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REVIEW

GLP-1(28-36)amide, the Glucagon-like peptide-1 metabolite: friend, foe, or pharmacological folly?

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Abstract: The glucagon-like peptide-1 (GLP-1) axis has emerged as a major therapeutic target for the treatment of type 2 diabetes. GLP-1 mediates its key insulinotropic effects via a G-protein coupled receptor expressed on β-cells and other pancreatic cell types. The insulinotropic activity of GLP-1 is terminated via enzymatic cleavage by dipeptidyl peptidase-4. Until recently, GLP-1-derived metabolites were generally considered metabolically inactive; however, accumulating evidence indicates some have biological activity that may contribute to the pleiotropic effects of GLP-1 independent of the GLP-1 receptor. Recent reports describing the putative effects of one such metabolite, the GLP-1-derived nonapeptide GLP-1(28-36) amide, are the focus of this review. Administration of the nonapeptide elevates cyclic adenosine monophosphate (cAMP) and activates protein kinase A, β-catenin, and cAMP response-element binding protein in pancreatic β-cells and hepatocytes. In stressed cells, the nonapeptide targets the mitochondria and, via poorly defined mechanisms, helps to maintain mitochondrial membrane potential and cellular adenosine triphosphate levels and to reduce cytotoxicity and apoptosis. In mouse models of diet-induced obesity, treatment with the nonapeptide reduces weight gain and ameliorates associated pathophysiology, including hyperglycemia, hyperinsulinemia, and hepatic steatosis. Nonapeptide administration in a streptozotocin-induced model of type 1 diabetes also improves glucose disposal concomitant with elevated insulin levels and increased β-cell mass and proliferation. Collectively, these results suggest some of the beneficial effects of GLP-1 receptor analogs may be mediated by the nonapeptide. However, the concentrations required to elicit some of these effects are in the micromolar range, leading to reservations about potentially related therapeutic benefits. Moreover, although controversial, concerns have been raised about the potential for incretin-based therapies to promote pancreatitis and pancreatic and thyroid cancers. The effects ascribed to the nonapeptide make it a potential contributor to such outcomes, raising additional questions about its therapeutic suitability. Notwithstanding, the nonapeptide, like other GLP-1 metabolites, appears to be biologically active. Increasing understanding of such noncanonical GLP-1 activities should help to improve future incretin-based therapeutics.

Keywords: diabetes, incretins, metabolites, insulinotropism

Introduction

Type 2 diabetes is a multifactorial disease from every perspective. It can have a number of causes and is usually a combination of genetic predisposition, poor diet, lack of exercise, and overweight or obesity. Type 2 diabetes is associated with a number of microvascular and macrovascular complications, including peripheral, cardiovascular, and cerebrovascular disease, neuropathy, nephropathy, retinopathy, and myopathy. Given these debilitating consequences, it is truly of concern that approaching half a billion

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people in the world, 347 million in 2008, have diabetes.[1] There is clearly a pressing need for a solution to the problem, both prophylactic and therapeutic.

There are a number of organs involved in the etiology of the disease, including the pancreas, skeletal muscle, liver, adipose tissue, gut, brain, and kidney.[2] Given the complicated pathogenesis of type 2 diabetes, it is not surprising that the drug classes available have a range of target organs and effects, including decreasing hepatic glucose output, increasing insulin secretion, increasing tissue glucose uptake, inhibiting carbohydrate digestion, increasing satiety, and decreasing appetite.[2] All of these have been targeted with varying degrees of success. An algorithm for initiating and then combining diabetes therapies is recommended by many diabetes advisory bodies around the world.[3,4]

One axis that has emerged as a major target for diabetes therapy is the glucagon-like peptide-1 (GLP-1) axis. Therapies currently marketed in this area include oral and injectable agents that target GLP-1, an insulin secretagogue (incretin) with a number of additional beneficial effects, including induction of weight loss and absence of hypoglycemia, making them popular as part of a multidrug approach to type 2 diabetes.[4,5] However, concern regarding their side effects is growing, and there is interest in development of next-generation therapies. The identification of improved therapies requires increased understanding of the molecular details on which to build a scaffold to construct effective drugs.

Background

Briefly, the incretin effect relates to the increase in insulin secretion from an oral glucose load versus a parenteral intravenous glucose load.[6] It is mediated via the action of two proteins secreted from the gastrointestinal tissue, ie, GLP-1, and glucose-dependent insulinotropic polypeptide (GIP), both of which increase glucose-stimulated insulin secretion from pancreatic β-cells. The biology of the incretin hormones, GLP-1 and GIP,[6] the rationale behind GLP-1 as a therapeutic target,[7] the currently available GLP-1-based therapies,[8] and the potential pathological pitfalls[9] have been extensively discussed elsewhere. Given that people with type 2 diabetes do not respond to GIP,[7] the focus of incretin therapeutic strategies has been on GLP-1. In addition to its insulin secretagogue effects at the level of the pancreas, GLP-1 has beneficial effects on many other organs (Table 1). These include delaying gastric emptying, increasing insulin gene expression, increasing β-cell mass (at least in young

Table 1 Reported effects of GLP-1, GLP-1 analogs, DPP-4 inhibitors, and GLP-1(28-36)amide

Target	GLP-1	GLP-1 analogs	DPP-4 inhibitors	GLP-1(28-36)amide
Pancreas	↑ proinsulin synthesis[64,65] ↓ proinsulin/insulin ratio[66] Glucose-dependent stimulation of insulin secretion[67]	↑proinsulin synthesis[64] ↓ proinsulin/insulin ratio[65] Glucose-dependent stimulation of insulin secretion[70,71]	Not determined ↓ proinsulin/insulin ratio[68] Glucose-dependent stimulation of insulin secretion[72]	Not determined Not determined No effect on glucose-dependent stimulation of insulin secretion[17]
	↓ glucagon secretion[67] ↑ β-cell mass[73]	↓ glucagon secretion[70,71] ↑ β-cell mass[75]	↓ glucagon secretion[72] ↑ β-cell mass[75]	Not determined ↑ β-cell mass and the number of proliferating β-cells in mice[17]
	↓ stressor-induced apoptosis[76,77]	↓ stressor-induced apoptosis[78]	Possible ↓ in stressor-induced apoptosis[79]	↓ stressor-induced apoptosis[16] ↑ intracellular ATP levels and enhances cell viability in human islet and INS-1 cells under stressed conditions[16]
Liver	Exerts insulin-sensitizing actions[80] ↓ glucose secretion[81] ↑ glycogen production[82]	Exerts insulin-sensitizing actions[80]	Not determined	Suppression of ROS formation and protection against falls in ATP levels induced by stressed conditions in isolated mouse hepatocytes[51] ↓ glucose production in DIO mouse hepatocytes[51] ↓ liver triglyceride accumulation in mice fed a VHFD[19]
Stomach	↓ gastric emptying[83]	↓ gastric emptying[71,85]	Minimal ↓ in gastric emptying[71]	Not determined
Heart	Cardioprotective[85]	Cardioprotective[86]	Cardioprotective[87]	Cardioprotective[18]
Brain/gut	↑ satiety[88]	↑ satiety[89]	Possible ↑ satiety[90]	↑ energy intake in mice[19]
Weight	↑ weight loss[91]	↑ weight loss[92,93]	No effect on weight loss[94]	↓ weight gain in mice fed a VHFD[19]
Brain	Neuroprotective[95]	Neuroprotective[95]	Neuroprotective[96]	Not determined

Abbreviations: ATP, adenosine triphosphate; DIO, diet-induced obesity; DPP-4, diaminopeptidyl peptidase IV; GLP-1, glucagon-like peptide-1; ROS, reactive oxygen species; VHFD, very high fat diet.

rodents), decreasing glucose secretion, increasing satiety, cardioprotection and neuroprotection, and increasing insulin sensitivity.[6] Whilst the latter remains somewhat contentious,[10] in light of the beneficial effects listed above, targeting the GLP-1 axis has become a favored therapeutic strategy.

GLP-1 structure and metabolites

In pancreatic α-cells, post-translational cleavage of proglucagon typically produces glucagon. However, and in marked contrast, proglucagon is cleaved in intestinal L-cells to generate a number of peptides, including GLP-1(1-37). It is then further cleaved by prohormone convertase type 1 into its active form GLP-1(7-37).[11] Approximately 80% of GLP-1(7-37) is cleaved of its final glycine with subsequent amidation of the penultimate arginine, resulting in the generation of GLP-1(7-36) amide, and this represents the major secretory product.[12] Once in the circulation, GLP-1(7-36)amide has a half-life of less than 2 minutes, being subject to rapid cleavage between positions 8 and 9 by the ubiquitously expressed enzyme diaminopeptidyl peptidase IV (DPP-4), which gives rise to GLP-1(9-36)amide.[6] This peptide has no discernible insulinotropic effect and is considered to be an inactive degradation product.[13] Whilst some still consider the GLP-1(9-36)amide and further GLP-1 metabolites, including GLP-1(28-36)amide and GLP-1(32-36) amide, to be inert, at least at physiological concentrations,[9,14] an increasing body of evidence suggests that these metabolites have beneficial cardioprotective and glucoregulatory actions when administered pharmacologically[15–23] and, importantly, that these effects occur independent of the GLP-1 receptor (GLP-1R).[24,25] As such, the therapeutic potential of these metabolites is garnering increasing interest, even though they are subject to rapid renal clearance and have a half-life of less than 5 minutes.[26,27]

Receptors

The actions of GLP-1 and its therapeutic analogs are mediated largely via the GLP-1R, which has been reviewed extensively elsewhere.[28] Briefly, the GLP-1R is a G protein-coupled receptor that belongs to the secretin-like family of G protein-coupled receptors (also known as family B). Although expression of the GLP-1R has been reported in multiple cell types and tissues,[29] technical and methodological issues mean that such findings should be interpreted with caution.[9] What has been demonstrated unequivocally is that the GLP-1R is essential for the insulinotropic action of GLP-1. Mice lacking the GLP-1R had reduced insulin levels and elevated blood glucose following an oral glucose challenge when compared with controls.[30] In addition, intracerebroventricular administration of GLP-1 inhibited feeding in wild-type mice, but not in GLP-1R null mice. Perhaps surprisingly, GLP-1R null mice showed no significant alteration in food intake, although there was a trend toward reduced feeding at 2 and 6 hours after a 20-hour fast. They also showed increased blood glucose levels following an intraperitoneal, as opposed to oral, glucose challenge.[30] While the former findings demonstrated the importance of the GLP-1R in the actions of GLP-1, the latter findings provide an early indication of the additional glucoregulatory effects of GLP-1 or its metabolites.

GLP-1 based therapies

Two major classes of GLP-1 based therapies have been developed. One class is the DPP-4 inhibitors, also known as gliptins, such as sitagliptin (Januvia®; Merck and Co, Whitehouse Station, NJ, USA) and vildagliptin (Galvus®; Novartis, Basel, Switzerland), that extend the half-life of a patient's endogenous GLP-1 and GLP-1R agonists. The other class is the GLP-1 mimetics, such as exenatide (Byetta®; Amylin Pharmaceuticals, LLC, San Diego, CA, USA, and AstraZeneca Pharmaceuticals LP, Wilmington, DE, USA) and liraglutide (Victoza®; Novo Nordisk, Princeton, NJ, USA), which are cleavage-resistant analogs with an extended circulating half-life. Liraglutide is a fatty acid derivative of human GLP-1 modified with a glutamic acid spacer and a C-16 fatty acid on Lys26 and a Lys to Arg substitution at position 34,[31] giving it an extended half-life of 12.6 hours.[32] Exenatide is a synthetic version of exendin-4,[33] a hormone originally isolated from the saliva of the Gila monster, with properties similar to those of human GLP-1 with the serendipitous trait of DPP-4 resistance due to the presence of several nonconserved amino acids and a C-terminal extension. There are several important differences between the two classes. First, the GLP-1R agonists have the distinct disadvantage of being administered by subcutaneous injection whilst the DPP-4 inhibitors are typically taken orally. Second, the magnitude of the effect on the circulating concentration of what may be considered "active GLP-1" is markedly different, with DPP-4 inhibitors generally raising concentrations of endogenous GLP-1(7-36)amide by 2–4-fold whilst circulating levels of GLP-1R agonists can be 10-fold greater than endogenous levels.[14] Third, and at least partly related to the second point, the beneficial effects of the GLP-1R agonists are often greater than those of the DPP-4 inhibitors, especially in the case of weight loss and delayed gastric emptying (see Table 1 for an overview and Table 2

Table 2 Details of approved drugs targeting the GLP-1 axis

Target	GLP-1R agonist				DPP-4 inhibitor
Generic name	**Exenatide[97,98]**	**Exenatide (extended-release)[97,99]**	**Liraglutide[100]**	**Lixisenatide[101]**	**Alogliptin[102]**
Trade name	**Byetta®**	**Bydureon®**	**Victoza®**	**Lyxumia®**	**Nesina®**
Route of administration	Subcutaneous injection twice daily	Subcutaneous injection once weekly	Subcutaneous injection once daily	Subcutaneous injection once daily	Oral
Dose/day	10 μg	2 mg exenatide once weekly	0.6 mg	20 μg	25 mg
C_{max}	211 pg/mL	Multiphasic release over an approximately 10-week period	35 ng/mL	56.7 pg/mL	145.5 ng/mL
t_{max} (hours)	2.1	ND	8–12	1–3.5	1.25
$t_{1/2}$ (hours)	2.4	2.4	13	1.5–4.5	21
V_d	28.3 L	28.3 L	11–17 L	90–140 L	417 L
CL/F	9.1 L/hour	9.1 (L/hour)	1.2 (L/hour)	20–67 L/hour	10.43 L/hour
$AUC_{0-24/\infty}$	1,036 pg·hour/mL ($AUC_{0-\infty}$)	ND	960 ng·hour/mL ($AUC_{0-\infty}$)	ND	1,058 ng·hour/mL (AUC_{0-24h})
PPB (%)	ND	ND	>98	55	20
Adverse effects	**Common (>1%)** GI, injection site hematoma, URTI, dizziness gastroenteritis, NP **Rare (<0.1%)** Angioedema, skin disorders, alopecia, somnolence, Pancreatitis[115]	**Common (>1%)** GI, NP, headache, injection site pruritus **Rare (<0.1%)** Angioedema, skin disorders, alopecia, somnolence Pancreatitis[115]	**Common (>1%)** GI, headache, anti-liraglutide antibody formation **Rare (<0.1%)** Unknown* Pancreatitis[115]	**Common (>1%)** GI, influenza, URTI, UTI, viral infection, dizziness, somnolence, back pain, injection site pruritus **Rare (<0.1%)** Unknown* Pancreatitis[115]	**Common (>1%)** Headache, diarrhea, pruritus, myalgia **Rare (<0.1%)** Hepatic dysfunction Pancreatitis[115]

Notes: Terminal half-life is the time required for the plasma concentration of a drug to decrease 50% in the final stage of its elimination. GI effects may include nausea, vomiting, diarrhea, constipation, and dyspepsia. Unknown*, cannot be estimated from available data. Byetta®; Amylin Pharmaceuticals, LLC, San Diego, CA, USA, and AstraZeneca Pharmaceuticals LP, Wilmington, DE, USA; Bydureon®; Amylin Pharmaceuticals, LLC, San Diego, CA, USA, and AstraZeneca Pharmaceuticals LP, Wilmington, DE, US; Victoza®; Novo Nordisk, Princeton, NJ, USA; Lyxumia®; Sanofi, Paris, France; Nesina®; Takeda Pharmaceutical Company, Chūō-ku, Osaka, Japan; Trajenta®; Boehringer Ingelheim Pty Limited, Ingelheim am Rhein, Germany; Onglyza®; Bristol-Myers Squibb, Manhattan, New York City, USA; Januvia®; Whitehouse Station, NJ, USA; Galvus®; Novartis International AG, Basel, Basel-Stadt, Switzerland; Suiny®; Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Aichi, Japan; Tenelia®; Mitsubishi Tanabe Pharma Corporation, Chūō-ku, Osaka, Japan, and Daiichi Sankyo Co., Ltd., Chūōō-ku, Tokyo, Japan; Zemiglo®; LG Life Sciences, Seoul, Korea.

Abbreviations: AUC, area under the curve; C_{max}, mean peak concentration; t_{max}, time to reach mean peak concentration; $t_{1/2}$, half-life; V_d, mean apparent volume of distribution; CL/F, apparent clearance; AUC_{0-t}, area under the curve; PPB, plasma protein binding (% bound); GI, gastrointestinal; NP, nasopharyngitis; URTI, upper respiratory tract infection; UTI, urinary tract infection; CPK, creatine phosphokinase; ND, not defined; GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; DPP-4, diaminopeptidyl peptidase IV.

for a summary of details of approved drugs targeting the GLP-1 axis).

A major drawback of the GLP-1R agonists is the need for daily parenteral administration, and this has underpinned substantial efforts to prolong the half-life of these agents. To that end, a once-weekly formulation of exenatide (Bydureon®; Amylin Pharmaceuticals, LLC, San Diego, Ca, USA) has been produced. This is composed of biodegradable microspheres that allow sustained release of exenatide and has been shown to improve glucose control compared with twice-daily exenatide.[34] Given that the GLP-1R agonists have been reported to show most, if not all, of the positive effects attributed to endogenous GLP-1, the question needs to be asked regarding whether the space for drug design and development around GLP-1 is full.

Side effects of GLP-1-based therapies

The documented side effects of the GLP-1R agonists include a number of gastrointestinal adverse effects such as nausea, vomiting, and diarrhea,[14] with the severity of these symptoms leading to the withdrawal of one agent, taspoglutide.[35] More significantly perhaps, there has been ongoing debate about a reported association between the DPP-4 inhibitors, GLP-1R agonists, and pancreatitis. In October 2007, the US Food and Drug Administration (FDA) put out an alert of a suspected association between exenatide and acute pancreatitis. Since then there have been a number of studies looking at the correlation between GLP-1-based therapies and pancreatitis, with varying results.[36–46] Two recent studies, one analyzing the

Linagliptin[103,105]	**Saxagliptin**[105,106]	**Sitagliptin**[107,108]	**Vildagliptin**[109]	**Anagliptin**[110]	**Teneligliptin**[111]	**Gemigliptin**[112–114]
Trajenta® Oral	**Onglyza®** Oral	**Januvia®** Oral	**Galvus®** Oral	**Suiny®** Oral	**Tenelia®** Oral	**Zemiglo®** Oral
5 mg	5 mg	100 mg	200 mg	100 mg	20 mg	50 mg
9.55 nM	24 ng/mL	950 nM	1,223 ng/mL	476 ng/mL	176.50 ng/mL	43.5 ng/mL
1.5 12 1,110 L ND ND	2 2.5 123 L ND 78 ng·hour/mL ($AUC_{0-\infty}$)	1–4 12.4 198 L ND 8.52 μM·hour	1.5 2.96 71 L 44.96 (L/hour) 4,588 ng·hour/mL ($AUC_{0-\infty}$)	1.8 4.37 2,470 (mL/kg) 620 (mL/hour/kg) 2,110 ng·hour/mL ($AUC_{0-\infty}$)	1 26.1 ND 12.2 (L/hour) 1,772.7 ng·hour/mL ($AUC_{0-\infty}$)	4.5 30.8 ND ND 503 ng·hour/mL (AUC_{0-24h})
75%–99% (concentration-dependent)	Negligible	38	9.3	37.1–48.2 (10–100,000 ng/mL)	<80	Not extensively bound
Common (>1%) NP **Rare (<0.1%)** Unknown* Pancreatitis[115]	**Common (>1%)** Headache URTI, UTI **Rare (<0.1%)** Unknown* Pancreatitis[115]	**Common (>1%)** NP, URTI **Rare (<0.1%)** Unknown* Pancreatitis[115]	**Common (>1%)** NP, headache, dizziness, back pain, diarrhea **Rare (<0.1%)** Angioedema, elevated liver enzymes, hepatitis Pancreatitis[115]	Information is too limited to characterize the incidence of adverse events Pancreatitis[115]	**Common (>1%)** GI disorder, skin disorder, NP **Rare (<0.1%)** Unknown* Pancreatitis[115]	**(In 7% of study group)** Constipation, NP, URTI, increase in blood CPK, hypoglycemia and rash **Rare (<0.1%)** Unknown* Pancreatitis[115]

FDA Adverse Event Reporting System database[43] and the other analyzing the database of a large health insurer,[44] demonstrated an increased risk of acute pancreatitis with the GLP-1-based therapies, exenatide and sitagliptin. A more recent study has added further weight to the safety concerns regarding GLP-1-based therapies. Butler et al compared 34 human donor pancreata, eight being from patients with type 2 diabetes on GLP-1-based therapy, 12 from patients with type 2 diabetes, and 14 from controls.[47] They found that the pancreata from patients with type 2 diabetes treated using GLP-1-based therapies had an approximate 40% increase in pancreatic mass over controls. While both β-cell and α-cell mass increased markedly, cell diameter did not, prompting the authors to suggest a mechanism of hyperplasia rather than hypertrophy. Of further concern, and in relation to a possible association between GLP-1-based therapies and pancreatic cancer, is that the pancreata of three of the patients with type 2 diabetes treated with GLP-1-based therapies had microadenomas and, of these patients, one also had a neuroendocrine tumor. The validity and reproducibility of the findings of this relatively small study have been questioned rigorously, with major concerns regarding the lack of appropriate matching of diabetic controls and those treated with an incretin.[9] There is also a large body of preclinical and clinical evidence against such an association from independent studies.[9] Whether this study should affect the prescribing of GLP-1-based therapies is a matter of ongoing and rather heated debate.[48–50] Notwithstanding, the report serves to highlight the need for continuing pharmacovigilance

studies and provides added impetus to improve existing GLP-1-based therapies and/or identify newer more selective therapies to maximize the beneficial effects whilst minimizing the deleterious side effects.

Of interest in this regard are the GLP-1 metabolites. The effects of GLP-1(9-36)amide, which is the major circulating form were recently and extensively reviewed by Tomas and Habener,[15] who described in vitro, preclinical, and clinical evidence supporting a beneficial, insulin-like effect of the GLP-1(9-36)amide.[23,51–53] The remainder of this review focuses on GLP-1 nonapeptide, the GLP-1(28-36)amide metabolite and a major product derived from the cleavage of GLP-1 by the neutral endopeptidase NEP24.11 (also known as neprilysin, common acute lymphoblastic antigen [CALLA], and cluster of differentiation 10).[54] Several recent studies have reported beneficial effects of this metabolite in a range of complementary in vitro cell systems and preclinical mouse models,[16–20,55] prompting growing interest in the possibility that it may represent a potential therapeutic agent. We will discuss these studies and highlight areas that warrant further investigation and clarification.

Therapeutic potential of GLP-1 nonapeptide

Over the past few years, there have been several reports showing the beneficial effects of GLP-1 nonapeptide in both in vitro and in vivo settings,[16–20,55] primarily from the research teams led by Habener[16,19,20] and Jin.[17,55] As described below, investigations from these two groups focus mainly on the hepatic and pancreatic effects of the GLP-1 nonapeptide and are largely complementary. However, they differ significantly in terms of the molecular mechanisms presented.

In vitro effects of the GLP-1 nonapeptide

In in vitro studies from Habener's laboratory, 5-carboxyfluorescein-labeled GLP-1(28-36)amide stained the mitochondria of a subpopulation of primary hepatocytes isolated from mice with diet-induced obesity.[20] Further investigations demonstrated that treatment with the GLP-1 nonapeptide across a broad range of concentrations (10 nM–10 μM) for 3.5 hours suppressed glucose production. Moreover, treatment of isolated hepatocytes or H4IIE cells with 10–100 nM GLP-1 nonapeptide decreased the formation of reactive oxygen species, reduced oxidative stress, and protected cellular adenosine triphosphate levels in the face of 24-hour exposure to H_2O_2 or t-butyl hydroperoxide.[20] In INS-1 β-cells and isolated human islets, treatment with GLP-1(28-36)amide at 1–10 μM, but not 100 nM, for 2–4 days had cytoprotective activity in the face of glucolipotoxic conditions representative of the pro forma type 2 diabetes state.[16] In keeping with observations in the hepatocytes, 5-carboxyfluorescein-labeled GLP-1(28-36)amide stained the mitochondria of stressed INS-1 β-cells. In addition, the mitochondrial membrane potential was protected and cellular adenosine triphosphate levels were increased, whilst cytochrome C release, caspase activation, and apoptosis were all reduced.[16] In both cases, the actions of GLP-1 nonapeptide were unaffected by cotreatment with 10-fold higher concentrations of the GLP-1R antagonist exendin-4(9-36), leading to the conclusion that the effects were independent of the GLP-1R[16,20] and prompting speculation that alternative receptors, possibly distinct G protein-coupled receptors, may be involved.[15]

The studies reported by Shao et al[17] and Ip et al[55] have also explored the effects of the nonapeptide on β-cells and hepatocytes. They demonstrated that in INS-1 β-cells, as well as isolated rat islets, relatively acute treatment with GLP-1 nonapeptide for 30 minutes at 50 nM increased cyclic adenine monophosphate (cAMP) levels, protein kinase A activity, and phosphorylation of downstream substrates, including cAMP response-element binding protein (CREB) and cAMP-dependent transcription factor-1 (ATF-1) as well as phosphorylation and nuclear translocation of β-catenin.[17] Insulin secretion from INS-1 cells and rat islets was enhanced, and longer-term treatment (48 hours) with the GLP-1 nonapeptide increased the growth of INS-1 cells, concomitant with increased cyclin D1 expression.[17] In isolated mouse hepatocytes or HepG2 cells, acute treatment with 100 nM GLP-1 nonapeptide promoted increased cAMP levels and phosphorylation of protein kinase A substrates, namely CREB, ATF-1, and β-catenin.[55] Moreover, after 8 hours of treatment, expression of the gluconeogenic genes, *Pck1* and *G6pc*, was reduced.[55] In both cell types, inhibition of protein kinase A using the H89 inhibitor blocked the effects of the GLP-1 nonapeptide.[17,55]

Taken together, these studies suggest that the nonapeptide mediates effects in hepatocytes and β-cells via conserved mechanisms involving direct targeting of the mitochondria and activation of the cAMP/protein kinase A signaling network.[16,17,19,20,55] These effects, in turn, may help to ameliorate oxidative stress and improve cell survival and cellular function, and thereby enhance insulin sensitivity, insulin production, and overall metabolism. We have made a point of detailing the concentration of GLP-1 nonapeptide used in each experimental approach in order to highlight the broad range of peptide concentrations employed. Although the minimal concentration required to elicit a response was not always determined empirically, this was the case in

several experiments including those where 10 μM GLP-1 nonapeptide was required.[16] It is noteworthy that the beneficial effects of 10 μM GLP-1 nonapeptide were typically equipotent to those observed for exendin-4 used at only 10 nM,[16] raising doubts about the physiological or, perhaps more importantly, therapeutic relevance, of such effects.[14] One possibility is that the stability of the GLP-1 nonapeptide may be relatively limited, such that in the longer-term experiments (2–4 days), its efficacy may appear modest. Consistent with such a scenario, a recent report indicates that the GLP-1 nonapeptide is rapidly metabolized in mouse and human hepatocytes (elimination half-life 13 minutes and 24 minutes, respectively) to give a number of cleavage products.[56] These findings also raise the intriguing possibility that the effects ascribed to the GLP-1(28-36)amide may be, at least partly, mediated by the actions of downstream metabolites.

Interestingly, regulated secretion of GLP-1(7-36)amide by α-cells has been reported, with elevated glucose levels increasing secretion of GLP-1(7-36)amide from a pancreatic α-cell line, αTC1-6, as well as from isolated human and rat islets.[57,58] NEP24.11, which cleaves the GLP-1(7-36)amide to liberate the nonapeptide, is also expressed in islets.[58,59] Thus, it seems plausible to speculate that there may be local regulated production of GLP-1(7-36)amide and its metabolites, including the nonapeptide, in islets and that dysregulation of these processes may contribute to the etiology of type 2 diabetes.

In vivo effects of the GLP-1 nonapeptide

Tomas et al were the first to investigate the effects of continuous delivery of the nonapeptide on weight gain and various metabolic parameters in C57bl/6 mice made obese by maintenance on a very high-fat (60%) diet.[19] The peptide was infused continuously via a mini-osmotic pump for 3–11 weeks at a rate (18.5 nmol/kg body weight per day) estimated to achieve a concentration of around 100 pM, which is comparable with that reported following GLP-1(7-36)-amide infusion. Administration of the GLP-1 nonapeptide resulted in a significant decrease in total body weight from 9 weeks and a reduction in weight gain to around 50% of that observed in control animals. Dual-energy X-ray absorptiometry indicated that the significant reduction in weight gain was due to a significant reduction in fat mass. Fasting glucose and insulin levels as well as hepatic steatosis were all significantly reduced.

In subsequent studies, Ip et al administered GLP-1 nonapeptide via daily intraperitoneal injection (18 nmol/kg) for up to 6 weeks.[55] After 4 weeks of treatment, obese mice receiving the nonapeptide showed a trend toward lower body weight (being around 10% lighter than controls) and weight gain was significantly reduced, to around 20% of that observed in control mice. Although they did not measure fat mass directly, this difference likely reflects a significant difference in fat accrual because expansion of fat tissue represents the major cause of weight gain in adult mice. Intraperitoneal glucose tolerance tests revealed no obvious differences between the groups, although there was a slight trend toward reduced glucose excursions in the GLP-1 nonapeptide-treated mice. Further investigations involving intraperitoneal pyruvate tolerance tests revealed a clear reduction in glucose excursions in the treated mice, suggesting improved regulation of hepatic glucose production. Consistent with the latter, analysis of hepatic gluconeogenic gene expression demonstrated reduced expression of *Pck1* and *G6pc*.[55]

It is noteworthy that in both reports the authors presented data to suggest energy intake was either increased[19] or unaffected[55] upon treatment with nonapeptide, leading to the suggestion that energy expenditure must be increased.[19] However, in both reports, the data were presented in terms of kilocalories per gram of body weight.[19,55] The practice of using body weight as a denominator in analysis of energy balance leading to an overestimate of the role of energy expenditure is a recurring problem,[60] reflecting the complexity of how best to approach the analysis of mouse metabolism.[61] A key requirement, emphasized in the latter perspective,[61] is the need for studies of sufficient sample size, highlighting further deficiencies in the reports.[19,55]

Furthermore, although both reports demonstrated improvements in various metabolic parameters after treatment with the nonapeptide, such improvements may be, at least partly, explained by the reduction in weight gain, which reflected a significant reduction in fat mass.[19]

The different temporal responses of key metabolic tissues to high-fat diet-induced insulin resistance have been described in some detail, with the emerging picture indicating that the liver and fat are acutely responsive when compared with skeletal muscle, which is more refractory.[62] Thus, the finding that administration of the nonapeptide promoted a significant improvement in pyruvate tolerance prior to a significant improvement in whole body glucose tolerance supports the concept that regulation of hepatic glucose production represents a relatively early step in the metabolic improvements observed following treatment with nonapeptide, given the pyruvate tolerance reflects hepatic glucose regulation whilst the glucose tolerance test is largely dependent on skeletal muscle. Finally, it remains possible that the observed metabolic improvements may simply reflect an indirect effect of decreased body weight and fat mass, rather than a direct effect of the GLP-1 nonapeptide. Future studies involving pair-fed controls should help to elaborate whether

this is the case. Additional studies are also required to define the effect, or lack thereof, of the GLP-1 nonapeptide on energy intake, and will need to be larger[61] than those reported to date.

Shao et al have also investigated the effects of nonapeptide in the context of type 1 diabetes using a streptozotocin mouse model.[17] GLP-1 nonapeptide 18 nmol/kg or exendin-4 24 nmol/kg was administered by daily intraperitoneal injection for 9 weeks. After 6 weeks, intraperitoneal glucose tolerance tests revealed no difference between treated or control mice although repeated testing after 9 weeks indicated improved glucose tolerance upon treatment with GLP-1 nonapeptide. Fasting insulin levels were increased as was β-cell proliferation and β-cell mass. Consistent with this, fasting glucose levels were significantly reduced. Similar effects were observed in exendin-4-treated mice.[17]

Further investigations in mouse models of diet-induced obesity and type 1 diabetes provide additional support for the molecular mechanisms described in vitro. Whilst the mitochondrial targeting and related effects of the nonapeptide described by Ip et al appear distinct from the activation of cAMP, protein kinase A, and downstream effectors reported by Shao et al, the latter have suggested that the nonapeptide may activate a compartmentalized cAMP pathway in the mitochondria.[19,55] Support for such a model comes from independent observations describing the activation of mitochondrial protein kinase A in neurons.[63] Further, the increase in protein kinase A phosphorylation in response to nonapeptide is relatively slow compared with that induced by GLP-1 or glucagon via classic G protein-coupled receptors, consistent with an alternative mechanism of activation.[55]

Summary and some outstanding questions

Emerging evidence indicates that GLP-1 nonapeptide is biologically active and has pharmacological effects in vitro and in vivo. Reported effects include inhibition of weight gain in mouse models of obesity with concomitant improvements in associated metabolic parameters, especially hepatic parameters, and increasing insulin levels and β-cell mass and proliferation in a mouse model of type I diabetes. These effects appear to be mediated, at least in part, by mitochondrial targeting of the nonapeptide, which correlates with an improved mitochondrial membrane potential and adenosine triphosphate levels, and reduced apoptosis as well as activation of the cAMP/protein kinase A/CREB cascade. Whilst these effects appear to be largely independent of the GLP-1R, further investigations are required to establish this unequivocally, and if correct, elaborate the outstanding molecular details. In addition, the role of mitochondrial targeting of the nonapeptide needs to be defined. It has been suggested that the nonapeptide may contain key amino acids that constitute a mitochondrial targeting sequence.[20] Are these sufficient? Does increased mitochondrial staining in stressed cells represent increased uptake of the nonapeptide or increased intracellular retention, or both? What are the molecular drivers for this phenomenon? Furthermore, does administration of the nonapeptide reduce weight gain simply by decreasing food intake and, if so, what are the underlying mechanisms? Additionally, could the nonapeptide contribute to reported adverse effects of the GLP-1R agonists, including the recognized gastrointestinal problems as well as the more contentious issue of acute pancreatitis? Finally, it will be important to establish whether endogenous nonapeptide is present in tissues and/or the circulation, because this would support a physiological as well as pharmacological role. Additional, adequately powered studies are required to address these and other questions that will help to provide a greater understanding of the contribution of the nonapeptide to existing incretin therapeutics as well as future therapies.

Disclosure

The authors report no conflicts of interest in this work.

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