes and reduced β cell function. Although the rapid degradation of native GLP-1 represented an initial impediment to the development of GLP-1-based pharmaceuticals, recent studies suggest that a number of strategies may be implemented to circumvent this problem *in vivo*. Accordingly, new drugs that promote sustained activation of GLP-1 receptor signaling may represent important additions to the current pharmaceutical agents available for the treatment of diabetes mellitus.

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