Pharmacology, Physiology, and Mechanisms of Incretin Hormone Action

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Incretins are gut hormones that potentiate insulin secretion after meal ingestion in a glucose-dependent manner. The two best-studied incretins, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), exert their insulinotropic actions through distinct G-protein-coupled receptors highly expressed on islet β cells. The GLP-1 and GIP receptors are also widely expressed in nonislet cells (Figure 1) and also exert indirect metabolic actions (Figure 2); hence, there is considerable interest in identifying extrapancreatic actions of incretin hormones. Two strategies encompassing potentiation of incretin receptor signaling have been pursued for the treatment of type 2 diabetes. Activation of GLP-1 and GIP receptors also leads to nonglycemic effects in multiple tissues, through direct actions on tissues expressing incretin receptors and indirect mechanisms mediated through neuronal and endocrine pathways. Here we contrast the pharmacology and physiology of incretin hormones and review recent advances in mechanisms coupling incretin receptor signaling to pleiotropic metabolic actions in preclinical studies. We discuss whether mechanisms identified in preclinical studies have potential translational relevance for the treatment of human disease and highlight controversies and uncertainties in incretin biology that require resolution in future studies.

Introduction to Incretin Biology
Incretins are gut hormones that potentiate insulin secretion after meal ingestion in a glucose-dependent manner. The two best-studied incretins, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), exert their insulinotropic actions through distinct G-protein-coupled receptors highly expressed on islet β cells. The GLP-1 and GIP receptors are also widely expressed in nonislet cells (Figure 1) and also exert indirect metabolic actions (Figure 2); hence, there is considerable interest in identifying extrapancreatic actions of incretin hormones. Two strategies encompassing potentiation of incretin receptor signaling have been pursued for the treatment of type 2 diabetes. Activation of GLP-1 and GIP receptors also leads to nonglycemic effects in multiple tissues, through direct actions on tissues expressing incretin receptors and indirect mechanisms mediated through neuronal and endocrine pathways. Here we contrast the pharmacology and physiology of incretin hormones and review recent advances in mechanisms coupling incretin receptor signaling to pleiotropic metabolic actions in preclinical studies. We discuss whether mechanisms identified in preclinical studies have potential translational relevance for the treatment of human disease and highlight controversies and uncertainties in incretin biology that require resolution in future studies.

Synthesis and Secretion of Incretins

GLP-1
GLP-1 is synthesized in and secreted from enteroeendocrine L cells found throughout the small and large intestine, after post-translational processing of proglucagon by prohormone conver-
**GIP**

GIP is a 42 amino acid peptide synthesized in and secreted from enteroendocrine K cells located primarily in the duodenum and proximal jejunum, and CNS production of GIP has also been described. GIP messenger RNA (mRNA) and protein have been localized to the α cell in mouse and human islets; however, the prohormone is processed by PC2 (rather than PC1/3 as seen in the K cell) to yield a 30 amino acid protein (GIP1–30) (Fujita et al., 2010). GIP1–30 increases insulin secretion in perfused mouse pancreata, and immunoneutralization of GIP decreased glucose-stimulated insulin secretion in isolated mouse islets, consistent with the local release of an insulinotropic GIP peptide from α cells (Fujita et al., 2010). Ectopic expression of biologically active GIP has also been localized to β cells in mice with targeted inactivation of the Gcg gene (Fukami et al., 2013).

The physiological importance of local GIP production in islets under different conditions is difficult to ascertain, and awaits studies of mice with targeted inactivation of the GIP gene in α and/or β cells. Although the molecular control of GIP biosynthesis in gut K cells is poorly understood, analysis of gene expression in isolated K cells identified regulatory factor X 6 (Rfx6) as an important determinant of GIP biosynthesis and secretion from K cells (Suzuki et al., 2013) and increased Rfx6 expression in K cells correlates with increased GIP secretion in high-fat-fed mice.

**Biological Actions of Incretins**

**Pancreas**

GLP-1 increases insulin and inhibits glucagon secretion in a glucose-dependent manner (Drucker, 2006). GLP-1 also increases insulin synthesis, confers glucose sensitivity to glucose-resistant β cells, stimulates β cell proliferation and neogenesis, and inhibits β cell apoptosis. The mechanisms through which GLP-1 inhibits glucagon secretion from α cells are controversial. Although a small subset of α cells express the GLP-1R, GLP-1 may inhibit glucagon secretion through one or more β-cell-derived products, such as insulin, GABA, or zinc. Nevertheless, GLP-1R agonists robustly inhibit glucagon secretion in C-peptide-negative subjects with T1DM (Dupré et al., 2004), illustrating that the β cell is not essential for transducing the glucagonostatic actions of GLP-1. Evidence from experiments employing somatostatin receptor 2 (SSRT2) antagonists and Ssrt2⁻/⁻ mice strongly suggests that the inhibitory actions of GLP-1 on α cells are indirect and mediated through somatostatin-dependent mechanisms (de Heer et al., 2008).

**GLP-1R Signaling in the β Cell.** TCF7L2, a transcription factor activated by the Wnt/β-catenin pathway, plays a central role in β cell physiology, and gene variations of Tcf7l2 are the strongest known genetic risks factors for β cell dysfunction and T2DM (Grant et al., 2006). Exendin-4 (Ex-4; 2 nM) stimulated canonical Wnt signaling in INS-1 cells and isolated mouse islets, actions

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**Figure 1. Direct Pharmacological Actions of GLP-1R Agonists**

GLP-1R agonists act directly via the GLP-1R on pancreatic islets, heart, intestine, subpopulations of immune cells, kidney, and brain.
GLP-1R stimulation increased the phosphorylation and stabilization of β-catenin, through a cAMP/PKA mechanism sensitive to AKT and ERK1/2 inhibition, and enhanced β-catenin/TCF7L2-mediated transcription of cyclin D1 mRNA, leading to increased β cell proliferation (Figure 4); the GLP-1R-dependent stimulation of β cell proliferation was abolished by small interfering RNA (siRNA) targeting β-catenin or after transfection of a dominant-negative TCF7L2 retrovirus in INS-1 cells (Liu and Habener, 2008). Notably, levels of Wnt-4 RNA and protein are robustly increased by Ex-4 in murine islets and INS-1 cells, and knockdown of Wnt-4 attenuated the GLP-1R-dependent stimulation of cell proliferation (Heller et al., 2011). Similarly, basal TCF7L2 and incretin receptor expression assessed by immunohistochemistry was reduced in islets from diabetic human pancreas. Knockdown of Tcf7l2 mRNA transcripts decreased levels of Glp1r mRNA and immunoreactive GLP-1R protein in human islets from nondiabetic subjects and abrogated the GLP-1-dependent (100 nM) potentiation of insulin secretion in perfusion experiments (Shu et al., 2009). Consistent with these findings, mice with pancreas-specific deletion of Tcf7l2 exhibit impaired β cell function, reduced islet Glp1r expression, and defective GLP-1-stimulated insulin release from isolated islets (da Silva Xavier et al., 2012). Murine islets deficient in chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) expression also exhibit reduced levels of Glp1r and Pdx1 mRNA transcripts, decreased β cell mass, and defective GLP-1R-dependent induction of β-catenin and target genes such as cyclin D1 and axin 2 (Boutant et al., 2012). Hence, the TCF7L2/Wnt pathway represents an important target for GLP-1 action in β cells.

Human nondiabetic subjects with SNPs in the Tcf7l2 gene (TT or TC at rs7903146) exhibit reduced insulin secretion in response to oral but not intravenous glucose, with no differences in circulating GLP-1 or GIP levels or insulin sensitivity, consistent with the notion that reduction in TCF7L2 impairs β cell incretin responsivity (Villareal et al., 2010). Nondiabetic carriers of the diabetes-associated Tcf7l2 variant allele exhibit normal β cell function in response to exogenous GLP-1 infusion during a hyperglycaemic clamp (Smushkin et al., 2012). In contrast, insulin secretion in response to oral glucose or a mixed meal was reduced in nondiabetic carriers of rs7903146 or rs12255372, and the insulinotropic response to exogenous GLP-1 and GIP was impaired during a hyperglycaemic clamp (Pilgaard et al., 2009; Schäfer et al., 2007). Although TCF/Wnt signaling was implicated in the control of intestinal proglucagon gene expression (Yi et al., 2005), the available evidence indicates that Tcf7l2 risk variants are associated with normal GLP-1 secretion but impaired GLP-1 action in human subjects. β-arrestin-1 is required for GLP-1R signaling in the β cell (Figure 4). β-arrestin-1 associates with the GLP-1R in INS-1 cells exposed to GLP-1 (100 nM for 3–10 min) (Sonoda et al., 2008).
Knockdown of β-arrestin-1 with siRNA reduced GLP-1 stimulated (100 nM) cAMP production and induction of IRS2 and decreased CREB and ERK1/2 phosphorylation, severely blunting the ability of GLP-1 to augment glucose-stimulated insulin secretion in INS-1 cells. Although β-arrestin-1 controls membrane receptor internalization and desensitization, knockdown of β-arrestin-1 had no effect on GLP-1R surface expression (Sonoda et al., 2008). β-arrestin-1 is also required for the GLP-1-mediated stimulation of the second wave of biphasic ERK1/2 phosphorylation (Quoyer et al., 2010). GLP-1R activation promoted β-arrestin-1 association with ERK1/2, leading to sustained activation and sequestration of phosphorylated ERK1/2 in the cytoplasm (Quoyer et al., 2010). Maintenance of cytoplasmic levels of phosphorylated ERK1/2 activated p90 ribosomal S6 kinase (p90RSK) and led to the phosphorylation and inactivation of BAD (Bcl-xL/Bcl-2-associated death promoter homolog), linking GLP-1R signaling to increased cell survival (Quoyer et al., 2010). GLP-1 failed to prevent apoptosis or stimulate insulin secretion in MIN-6 cells treated with β-arrestin-1 siRNA or in islets from β-arrestin−1−/− mice (Quoyer et al., 2010). Hence, β-arrestin-1 is required for GLP-1R-dependent enhancement of insulin secretion and β cell cytoprotection.

Insulin-like growth factor (IGF) signaling also regulates the biological actions of GLP-1 in the β cell (Figure 4). GLP-1 (100 nM, 18 hr) increased the expression of IGF-1R protein, but not mRNA, in MIN-6 cells and mouse islets (Cornu et al., 2010), actions mediated through cAMP- and PKA-dependent mechanisms. Knockdown of IGF-1R prevented the GLP-1 induction of Akt and BAD phosphorylation and blunted the antiapoptotic actions of GLP-1 in cytokine-treated MIN-6 cells and mouse islets. The ability of GLP-1 to phosphorylate Akt correlated with the glucose-stimulated release of IGF-2, as both diazoxide (200 uM) and nimodipine (1 uM) reduced GLP-1-mediated secretion of IGF-2 and blocked Akt phosphorylation (Cornu et al., 2009). Furthermore, reduction of IGF-2 action by RNA silencing or blocking antisera rendered MIN-6 cells and mouse islets insensitive to GLP-1-mediated reduction in cytokine-induced apoptosis (Cornu et al., 2009). GLP-1 stimulation of the IGF-1R/IGF-2 pathway enhances β cell proliferation, whereas GLP-1 (100 nM, 48 hr) failed to increase β cell proliferation in IGF1r−/− mouse islets and in islets treated with either IGF2 short hairpin RNA or antisera (Cornu et al., 2010). Thus, GLP-1R signaling promotes cell survival by increasing the release of IGF-2 and stimulation of an IGF-1R/IGF-2 autocrine loop.

Identification of a Gut-Brain GLP-1 Axis. GLP-1 stimulates insulin secretion and regulates glucose concentrations through both pancreatic and extrapancreatic mechanisms (Figure 3). The hepatoportal region is exposed to higher concentrations of active GLP-1 compared to the systemic circulation and activation of hepatoportal glucose sensor(s) increases glucose
disposal in rodents. Intragastric infusion of glucose at levels insufficient to increase systemic glucose concentrations differentially regulates c-Fos expression in neuronal subpopulations and increased muscle glycogen synthesis; these actions of intragastric glucose were diminished after central infusion of Ex-9 and were absent in \textit{Glp1r}/-/- mice. Furthermore, the ability of intragastric glucose to activate Fos expression in the CNS in a GLP-1R-dependent manner was markedly diminished in high-fat-fed insulin-resistant mice (Knauf et al., 2008b).

\textit{Glp1r} mRNA transcripts have been identified in the rat nodose ganglion, which contains vagal afferent nerves, and an immunoreactive GLP-1R protein was detected by western blotting and immunohistochemistry in the portal vein (Vahl et al., 2007). The importance of the portal GLP-1R is revealed by data showing that low dose intraportal but not intrajugular systemic infusion of the GLP-1R antagonist [des-His(1),Glu(9)] exendin-4 impaired glucose tolerance. Portal infusion of glucose led to substantially greater glucose clearance relative to the same amount of glucose administered via the femoral vein in mice, and attenuation of portal GLP-1R signaling with Ex-9 prevented activation of the hepatoportal glucose sensor (Burcelin et al., 2001). Similarly, portal infusion of glucose into \textit{Glp1r}/-/- mice failed to preferentially activate glucose clearance. Local potentiation of incretin action through selective inhibition of intestinal but not systemic DPP-4 activity is also sufficient to enhance glucose tolerance, independent of changes in plasma GIP, GLP-1, or insulin, in association with enhanced vagal nerve discharge (Waget et al., 2011). Hence, enteral nutrients activate a functional GLP-1R in the portal vein, initiating a vagal reflex circuit to control whole-body glucose disposal, independent of increases in insulin secretion. Whether this mechanism is similarly important for enteral glucose disposal in humans is difficult to ascertain.

A role for CNS GLP-1R signaling in the control of glucose homeostasis has been proposed. Intracerebroventricular (i.c.v.) Ex-4 (0.5 pmol/kg/min) increased plasma insulin levels during a hyperglycaemic clamp in wild-type (WT) but not in \textit{Glp1r}/-/- mice, whereas i.c.v. Ex-9 increased muscle glucose utilization independent of muscle insulin action though mechanisms requiring intact vagal innervation (Knauf et al., 2005). Similarly, \textit{Glp1r}/-/- mice exhibited increased glucose disappearance, enhanced glucose disposal in muscle, and increased muscle glycogen levels during insulin clamps and fail to regulate glucose appropriately during acute exercise (Ayala et al., 2009). Nevertheless, the hyperglycemia arising in \textit{Glp1r}/-/- mice during exercise was due to impaired suppression of hepatic glucose production and not to defective muscle glucose uptake. Ex-4 infused into the brain for 3 hr decreased whole-body glucose utilization in the awake, free-moving mouse clamped under hyperinsulinemic-hyperglycemic conditions, in association with

**Figure 4. GLP-1 Signal Transduction Pathways in the Pancreatic \( \beta \) Cell**

Recent studies delineating roles for \( \beta \)-arrestin-1, \( \beta \)-catenin, and IGF-1R signaling in the effects of GLP-1 on insulin secretion, \( \beta \) cell growth, and function are illustrated.
reductions in peripheral blood flow (Cabou et al., 2008). These actions of CNS GLP-1R signaling were blocked by coadministration of Ex-9 and eliminated in Glp1r−/− mice. Furthermore, chronic i.c.v. administration of Ex-9 blocked the development of hyperinsulinemia and insulin resistance in high-fat-fed mice, independent of changes in food intake or body weight, consistent with development of increased energy expenditure pursuant to reduction in brain GLP-1R signaling (Knauf et al., 2008a). These observations are concordant with the phenotypes of resistance to diet-induced obesity, increased activity, and enhanced energy expenditure in high-fat-fed Glp1r−/− mice (Hansotia et al., 2007). The central GLP-1R-dependent reduction of femoral blood flow was associated with reduced hypothalamic reactive oxygen species (ROS) and increased vagus nerve activity; provision of ROS donor reversed the effect of brain GLP-1 signaling on peripheral blood flow (Cabou et al., 2008). Under conditions of a hyperglycemic-hyperinsulinemic clamp, central Ex-4 not only reduced femoral blood flow, but also impaired peripheral insulin sensitivity, actions associated with enhanced hypothalamic membrane protein kinase C-δ (PKC) translocation. Conversely, under the same conditions, femoral blood flow and insulin sensitivity were enhanced in Glp1r−/− mice or in WT mice with brain infusion of Ex-9. The central action of Ex-4 on blood flow and insulin sensitivity were mimicked by phorbol-12-myristate-13-acetate (PMA) and blocked by calphostin C, a nonspecific PKC inhibitor, as well as rottlerin, a specific PKC-δ antagonist (Cabou et al., 2011). Furthermore, hypothalamic PKC-δ activity was increased in high-fat-fed diabetic WT mice, and intrahypothalamic rottlerin enhanced both femoral artery blood flow and insulin sensitivity.

The available evidence suggests that enteric glucose absorption activates GLP-1R-sensitive CNS networks that promote enhanced glucose disposal. Whether GLP-1R-dependent CNS pathways are similarly activated by other nonglucose macronutrients remains uncertain; however, fructose attenuates the anorectic actions of exendin-4 in the CNS via an AMPK-dependent pathway (Burmeister et al., 2013), evidence for cross-talk in nutrient sensing GLP-1R-dependent pathways controlling food intake. Furthermore, brain GLP-1R signaling controls peripheral blood flow and insulin sensitivity predominantly under hyperinsulinemic, hyperglycemic conditions. The relative importance of central versus peripheral GLP-1 action for control of glucose homeostasis remains uncertain as transgenic rescue of GLP-1R expression in pancreatic ducal and β cells under the control of the Pdx-1 promoter normalized glucose intolerance in nondiabetic Glp1r−/− mice and completely restored the insulinitropic and β cell proliferative effects of exogenous Ex-4 (Lamont et al., 2012). Unexpectedly however, transgenic mice with selective β cell rescue of the human GLP-1R did not exhibit impaired glucose tolerance after peripheral administration of the GLP-1R antagonist Ex-9. Hence, these findings, together with studies of GLP-1 action in the brain, emphasize the importance of both the β cell GLP-1R and neural-GLP-1R-dependent communication, as key contributors to GLP-1R-dependent regulation of glucose homeostasis. Whether the CNS GLP-1R system is similarly important for control of blood flow or glucoregulation in humans awaits the development of CNS penetrant versus nonpenetrant GLP-1R agonists suitable for use in human subjects.

GLP-1 Actions Promoting Cell Growth. GLP-1 rapidly stimulates β cell proliferation and engages proliferative and antiapoptotic pathways in the rodent β cell, resulting in expansion of β cell mass in preclinical studies (Drucker, 2003). Furthermore, GLP-1R signaling enhances β cell survival in human islets in vitro and in islet transplant studies of rodent and human islets in animals in vivo (King et al., 2005). These observations have prompted investigation of GLP-1R agonists as adjuvant therapy in human subjects receiving islet cell transplants. However, compelling evidence for benefit of GLP-1R agonists in these patients remains elusive. Although exenatide improved glycemia and enhanced β cell function in some islet graft recipients (Faradji et al., 2008), the human studies to date are small, nonrandomized, and of short duration. Furthermore, GLP-1R agonists are poorly tolerated in many subjects with T1DM and islet transplants, and native GLP-1 failed to suppress glucagon secretion from islets transplanted into human recipients (Rickels et al., 2009). Notably, the proliferative actions of GLP-1R agonists are markedly attenuated or absent in older rodents, likely reflecting epigenetic alterations in the capacity for β cell mitosis, downregulation of p27, and increased expression of 16Kldc, a negative regulator of cyclin dependent kinase 4 (Rankin and Kushner, 2009; Tschen et al., 2009, 2011). Similarly, the capacity for human islets from older donors to exhibit a proliferative response to GLP-1R agonists remains unclear and appears to be limited (Tian et al., 2011).

Ex-4 also induced expansion of pancreatic ductal glands in the rat pancreas, and 12 weeks of Ex-4 administration increased pancreas weight, in association with histological findings of chronic pancreatitis in mice with pancreatic-specific expression of the oncogene Kras (Gier et al., 2012). In contrast, GLP-1R activation inhibited cell growth and augmented apoptosis in murine CT26 colon cancer cells that express an endogenous functional GLP-1R (Koehler et al., 2011), highlighting that the growth-modulating properties of GLP-1R signaling are highly cell specific. Sustained GLP-1R activation also increased the mass of the mucosal epithelium in the rodent small bowel and, to a lesser extent, in the colon through incompletely defined mechanisms (Simonsen et al., 2007). GLP-1R expression has been identified in rodent thyroid C cells; acute GLP-1R activation increases calcitonin levels in rodents and sustained activation of GLP-1R signaling produces C cell hyperplasia and medullary thyroid cancer in rodents, to a greater extent in rats than mice (Bjerre Knudsen et al., 2010). The proliferative actions of GLP-1R agonists were not associated with RET or MAP kinase activation in murine C cells. However C cell GLP-1R density and proliferation in response to GLP-1R agonists is highly species specific, and GLP-1R agonists do not promote C cell proliferation or increases in calcitonin levels in primate studies after 87 weeks of exposure (Bjerre Knudsen et al., 2010). Furthermore, the available clinical data from studies of diabetic or nondiabetic obese subjects receiving continuous treatment with liraglutide for up to 2 years do not reveal significant increases in calcitonin levels over time (Hegeđüs et al., 2011). Nevertheless, the engagement of proliferative signaling pathways in multiple tissues after administration of GLP-1R agonists suggests that ongoing scrutiny and monitoring of patients treated for prolonged periods of time with GLP-1R agonists for the possible development of malignancy is appropriate. The low incidence of pancreatic cancer...
(10–60:100,000) and even lower incidence of medullary thyroid cancer (less than 1:100,000) in human subjects suggests that even meta-analyses of ongoing randomized controlled clinical trials of diabetic subjects treated with GLP-1R agonists to assess cardiovascular safety (Ussher and Drucker, 2012) will be insufficiently powered to clarify any potential relationship between incretin-based therapies and cancer incidence. 

GLP-1 Action on Pancreatic β Cells and Glycemic Control in Humans. GLP-1 induces glucose competence in nonresponsive β cells ex vivo (Holz et al., 1993) and enhances and/or restores glucose sensing and sulfonylurea sensitivity to diabetic human β cells in vivo (Gutniak et al., 1996), through poorly understood mechanisms. Conversely, the strict glucose-dependence of GLP-1 action on the human β cell is abrogated by concomitant administration of sulfonylureas (de Heer and Holst, 2007). The robust stimulation of β cell proliferation in rodents coupled with cytotoxic protective actions of GLP-1R signaling in rodent and human islets fostered considerable interest in whether incretin-based therapies might exert durable actions in subjects with T2DM. Although 2 years of treatment with the GLP-1R agonist lixisenatide produced significantly greater reductions in HbA1c relative to the active comparator glimepiride, only 43% of the originally randomized population completed the 2 year trial (Garber et al., 2011). Furthermore, while a substantial subset of liraglutide-treated patients maintained good glycemic control over the 2 year treatment period, a considerable proportion, ~20% of patients failed to maintain glycemic control on liraglutide (Garber et al., 2011). Similarly, while more diabetic patients treated with twice daily exenatide maintained adequate glycemic control relative to subjects treated with glimepiride in an open label study, only 138 out of the initial 515 patients randomized to exenatide completed the study, and 39% of exenatide-treated patients failed to maintain adequate glycemic control (Gallwitz et al., 2012). Bunck and colleagues compared β cell function using sequential glucose clamp studies in a small number of subjects randomized to intensive therapy with exenatide at higher than clinically approved doses, versus insulin glargine, for 3 years. No meaningful differences were seen in HbA1c or multiple measures of β cell function across different treatment groups (Bunck et al., 2011). Although exenatide-treated subjects exhibited an increased disposition index 4 weeks after cessation of therapy, whether this difference reflects a beneficial direct effect of exenatide on β cell function or simply the indirect benefit of a substantial 7.9 kg weight loss in subjects receiving exenatide cannot be determined (Bunck et al., 2011). Hence, these and other clinical studies do not support the concept that treatment with GLP-1R agonists improves functional β cell mass over time in human diabetic subjects.

GIP Action in the Pancreas. GIPR activation in the β cell increases insulin secretion through cAMP/PKA and cAMP/Epa2 pathways, whereas Gipr−/− mice exhibit impaired oral but normal intraperitoneal glucose tolerance, consistent with the known incretin action of GIP (Miyawaki et al., 1999). The mechanisms mediating defective GIP responsivity in experimental and clinical diabetes involve downregulation of GIP receptor expression and/or attenuation of receptor signaling. Partial pancreatectomy resulted in hyperglycemia and reduced islet Gipr and Gipr1 mRNA and protein expression in rats after 4 weeks, which was prevented by reducing hyperglycemia with phlorizin (Xu et al., 2007). The β cell Gipr is transcriptionally upregulated by PPAR-γ, and Gipr mRNA transcripts are reduced in islets from Ppareg−/− mice, potentially linking glucotoxity to reduced PPAR-γ activity and decreased Gipr expression (Gupta et al., 2010). Loss of GIP responsivity and Gipr expression was associated with ubiquitination and GIP receptor degradation under conditions of hyperglycemia in rodent and human islets. In contrast, the Gipr1r but not the Gipr, appears to be downregulated by nonesterified fatty acids in rodent islets. Although exogenous GIP fails to stimulate insulin secretion in most hypoglycemic subjects with T2DM, a 4 week course of insulin therapy improved glycemic control (HbA1c of ~7%) and restored the insulinoportunistic response to exogenous GIP in human diabetics (Højberg et al., 2009). The resistance to GIP action in diabetic subjects is not restored by acute coadministration of xenin-25, a neurotensin-related peptide that potentiates the insulinoportunistic actions of GIP in nondiabetic subjects (Wice et al., 2012). Human subjects with a SNP (rs10423928) in the Gipr gene exhibit reduced islet Gipr mRNA transcripts, secrete less insulin in response to exogenous GIP (240 pmol/kg/hr), and display impaired incretin responses and elevated 2 hr glucose levels during an OGTT (Lyssenko et al., 2011; Saxena et al., 2010). Hence, genetic and adaptive changes in Gipr expression may contribute to the pathophysiology of the impaired incretin response in susceptible individuals.

GIP exhibits antiapoptotic actions (Figure 5) in INS-1 cells and isolated mouse islets in association with phosphorylation of AKT (Ser473), inhibition of apoptosis signal regulating kinase-1 activation, and enhanced EPAC2 signaling independent of PI3K or PKA activation (Widenmaier et al., 2009). GIP (100 nM) increased the expression of the antiapoptotic protein Bcl-2 in INS-1 cells through PKA-dependent phosphorylation of CREB. Although D-[ala2]GIP (24 nM/kg) administration reduced levels of cleaved caspase-3 in islets of STZ-treated mice, Gipr−/− mice did not exhibit enhanced susceptibility to STZ-induced β cell apoptosis (Maida et al., 2009). The differential sensitivity of Gipr1−/− but not Gipr−/− β cells to apoptotic injury may be explained by coupling of basal GLP-1R (but not GIPR) signaling to genes and proteins essential for β cell survival (Maida et al., 2009).

GIP receptors have been localized to rodent and human α cells and GIP infusion (20 ng/kg/min) increased plasma levels of glucagon and enhanced glucose excursion during a meal test in subjects with T2DM (Chia et al., 2009). Whether GIPR activation can provide further glucoregulatory benefit on top of treatment with GLP-1R agonists is uncertain. Transient GIP (4 pmol/kg/min) infusion failed to significantly augment the insulinoportunistic effects of GLP-1 (1.2 pmol/kg/min) and reversed the glucagonostatic action of GLP-1 (Mentis et al., 2011). Hence, whereas the insulinoportunistic actions of GIP appear consistently diminished in the setting of moderate hyperglycemia, GIP retains its ability to stimulate glucagon secretion under hyperglycaemic conditions, through incompletely understood mechanisms.

Liver

GLP-1R agonists inhibit hepatic glucose production (HGP) and reduce hepatic lipid content; however, whether hepatocytes express the GLP-1R is controversial. Analysis of liver RNA by PCR with primers that span the Glp1r coding region demonstrates that murine hepatocytes do not express full-length (1.4 kb) Glp1r mRNA transcripts (Panwani et al., 2013), nor do they
exhibit a cyclic AMP response to GLP-1R agonists (Flock et al., 2007). Conversely, \(\text{Glp1r}\) mRNA transcripts (453 bp product) were detected by RT-PCR, and western blot analysis identified a putative GLP-1R-immunoreactive protein of \(\sim 56\) kDa with protein extracts from rat hepatocytes (Svegliati-Baroni et al., 2011). GLP-1R expression was also reported in human liver by RT-PCR (142 bp product) and western blotting; however, specific GLP-1R binding sites were not detected in human liver sections by receptor autoradiography (Körner et al., 2007). The discrepant findings may reflect interspecies variability, and/or technical issues related to antisera sensitivity and specificity, and detection of partial but not full-length \(\text{Glp1r}\) complementary DNAs by PCR. Characterization of the sensitivity and specificity of three commonly utilized GLP-1R antisera revealed that although these antisera detect immunoreactive proteins of a size consistent with that of the GLP-1R, similar proteins were detected in extracts from \(\text{Glp1r}^{-/}\) mice and none of these antisera was sufficiently sensitive to recognize the GLP-1R by conventional or enhanced immunoprecipitation/immunoblotting western blot analysis (Panjwani et al., 2013). Whether human hepatocytes express the GLP-1R also remains uncertain.

GLP-1R agonists inhibit endogenous glucose production (EGP) in preclinical studies through mechanism(s) that remain unclear. GLP-1 may regulate HGP indirectly through stimulation of insulin release or via the brain through central mechanisms (Figure 2). GLP-1 suppressed hepatic glucose production when administered into the lateral ventricle (0.01 \(\mu\)g/min) of male mice during a 2 hr hyperinsulinemic-euglycemic clamp, whereas i.c.v. infusion of Ex-9 (0.01 \(\mu\)g/min) blunted the GLP-1-dependent suppression of HGP (Burmeister et al., 2012). Inhibition of EGP was seen after GLP-1 administration into the arcuate nucleus of nondiabetic rats, but GLP-1 had no effect on EGP after injection into the third ventricle or paraventricular nucleus (Sandoval et al., 2008). However, administration of GLP-1 into the third ventricle enhanced insulin secretion during an intravenous glucose tolerance test. In contrast, arcuate GLP-1 injection had no effect on food intake, which was suppressed by GLP-1 injection into the paraventricular nucleus (Sandoval et al., 2008). Conversely, phosphorylation of Akt and GSK-3\(\beta\) in the liver and suppression of hepatic glucose output during a hyperinsulinemic-euglycemic clamp was defective in \(\text{Glp1r}^{-/}\) mice (Ayala et al., 2009). Thus, pharmacological and physiological GLP-1R signaling suppresses EGP in animals in part through actions on the central nervous system.

The GLP-1-mediated suppression of EGP in clinical studies has been attributed to changes in glucagon and insulin levels. However, the majority of these studies did not control for changes in plasma insulin levels pursuant to GLP-1R activation, or maintained circulating levels of insulin at concentrations known to independently suppress hepatic glucose output \((\sim 150–175\ pM)\). In contrast, GLP-1 suppressed EGP when infused (1.2 pmol/kg/min) into nondiabetic subjects during a

Figure 5. Biological Actions of GIP
The direct and indirect actions of GIP on target tissues are illustrated.
2 hr pancreatic clamp where plasma insulin levels were maintained at concentrations that do not suppress EGP (~25 pM) (Seghieri et al., 2013), demonstrating that GLP-1 can inhibit hepatic glucose production independent of changes in insulin and glucagon. Although experimental conditions in this study transiently mimicked a diabetic state (elevated plasma glucose and glucagon:insulin ratios), whether GLP-1 also suppresses EGP in diabetic subjects independent of changes in islet hormones requires clarification.

GLP-1R agonists reduced hepatic steatosis and decreased plasma levels of liver enzymes in high-fat-fed or genetically obese rodents (Ding et al., 2006; Mells et al., 2012); however, the reduction in hepatic steatosis was frequently associated with weight loss. In contrast, treatment with the GLP-1R agonist AC3174 (30 μg/kg/day for 4 weeks) produced reductions in liver weights and hepatic lipid content similar to those achieved in pair-fed mice fed a high-trans-fat diet (Trevaskis et al., 2012). Acute central activation of GLP-1R signaling rapidly reduced hepatic triglyceride content under hyperinsulinemic conditions in high-fat-fed mice (Burmeister et al., 2012). However, hepatic triglyceride secretion was reduced in the absence of peripheral hyperinsulinemia after i.c.v. Ex-4 administration in high-fat-fed mice (Panjwani et al., 2013). Although GLP-1R activation clearly reduces hepatic fat, possibly via effects on hepatic lipid synthesis and lipid oxidation, the precise mechanism(s) underlying GLP-1-mediated reduction in hepatic fat remain unclear.

GLP-1R agonists also reduce hepatic steatosis in humans. Exenatide (10 μg, twice daily) reduced ALT levels in subjects with T2DM (both male and female, A1C = 8.2% at start of therapy) in an uncontrolled, open-label study over a 3 year period (Klonoff et al., 2008). However, 84% of the subjects lost weight, and those that lost the most weight (~12.5 kg) showed the greatest improvement in alanine aminotransferase (ALT) levels, whereas ALT levels did not change in the absence of weight loss. Liraglutide (1.8 mg/day), in combination with metformin (1.5–2 g/day) increased the liver-to-spleen attenuation ratio, a surrogate measurement of reduced hepatic steatosis, and reduced ALT levels in T2DM subjects over 26 weeks (Jendle et al., 2009). However, liraglutide also caused weight loss (~3 kg) and decreased adiposity. Since GLP-1R agonists simultaneously reduce hepatic steatosis and body weight, it is difficult to attribute reduced hepatic steatosis to actions of GLP-1R agonists independent of weight loss. However, exenatide (10 μg twice-daily) plus pioglitazone (45 mg/day) reduced hepatic triglyceride content, decreased circulating levels of liver enzymes, and increased adiponectin levels in T2DM patients without changes in body weight over a 12 month period (Sathyarayana et al., 2011). Hence, it remains possible that GLP-1R activation regulates hepatic lipid synthesis, secretion, uptake, or oxidation, resulting in reduction of hepatic fat content independent of changes in body weight. Ongoing clinical trials are assessing the potential benefits of GLP-1R agonists in overweight human subjects with or without diabetes with pre-existing steatohepatitis.

**Adipose Tissue**

GLP-1 and White Adipose Tissue. GLP-1 actions have been studied in differentiated 3T3-L1 cells; however, whether GLP-1R expression is localized to adipocytes in vivo is unclear. GLP-1 binding sites were detected in solubilized membranes from both human and rat white adipose tissue (WAT) using radioligand binding assays, whereas others have been unable to detect Glp1r mRNA transcripts in human or rat WAT. Full-length Glp1r transcripts (1.4 kb) were detected in RNA from the stromal vascular fraction (SVF) of murine adipose tissue (Panjwani et al., 2013), and RT-PCR and immunohistochemistry detected GLP-1R expression in adipocytes and the SVF of human visceral adipose tissue from obese male and female subjects (Vendrell et al., 2011). However, the specificity of the GLP-1R antisera employed in these studies remains uncertain (Panjwani et al., 2013). Although central GLP-1R activation may indirectly increase brown adipose tissue (BAT) thermogenesis in rodents (see below), Glp1r expression has not been reported in adult murine BAT.

GLP-1 (10–100 nM, 4 hr) increased lipolytic rates in human primary adipocytes in vitro (Vendrell et al., 2011). However, GLP-1 infusion (1 μM, 80 min) into abdominal subcutaneous adipose tissue via in situ microdialysis failed to demonstrate a lipolytic effect in healthy human volunteers (Bertin et al., 2001). I.c.v. GLP-1 infusion (0.75 nmol/day for 7 days) decreased adiposity, whereas, i.c.v. infusion of Ex-9 (7.5 nmol/day for 7 days) increased fat mass in 8- to 10-week-old male mice (Nogueiras et al., 2009). Although central infusion of GLP-1 increased peripheral levels of total GLP-1, indicating leakage into the periphery, subcutaneous infusion of identical concentrations of GLP-1 (0.75 nmol/day) did not recapitulate the effects seen with central administration. The ability of central GLP-1R activation to reduce WAT mass was independent of changes in food intake and mediated through increased sympathetic outflow. Additionally, i.c.v. but not peripheral administration of GLP-1 decreased lipogenic gene expression (Fas, Scd-1, and Accα) and significantly reduced triglyceride content in WAT after 2 days (Nogueiras et al., 2009). In contrast, i.c.v. GLP-1 did not decrease body weight or alter lipogenic gene expression in WAT of triple beta-adrenoreceptor knockout mice, a murine model lacking a functional sympathetic nervous system. I.c.v. GLP-1 (0.75 nmol/day for 2 days) administered to high-fat-fed obese, wild-type mice failed to alter body mass or lipogenic gene expression in WAT, suggesting an obesity-induced resistance to CNS GLP-1 communicating with WAT. The available data indicate that the actions of GLP-1 on adipose tissue are predominantly indirect and modulated through increased sympathetic tone (Figure 2), leading to decreased lipogenesis and reduced triglyceride content. Whether one or more WAT depots express a functional GLP-1R in human subjects requires further experimentation.

**GLP-1 and Brown Adipose Tissue.** GLP-1 communicates with rodent BAT through the CNS to increase thermogenesis (Figure 2). Acute i.c.v. injection of GLP-1 (1 nmol) increased intrascapular BAT temperature in 12- to 14-week-old male mice, whereas peripheral GLP-1 administration had no thermogenic effect (Lockie et al., 2012). CNS administration of GLP-1 increased sympathetic nerve activity toward intrascapular BAT. Additionally, i.c.v. injection of oxyntomodulin (1 nM), a proglucagon-derivative peptide with mixed Gcgr/GLP-1R activity, mimicked the effects of GLP-1 on BAT sympathetic nervous system activity and increased expression of Pgc-1α and Ucp1 in BAT from wild-type, but not Glp1r<sup>−/−</sup> mice. Nevertheless, Glp1r<sup>−/−</sup> mice did not exhibit basal abnormalities in BAT.
thermogenesis in response to changes in ambient temperature, and compelling evidence that GLP-1R agonists increase energy expenditure in diabetic or obese human subjects is lacking. Hence, while central pharmacological GLP-1R activation increases BAT thermogenesis in rodents, the endogenous GLP-1R is not required for the thermogenic adaptive physiological response to cold. Furthermore, there is no compelling evidence that GLP-1R agonists increase thermogenesis in human subjects.

**GIP and White Adipose Tissue.** Activation of the rodent GIPR increases the uptake of lipids, enhances adipokine secretion, and promotes weight gain, expansion of adipose tissue depots, and insulin resistance (Figure 5) (Lamont and Drucker, 2008), whereas Gipr−/− mice are protected from high-fat-feeding-induced obesity (Miyawaki et al., 2002). Similarly, activation of GIPR expression in rodent or human adipocytes ex vivo induces cytokine and osteopontin expression and impairs insulin-sensitive glucose uptake ex vivo, and GIP levels correlate with body mass index (BMI) and impaired insulin sensitivity in human subjects (Ahlqvist et al., 2013). Attenuation of GIP action with immunoneutralizing antisera, or elimination of GIP-producing K cells, also produces resistance to diet-induced obesity in preclinical studies. Ablation of enteroendocrine K cells markedly impairs the incretin effect to a greater extent than in Gipr−/− mice, findings potentially explained by the importance of GIP as an endocrine factor stimulating GLP-1 secretion and by deficiency of xenin-25, which acts as a cofactor for GIP action on the β cell (Althage et al., 2008; Wice et al., 2010). Transgenic rescue of GIPR expression in adipocytes of Gipr−/− mice restores the weight gain response to high-fat feeding; surprisingly, however, the increase in body mass was attributed to expansion of lean body mass with no effect on the adipose tissue compartment (Ugleholdt et al., 2011). Although GIP in the absence of insulin has little consistent impact on adipocyte lipid metabolism, free fatty acids, or triglycerides in human subjects, the combination of GIP plus insulin had a greater effect to increase adipose tissue fatty acids, or triglycerides in human subjects, the combination of GIP plus insulin had a greater effect to increase adipose tissue blood flow in lean nondiabetic human subjects than did infusion of insulin or GIP alone (Asmar et al., 2010). In contrast, infusion of GIP alone (2 pmol/kg/min) increased insulin levels, lowered circulating levels of free fatty acids after 60 min, and decreased expression of 11β-HSD1 and adipose triglyceride lipase in adipose tissue without changes in triglyceride levels in healthy male nondiabetic subjects (Gögebakan et al., 2012). Human subjects with the Gipr SNP (rs10423928) have reduced BMI and lean body mass and waist circumference, consistent with a reduction in adipogenesis (Lyssenko et al., 2011). Whether this phenotype reflects decreased GIPR signaling in adipose tissue, reduced circulating insulin levels, or interactions between the β cell and adipose tissue is unclear. Additional evidence for potential complexity of GIPR signaling in human adipocytes is reflected by the detection of 64 splice variants of the Gipr via RT-PCR analysis of RNA from human abdominal subcutaneous tissue (Ahlqvist et al., 2013); only two of the 64 transcripts were predicted to encode a full-length GIPR protein. Hence, potential heterogeneity of GIP action in human adipose tissue may be accounted for in part by genetic variation in the Gipr.

**Nonmetabolic Actions of Incretins in the Brain**

GLP-1. GLP-1 is produced within the CNS and peripheral GLP-1 produced by the L cell (1) crosses the blood-brain barrier and (2) communicates with the brain via sensory afferent vagal neurons. The relative importance of these signaling pathways is difficult to ascertain. GLP-1 receptors are expressed throughout the brain, in regions that control glucose homeostasis, gut motility, food intake, aversive signaling, and cardiovascular function. As outlined below, GLP-1R signaling exerts neuroprotective and neurotrophic effects, with implications for the treatment of neurodegenerative diseases.

Alzheimer’s disease is characterized by deposits of amyloid β protein (Aβ) and the microtubule-associated protein Tau, resulting in senile plaques, neurofibrillary tangles, and progressive dementia. An acute bolus of Aβ i.c.v. (10–100 nmol) diminished long-term potentiation (LTP) induced by high-frequency stimulation, whereas a GLP-1R agonist (15 nmol, i.c.v.) prevented the Aβ-induced reduction of LTP in male rats (Gault and Hölscher, 2008a). Infusion of GLP-1 or Ex-4 (3.3–6.6 ng or 0.2 ng, respectively) into the lateral ventricles of lean male mice reduced endogenous levels of Aβ and treatment of rat primary hippocampal cultures (GLP-1, 5–20 nM; Ex-4, 100–500 nM; 24 hr) prevented neuronal death induced by Aβ (Perry et al., 2003). Exogenous Aβ also activates the JNK/TNF-α pathway, leading to impaired insulin action in hippocampal neurons cultured ex vivo; Ex-4 (25 nmol/kg/day, 3 weeks, intraperitoneal [i.p.]) prevented Aβ-induced JNK activation and decreased serine phosphorylation in hippocampal neurons from male amyloid precursor protein (APP)/PS1 transgenic mice, leading to improved brain insulin signaling, spatial memory, and memory retention (Bomfim et al., 2012). Conversely, Gip1r−/− mice demonstrated impaired synaptic plasticity and memory formation and blunted LTP in the hippocampus after pulse stimulation (Abbas et al., 2009). Consistent with the importance of the endogenous GLP-1R for control of memory and neuronal integrity, rescue of hippocampal GLP-1R expression with a recombinant adenovirus ameliorated the learning deficit and decreased kainic acid-induced neurotoxicity in Gip1r−/− mice (Ding et al., 2003). A randomized, double-blind clinical trial examining the effects of daily liraglutide (1.8 mg/day) on intracerebral amyloid deposits in the CNS of patients with Alzheimer’s disease is ongoing.

Parkinson’s disease is characterized by loss of dopaminergic signaling in nigrostriatal neurons, which also express the GLP-1R. GLP-1 or Ex-4 (0.1 μM) maintained cell viability during hypoxic injury in primary neuronal cultures isolated from rat cerebral cortical cells; these actions were prevented by Ex-9 (10 μM) and absent in neurons from Gip1r−/− mice (Li et al., 2009). Similarly, pretreatment with GLP-1 or Ex-4 (0.1 μM) reduced apoptosis and preserved survival of rat primary ventral mesencephalic cells exposed to dopaminergic toxin 6-hydroxydopamine (6-OHDA) (Li et al., 2009). The protective effects of GLP-1 were abolished by inhibitors of PKA or PI3K. Continuous Ex-4 administration (20 nM, 0.25 μl/hr, i.c.v.) for 7 days prevented neuronal death, maintained levels of brain dopamine, and preserved motor function in mice exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a chemically induced model of Parkinson’s disease. Acute treatment with Ex-4 (4 × 10 μg/kg, i.p.) prevented MPTP-induced microglial activation, decreased mRNA levels of TNF-α and IL-1β in the ventral midbrain, and preserved neuronal density in 8-week-old male C57BL/6 mice (Kim et al., 2009). Similarly, Ex-4 (0.5 μg/kg/day) given 7 days after i.c.v.
administration of the toxin 6-OHDA reversed the loss of dopaminergic neurons, restored dopamine levels, and improved behavioral test scores in rats.

Amyotrophic lateral sclerosis (ALS) is characterized by progressive degeneration of motor neurons in the CNS. Ex-4 (100 nM) improved cell viability and reduced apoptosis after hydrogen peroxide-induced oxidative stress in N18 neuronal cells, a cell line that resembles spinal cord cells (Li et al., 2012). Administration of Ex-4 (3.5 pmol/kg/min, subcutaneous [s.c.]) for 12 weeks preserved spinal cord structure, reduced caspase-3-positive neurons in the lumbar spine, and maintained motor neuron density and function in SOD1 G93A mice, a genetic model of ALS (Li et al., 2012).

GLP-1R activation reduces cell death after ischemic injury in the brain. Ex-4 (21 ng, i.c.v.) given 15 min prior to middle cerebral artery occlusion (MCAO) reduced infarct size in wild-type but not in Gipr<sup>-/-</sup> mice (Li et al., 2009). Intravenous administration of Ex-4 (10 µg/kg; i.v.) after middle cerebral artery injury reduced oxidative stress, inflammation, and the number of apoptotic cells in the ischemic boundary zone in 8-week-old male mice (Teramoto et al., 2011). However, whether GLP-1 elicits direct neuroprotective/neuroregenerative effects or acts in the brain through indirect mechanisms is unclear. Infusion of GLP-1 (1.2 pmol/kg/min) increased glucose clearance in the brain, reduced intracerebral glucose concentrations, and enhanced flow across the blood-brain barrier in a randomized, double-blind, placebo-controlled crossover study in nine healthy men undergoing a pancreatic clamp (Gejl et al., 2012). The enhanced cerebral clearance of glucose suggests blood flow to the brain was increased; however, cerebral blood flow was not directly measured. The potential for GLP-1 to elicit neuroprotective effects indirectly through alterations in cerebral blood flow or reductions in brain hyperglycemia requires additional investigation.

GIP. Gipr mRNA and protein are expressed in the hippocampus, cerebral cortex, and olfactory bulb in rats and in the hippocampus and neocortex of human subjects (Figueiredo et al., 2011). GIP-producing cells have been identified throughout the rat brain, with relatively higher mRNA and protein expression in the olfactory bulb, hippocampus, hypothalamus, thalamus, and cerebellum (Nyberg et al., 2007). Whether GIP crosses the blood-brain barrier in physiologically relevant concentrations is unclear; hence, the relative importance of central versus peripheral-derived GIP remains uncertain.

Exogenous administration of GIP (1.92 nmol/day for 5 days, i.c.v.) increased proliferation of hippocampal rat progenitor cells, whereas Gipr<sup>-/-</sup> mice demonstrated a reduction in proliferating cells in the hippocampal dentate gyrus (Nyberg et al., 2005). GIP also increased the proliferation of rat adult hippocampal progenitor (AHP) cells in a dose-dependent manner, actions prevented by antagonism of the GIPR. Acute GIP administration (15 nmol, i.c.v.) prevented the detrimental effects of Aβ on long-term potentiation in rats, consistent with actions of GIP to improve synaptic plasticity in the hippocampus (Gaut and Hölscher, 2008b). Chronically, [D-ALA2]GIP (25 nmol/kg twice daily for 28 days, s.c.) prevented the decrease in LTP induced by high-fat feeding in rats and improved object recognition memory. Treatment with [D-ALA2]GIP (25 nmol/kg/day for 35 days, i.p.) reduced Aβ deposits in the cortex and reduced oxidative stress and inflammation in both the cortex and hippocampus of 12- to 19-month-old APP/PS1 transgenic mice (Duffy and Hölscher, 2013). Conversely, Gipr<sup>-/-</sup> mice have impaired recognition and spatial learning and reduced LTP in the hippocampus (Faire et al., 2011). It still remains to be determined whether and how peripheral administration of GIPR agonists communicates with the brain. There are no data examining the potential consequences of modulating brain GIP receptor activity in humans.

Gipr mRNA transcripts have been localized to cells within the hypothalamus, pituitary, and adrenal cortex, and GIPR activation increases plasma corticosterone in rodents (Bates et al., 2012). Conversely, Gipr<sup>-/-</sup> mice exhibit reduced meal-related increases in plasma corticosterone, raising the possibility that alterations in glucocorticoid tone in rodents contribute to changes in adiposity after enhanced or reduced GIPR signaling. Nevertheless, glucocorticoid replacement at physiological levels did not reverse the metabolic phenotypes of Gipr<sup>-/-</sup> mice (Bates et al., 2012). Although ectopic GIPR expression in human adrenal tumors underlies the pathophysiology of food-induced Cush- ing’s syndrome in some patients, the importance of a GIPR-hypothalamic-pituitary-adrenal axis for human glucocorticoid secretion has not been established.

**Immune System**

Full-length (1.4 kb) Glp1r mRNA transcripts were identified in multiple immune cell subpopulations from C57BL/6 and NOD mice, including thymocytes, splenocytes, bone-marrow-derived cells, and regulatory T cells (Hadjiyanni et al., 2010). Furthermore, GLP-1R agonists increased lymphocyte CAMP formation in a GLP-1R- and Ex-9-dependent manner. No major differences in the number or localization, cell survival, or chemotaxis of different immune subpopulations were detected in lymphocytes from peripheral lymph nodes, spleen, or thymus of Glp1r<sup>-/-</sup> mice; however, Glp1r<sup>-/-</sup> lymphocyte subpopulations exhibited modest impairments in the proliferative response to mitogenic stimuli (Hadjiyanni et al., 2010). Although Glp1r expression has been reported in human macrophages and macrophage cell lines, murine macrophages (C57BL/6, ApoE<sup>-/-</sup>, and IL-10<sup>-/-</sup>) reflecting various activity states (M1 and M2 polarized) do not express full-length Glp1r mRNA transcripts (Panjwani et al., 2013). Invariant natural killer T (iNKT) cells, innate immune cells implicated in the pathogenesis of psoriasis, express Glp1r mRNA transcripts, and iraglutide (15 µg/ml) increased levels of cAMP and CREB phosphorylation and regulated cytokine production in iNKT cells (Hogan et al., 2011).

The anti-inflammatory actions of GLP-1R agonists in the CNS include suppression of proinflammatory cytokines and reduced microglial activation. Similar anti-inflammatory properties of GLP-1 have been reported in the pancreas and cardiovascular system. Ex-4 (100 nM, 6 hr), combined with the phosphodiesterase inhibitor rolipram (15 µM), suppressed the mRNA and protein levels and secretion of the proinflammatory cytokine CXCL10 in human islets treated with interferon-γ (IFN-γ) (Pugazhenthii et al., 2010). The anti-inflammatory effects of Ex-4 in islets were mediated through increased cAMP and were prevented by inhibition of adenyl cyclase (SQ22536, 100 µM), whereas forskolin (20 µM) and dibutylr-cAMP (1 mM) reproduced the actions of Ex-4. Administration of Ex-4 (24 nM, twice daily, i.p.) for 4 weeks reduced levels of mRNA transcripts for proinflammatory cytokines (Mcp-1, Tnfα, and Stat3) in the pancreas of high-fat-fed mice.
mice (Koehler et al., 2009), and activation or elimination of GLP-1R activity had no effect on the severity of caerulein-induced pancreatitis. Liraglutide (30 nM, 2.5 hr) attenuated apoptosis and decreased TNF-α-induced ROS production, PKC activation, and NF-κB activation in primary human endothelial cells (Shiraki et al., 2012). Liraglutide (10–100 nM) also reduced TNF-α (100 ng/ml, 24 hr)-induced caspase-3 activation in murine cardiomyocytes, actions abolished by Ex-9 (10 μM) (Noyan-Ashraf et al., 2009). Hence, GLP-1R activation reduces inflammation and promotes cell survival in multiple organs and cell types.

GLP-1R agonists ameliorate the severity of type 1 diabetes in mice, inducing disease remission in association with enhanced numbers of regulatory T cells. Continuous administration of GLP-1 for 4 or 8 weeks delayed or prevented the development of diabetes and insulitis, with increased β cell proliferation and reduced apoptosis being detected in GLP-1-infused mice (Zhang et al., 2007). Ex-4 (0.1–4 μg/day) delayed the onset of diabetes and reduced lymphocyte infiltration into the islets of female NOD mice when Ex-4 administration was started at 4 or 9 weeks of age (Hadiyanni et al., 2008). Furthermore, GLP-1 (10–100 μg/kg/day for 3 weeks) plus gastrin (1.5 μg/kg) restored normoglycemia and increased islet insulin content and β cell mass in diabetic, female NOD mice; however, neither GLP-1 nor gastrin alone were effective (Suarez-Pinzon et al., 2008).

GLP-1/gastrin treatment increased the number of TGF-β1-secreting lymphocytes and decreased INF-γ-secreting lymphocytes within islets transplanted into diabetic, female NOD mice. The transplanted islets demonstrated improved survival and decreased apoptosis after GLP-1/gastrin administration, resulting in the delayed onset of diabetes (Suarez-Pinzon et al., 2008). Thus, GLP-1R activation modulates lymphocyte activation to favor an anti-inflammatory phenotype that promotes islet function.

Clinically, 4 weeks of liraglutide treatment (1.2 mg/day) decreased the daily insulin dose and HbA1c levels in both C-peptide-positive and -negative subjects with T1DM (Kielgast et al., 2011). Liraglutide did not alter basal or glucagon-stimulated C-peptide levels (duration of diabetes ≥3.7 years) but did reduce the amount of time spent in hypoglycemia (<3.9 mM), and two liraglutide-treated C-peptide-positive subjects discontinued insulin therapy. The reduction in insulin dose achieved was proportional to the baseline C-peptide status prior to initiation of therapy (Kielgast et al., 2011). In contrast, treatment of subjects with long-standing (20+ years), C-peptide-positive T1DM with exenatide (at doses up to 40 μg/day) and insulin therapy with or without daclizumab in a crossover design did not result in augmentation of insulin secretion, despite the considerable weight loss (mean 4.2 kg) achieved on exenatide (Rother et al., 2009). Thus, more-extensive studies are warranted to investigate whether GLP-1R activation improves glucose homeostasis independent of glucagon secretion and gastric emptying and to determine whether therapy with GLP-1R agonists can improve or maintain β cell function in newly diagnosed patients with T1DM.

The finding that GLP-1R agonists modulate iNKT cell activity prompted assessment of the role of GLP-1R agonists in diseases such as psoriasis. GLP-1 treatment (150 μg/ml, 24 hr) in iNKT cells increased the production of IFN-γ and IL-4 under resting conditions but inhibited the production of these cytokines in acti-
doses of liraglutide (3 mg daily), the weight loss achieved is considerably less than that observed after gastric bypass surgery (GBS) (Astrup et al., 2009). Furthermore, the observation that clinical trials employing low calorie diets also produce major improvements in diabetes and considerable weight loss without changes in circulating GLP-1 further challenges the notion that increased levels of GLP-1 play an essential role in the amelioration of diabetes and obesity observed after bariatric surgery. Bradley and colleagues assessed the metabolic consequences of laparoscopic adjustable gastric banding, a condition not associated with increased plasma levels of gut hormones, with the Roux-en Y GBS, in nondiabetic obese subjects after comparable amounts (~20%) of weight loss. The improvement in β cell function and insulin resistance was primarily attributed to weight loss in both groups (Bradley et al., 2012), independent of the multiple different features of the two bariatric surgery procedures. The importance of GLP-1 action for metabolic improvements after bariatric surgery has been examined with murine models, including vertical sleeve gastrectomy (VSG). Although circulating levels of GLP-1 were significantly elevated in mice after VSG, food intake, weight loss, macronutrient selection, and improvements in β cell function and glucose homeostasis were similar after VSG in two different models of whole body inactivation of the Glp1r (Wilson-Pérez et al., 2013).

A subset of patients experience persistent severe recurrent hypoglycemia after bariatric surgery, eventually requiring subtotal or total pancreatectomy for symptom management; many of these patients have very high levels of GLP-1 after meal ingestion. In some patients, attenuation of the hypoglycemia may be achieved by prescription of small meals with low-carbohydrate composition or by suppression of rapid gut motility and GLP-1 secretion with somatostatin analogs. Although pancreas from some hyperinsulinemic hypoglycemic subjects exhibit enlarged islets and histological evidence of nesidioblastosis, these findings were not associated with enhanced GLP-1R expression in islets from affected individuals (Reubi et al., 2010). Subjects with recurrent hypoglycemia and hyperinsulinemia after meal ingestion after GBS often exhibit much higher levels of GLP-1; however, studies using Ex-9 to block GLP-1 action in GBS subjects did not reveal major differences in the contribution of GLP-1 to enhanced insulin secretion in patients with or without symptomatic hypoglycemia (Salehi et al., 2011). Hence, although very high GLP-1 responses after GBS promote hyperinsulinemia and hypoglycemia, additional factors beyond GLP-1 likely contribute to sensitization of β cell function and exaggerated insulin release in many subjects after GBS.

**GLP-1, the Exocrine Pancreas, and Pancreatitis**

Whether activation of GLP-1R signaling increases the risk of pancreatitis remains uncertain. Ex-4 (10 μg/kg/day for 75 days) modestly increased plasma lipase activity, an indirect reflection of pancreatitis, and enhanced focal histological inflammation in the pancreas of male, Sprague-Dawley rats (Nachnani et al., 2010). However, the animals lost significant amounts (30%) of body weight, an independent risk factor for pancreatitis. Several studies have failed to detect biochemical or histological evidence of pancreatitis in mice or rats continuously treated with GLP-1R agonists for several months. Administration of Ex-4 (0.072–0.72 nmol/kg) for 4 weeks did not alter plasma amylase or lipase activity in rats, and acute Ex-4 administration (0.072–0.72 nmol/kg, 6 hr) decreased the activity of both enzymes after chemically induced pancreatitis (caerulein, 10 μg/kg) in male Sprague-Dawley rats (Tatarkiewicz et al., 2010). Similarly, Ex-4 (7.2 nmol/kg/day for 4 weeks) attenuated increases in plasma amylase and lipase activity after induction of acute pancreatitis (caerulein, 5 × 10 μg/kg) in ob/ob mice and reduced acute inflammation and vacuolation in the exocrine pancreas (Tatarkiewicz et al., 2010). Liraglutide (75 μg/kg/day for 7 days) enhanced the expression of anti-inflammatory genes (REGIIIα and REGIIIβ) in wild-type but not in Gipr−/− mice (Koehler et al., 2009), whereas Ex-4 administered prior to or after the administration of caerulein (6 μg/kg) did not modify the severity of or recovery from pancreatitis. Thus, acute or sustained GLP-1R signaling does not promote enhanced susceptibility to pancreatitis.

Clinically, exenatide therapy did not increase the relative risk of pancreatitis compared with other antidiabetic agents in retrospective analyses of large health care claims databases (Dore et al., 2009; Garg et al., 2010). Nevertheless, acute exenatide administration inhibited cholecystokinin-stimulated gall bladder emptying in healthy nondiabetic subjects (Keller et al., 2012), and multiple isolated cases associating GLP-1R agonists with the development of acute pancreatitis have been reported. Furthermore, pancreatic enzyme levels may be increased in a subset of asymptomatic patients treated with GLP-1R agonists; hence, the actions of GLP-1R agonists on the function of the exocrine pancreas and biliary tract in diabetic or obese human subjects requires further study.

**Incretin Action in the Skeleton**

There is little evidence that GLP-1R agonists directly modify bone formation or resorption. In rodents, the GLP-1R is expressed on calcitonin-producing cells, and GLP-1R activation rapidly increases calcitonin secretion in rats and mice (Bjerre Knudsen et al., 2010). Conversely, Gipr−/− mice exhibit reduced levels of calcitonin mRNA transcripts in the thyroid, increased osteoclast activity, osteopenia, and increased bone resorption, findings reversed by calcitonin administration (Yamada et al., 2008). Although sustained GLP-1R activation produces favorable changes in markers of bone formation and resorption in clinical studies, there is no evidence that GLP-1R agonists produce changes in bone strength or bone quality in human subjects. GIP receptors have been detected in osteoblasts, and sustained activation of GIPR expression produces anabolic actions on bone mass in rodents. Conversely, Gipr−/− mice exhibit reduced osteoclast number, whereas exogenous GIP reduces osteoblast apoptosis in mice (Tsukiyama et al., 2006). Although bone resorption is reduced after meal ingestion in rodents and humans, acute GLP-1 or GIP administration had no effect on markers of bone turnover in postmenopausal women (Henriksen et al., 2003).

**Future of GIP and GLP-1 Research**

The therapeutic potential of manipulating GIP receptor signaling for the treatment of diabetes or obesity remains uncertain. GIPR agonists exhibit potent insulinotropic properties in preclinical studies, and human genome-wide association study data correlate loss-of-function SNPs in the Gipr gene with oral glucose intolerance. However, genetic or pharmacological inhibition of GIPR signaling in preclinical studies prevents or attenuates diet-induced obesity, and the same Gipr SNPs are associated with reduced BMI and waist circumference in human genetic
studies. Future experiments should address (1) mechanism(s) that regulate GIPR signaling in the diabetic islet resulting in enhanced glucagon and reduced insulin secretion and (2) the relative importance of GIPR signaling in the β cell versus the adipocyte for adipogenesis. The mechanisms and tissues important for GIPR-dependent control of body weight (β cell versus adipose tissue versus CNS) also require further clarification, as does the complexity of Gipr mRNA splicing and the expression of GIPR isoforms in different tissues (Ahlvist et al., 2013). The comparative activity of GIP-GLP-1 coagonists, which exhibit promising efficacy in preclinical studies (Tschöp and DiMarchi, 2012), awaits careful assessment in human clinical trials.

Although GLP-1 receptors are widely distributed in diverse extrapancreatic tissues and GLP-1R agonists produce favorable pleiotropic effects on many cells and tissues in preclinical studies, the majority of experiments in vivo do not employ active comparators that provide the same degree of glucose control or weight loss. Hence, the extent to which actions attributed to GLP-1 are direct, or indirect, arising secondary to changes in body weight, glucose, or insulin action, requires more careful elucidation. Furthermore, the majority of "gain-of-function" studies with GLP-1 (and GIP) in rodents and humans achieve circulating levels of these peptides generally far greater than what might be observed even during the postprandial state, requiring consideration of the pharmacological versus the physiological context of the observed action. Similarly, pharmacological levels of GLP-1 and GIP frequently increase insulin levels in acute and chronic preclinical studies, and few experiments employ insulin as an active comparator for analysis of incretin action in vivo.

Of comparable concern is the quality of the current reagents used in studies of incretin action. Ex-9 and most GIPR antagonists are in fact nonspecific and function as partial agonists at their respective receptors (Tschöp and DiMarchi, 2012). The majority of available incretin receptor antagonists and antagonists do not effectively or selectively penetrate the CNS, rendering uncertain the extent to which CNS incretin action observed after i.c.v. or intracerebral peptide administration may be extrapolated to human biology. Moreover, many studies employing i.c.v. or intracerebral peptide administration fail to assess the extent to which these peptides leak into the systemic circulation. The majority of antisera used to localize mouse and human GLP-1R expression do not in fact recognize the GLP-1R under commonly utilized experimental conditions (Panjwani et al., 2013; Pyke and Knudsen, 2013). These findings mandate a reevaluation of the mechanisms of GLP-1 action in different experimental paradigms as the literature contains dozens of publications using nonspecific antisera to infer GLP-1R expression in many tissues and cell types. Hence, the field requires development of better reagents and much more careful characterization of antisera employed for receptor localization studies. Ongoing studies employing highly selective incretin antagonists and cell- and tissue-specific inactivation of incretin receptor expression should advance our understanding of how GLP-1 and GIP exert their actions in different cell types.

The effective translation of preclinical data into comparable robust human observations is hampered by the predominant use of young mice and rats in short term preclinical studies. It is now widely recognized that young, healthy animals frequently exhibit profound differences in biological responses in the endocrine pancreas, bone marrow, and immune and cardiovascular systems relative to older subjects with advanced diabetes and atherosclerosis. The considerable body of preclinical data demonstrating induction of insulin gene expression, enhancement of β cell proliferation, reduction of β cell apoptosis, and expansion of β cell mass in young rodents (Drucker, 2003, 2006) has not been matched by robust data from carefully controlled clinical trials attempting to demonstrate preferential preservation of β cell function in human subjects (Drucker, 2011). Hence, more studies of incretin action in older animals with established diabetes, obesity, and cardiovascular disease seem warranted. The increasing use and success of incretin-based therapies to treat T2DM and reduce body weight is based on a robust body of scientific observations, spanning cellular and molecular biology, preclinical animal studies, and human clinical trials. Future advances in our understanding of incretin biology, and development of coagonists employing two or more incretin-related peptide epitopes within a single molecule (Day et al., 2009; Pocai et al., 2009), may expand the domain of incretin-based therapies into new therapeutic areas and have important implications for the safe and effective use of current incretin-based agents for the treatment of human disease.

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