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

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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].

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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

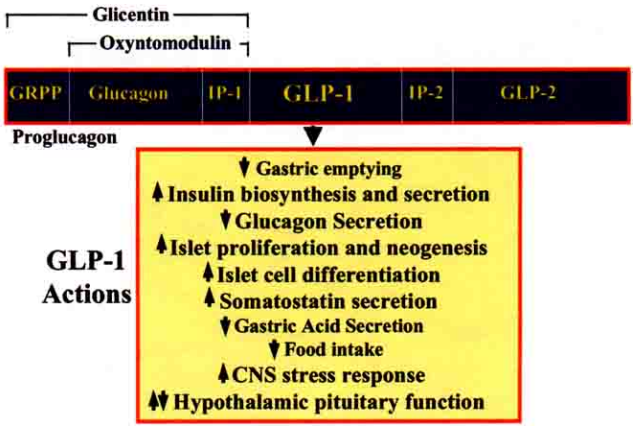

Fig. (1). The biological actions of GLP-1.

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

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

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

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.

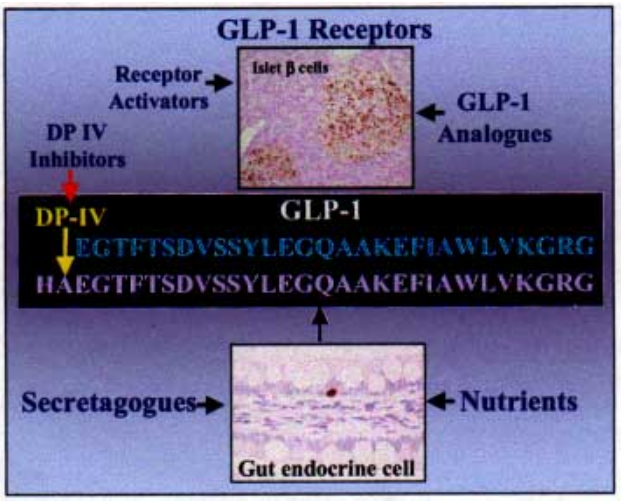

Fig. (2). Strategies for enhancing GLP-1 action in diabetic patients.

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

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

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

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