Cell Metabolism
Perspective

The Cardiovascular Biology of Glucagon-like Peptide-1

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Glucagon-like peptide-1, produced predominantly in enteroendocrine cells, controls glucose metabolism and energy homeostasis through regulation of islet hormone secretion, gastrointestinal motility, and food intake, enabling development of GLP-1 receptor (GLP-1R) agonists for the treatment of diabetes and obesity. GLP-1 also acts on the immune system to suppress inflammation, and GLP-1R signaling in multiple tissues impacts cardiovascular function in health and disease. Here we review how GLP-1 and clinically approved GLP-1R agonists engage mechanisms that influence the risk of developing cardiovascular disease. We discuss how GLP-1R agonists modify inflammation, cardiovascular physiology, and pathophysiology in normal and diabetic animals through direct and indirect mechanisms and review human studies illustrating mechanisms linking GLP-1R signaling to modification of the cardiovascular complications of diabetes. The risks and benefits of GLP-1R agonists are updated in light of recent data suggesting that GLP-1R agonists favorably modify outcomes in diabetic subjects at high risk for cardiovascular events.

Among several dozen peptides secreted from enteroendocrine cells (EECs), glucagon-like peptide-1 (GLP-1(7-36)amide and GLP-1(7-37), collectively referred to as GLP-1) has become the most extensively studied gut hormone with the greatest translational relevance. Initial studies of GLP-1 focused on its role as incretin hormone, stimulating glucose-dependent insulin secretion in the context of nutrient ingestion. Subsequent studies extended GLP-1 actions in islets to encompass control of proinsulin gene expression and biosynthesis, as well as β cell proliferation and survival (Drucker, 2006). Concomitant experiments identified that GLP-1 rapidly suppressed glucagon secretion from α cells, likely through indirect mechanisms via inhibitory factors released from β cells and through stimulation of local δ cell somatostatin secretion (Campbell and Drucker, 2013). The majority of evidence highlighting the importance of GLP-1 in the control of metabolism derives from physiological and pharmacological studies; however, human genetic studies associate variation within the coding region of the *GLP1R* with relative levels of glycemia, risk of type 2 diabetes (T2D), and insulin secretion (Wessel et al., 2015).

Activation of the GLP-1 receptor (GLP-1R) also leads to inhibition of gastric and small bowel motility, resulting in delayed nutrient absorption; acute and sustained GLP-1R signaling reduces appetite, leading to reduced food ingestion and weight loss. Some actions of GLP-1R agonists, most notably the inhibition of gastric emptying, are subject to rapid tachyphylaxis, detectable within hours (Meier, 2012); however, the glucose-dependent control of insulin and glucagon secretion and reduction of food intake are maintained following sustained activation of GLP-1R signaling. The first GLP-1R agonist was approved for the treatment of T2D in 2005 (Drucker and Nauck, 2006). Subsequently, multiple GLP-1R agonists, each with different structures, modes of administration, potential for immunogenicity, and unique pharmacokinetics, have been approved for the treatment of T2D, and one GLP-1 analog, liraglutide, is approved for the treatment of obesity (Drucker, 2015; Meier, 2012; Ussher and Drucker, 2014). Collectively, the interest in these new agents has fostered considerable research activity, and new information on mechanisms of action and clinical data on long-term safety continue to accumulate (Campbell and Drucker, 2013; Drucker, 2013, 2015; Meier, 2012; Sandoval and D'Alessio, 2015).

The goals for treatment of diabetes are patient specific and include reduction of glycemia while avoiding hypoglycemia, prevention of weight gain, and decreasing the burden of long-term complications. The complications arising secondary to diabetes are often subclassified based on microvascular or macrovascular pathophysiology (Forouhi and Wareham, 2014) and include neuropathy, retinopathy, nephropathy (largely microvascular), and both cerebrovascular and cardiovascular (CV) injury, predominantly manifesting as stroke, heart failure (HF), and myocardial infarction (MI). Sustained reduction in glycemia reduces rates of microvascular disease in subjects with T2D (American Diabetes Association, 2016). In contrast, the importance of glucose control for reducing macrovascular disease has been more difficult to ascertain, reflecting challenges in clinical trial design and insufficient long-term follow-up. Nevertheless, it is now widely accepted that reduction of glycemia over time does translate into meaningful reductions in rates of major CV outcomes in subjects with T2D (Holman et al., 2014).

Our understanding of the risks and benefits of GLP-1R agonists, together with their underlying mechanisms of action, has been informed by results of large clinical trials, smaller mechanistic clinical studies, and extensive preclinical experimentation. More recently, preliminary results of several CV outcome studies suggest that some GLP-1R agonists significantly reduce the risk of major CV complications, principally MI, and CV death. Here we review the biology of native GLP-1 and degradation-resistant GLP-1R agonists approved for the treatment of T2D, updating mechanisms and clinical concepts that have evolved since our most recent review in this journal (Campbell and Drucker,

2013). Results from preclinical and clinical studies of GLP-1 and degradation-resistant agonists are sometimes contradictory, perhaps reflecting differences in species, experimental methodology, and duration of drug administration, ultimately impacting pharmacokinetics and pharmacodynamics. Accordingly, we purposively provide, where appropriate, relevant study details illustrating how key experiments were done and highlighting experimental differences that may impact results obtained.

A major goal and unmet need of T2D therapy is the prevention of macrovascular disease, and recent clinical trial results with empagliflozin highlight the potential for anti-diabetic therapy to reduce the burden of CV disease (Zinman et al., 2015). Hence, we focus this Perspective on recent advances in knowledge illuminating how GLP-1R signaling modifies CV biology, risk factors, and CV outcomes. While mindful of related information surrounding the metabolic and CV biology of dipeptidyl peptidase-4 (DPP-4) and the DPP-4 inhibitors, this topic is itself extensive in scope and has been discussed elsewhere (Mulvihill and Drucker, 2014; Ussher and Drucker, 2014; White and Baker, 2016). Similarly, as recent reviews provide useful updates focused on incretin and EEC biology and control of metabolism, we refer the interested reader to the following resources for more general background information: Campbell and Drucker (2013), Drucker (2016), Gribble and Reimann (2016), Meier (2012), and Sandoval and D'Alessio (2015).

GLP-1: A Target and Mediator of the Inflammatory Response

The role of systemic and localized tissue inflammatory responses in the pathophysiology of diabetes, obesity, and cardiovascular disease (CVD) has received considerable attention (Libby and Hansson, 2015). More recently, the gut and its immune and EEC networks have been revealed as major targets for control of inflammation impacting the local gut mucosa, as well as distant organs including liver, adipose tissue, muscle, heart, blood vessels, and islets (Drucker, 2016; Winer et al., 2016). Indeed, anti-inflammatory agents selectively targeting specific mediators of the inflammatory response have shown therapeutic benefit in subjects with T2D and remain under active investigation (Donath, 2014). Increasing evidence, reviewed below, demonstrates that inflammatory stimuli increase GLP-1 secretion, and GLP-1, in turn, modulates inflammation in multiple sites, including the heart and blood vessels. Hence, understanding the importance of emerging networks linking inflammatory signals to the synthesis, secretion, and actions of GLP-1 may have direct relevance for the development of CVD.

Inflammatory Signals Regulate GLP-1 Synthesis, Secretion, and Action

Circulating levels of intestinal proglucagon-derived peptides (PGDPs), including GLP-1, were increased in rodents with experimental inflammation induced by injection of cytokines or lipopolysaccharide (LPS) (Ellingsgaard et al., 2011; Kahles et al., 2014; Nguyen et al., 2014). Levels of plasma GLP-1 were also markedly elevated ~6.9-fold in 155 critically ill human subjects assessed on admission to an intensive care unit (Kahles et al., 2014), and a smaller but significant 2-fold increase in circulating total GLP-1 levels was observed in 22 non-diabetic human subjects studied within 24 hr of cardiac surgery and cardiopulmonary bypass (Lebherz et al., 2016). In both clinical studies, levels of interleukin-6 (IL-6) were elevated and correlated with plasma levels of total GLP-1. In contrast, a 10 min infusion of low dose LPS (0.5 ng/kg) in healthy, young, non-obese human subjects robustly increased plasma levels of TNF-α, IL-6, and IL-1 receptor antagonist (IL-1ra), but plasma levels of GLP-1 remained unchanged over a 6 hr observation period (Ziegler et al., 2015).

The direct effects of inflammatory mediators on GLP-1 secretion encompass both inhibitory and stimulatory actions. Interleukin-1 (IL-1), IL-6, and LPS all acutely increased plasma levels of GLP-1 in mice; however, only IL-6 and LPS directly increased GLP-1 secretion from enteroendocrine L cells or the murine GLUTag cell line in vitro (Ellingsgaard et al., 2011; Kahles et al., 2014; Nguyen et al., 2014). IL-6 also increased pancreatic glucagon secretion in the context of concomitant stimuli such as LPS or hypoglycemic stress in mice, through mechanisms that involve direct effects on islet α cells, as well as via CNS augmentation of autonomic tone (Barnes et al., 2014). The IL-6-dependent induction of islet GLP-1 biosynthesis and secretion has been linked to reprogramming of gene expression in rodent and human islet α cells, enabling induction of Pcsk1 expression and production of intact GLP-1 from proglucagon (Ellingsgaard et al., 2011). Whether these actions of IL-6 are preserved in the islets of normal, diabetic, or obese human subjects within their normal pancreatic location in vivo is more difficult to determine and has not yet been definitively ascertained.

The incretin hormone glucose-dependent insulinotropic polypeptide (GIP) increased gut GLP-1 secretion in rodents and enhanced islet GLP-1 biosynthesis and secretion; mice and human islet α cells treated with GIP (10–100 nM) under conditions of low (2.8 mM) or high (16.7 mM) glucose exhibit islet production of GLP-1 through recruitment of islet-derived IL-6 and induction of Pcsk1 in α cells (Timper et al., 2016). However, GIP does not increase plasma levels of GLP-1 in normal or diabetic human subjects, and there are no data examining whether GIP augments GLP-1 secretion in humans with experimental or clinical inflammation. Evidence for the importance of a cytokine-PGDP axis in the rat and mouse brain derives from observations that centrally administered exendin-4 increased the expression of IL-6 and IL-1β in the hypothalamus (and IL-6 in the hindbrain), whereas pharmacological disruption or genetic attenuation of either IL-6 or IL-1β signaling reduced the delayed anorectic response to central or peripheral exendin-4 (Shirazi et al., 2013). IL-6 may also be important for the synthesis, secretion, and anorectic actions of GLP-1 in the brain, as almost 40% of the GLP-1+ neurons within the nucleus of the solitary tract express the IL-6 receptor α, and these PGDP+ neurons respond to exogenous IL-6 with an increase in cytosolic Ca^{2+} (Anesten et al., 2016).

Acute exposure to TNF-α increased GLP-1 secretion from human NCIH716 EECs; however, GLP-1 levels were not increased in mice after acute TNF-α injection (4 mg/kg) (Kahles et al., 2014), nor in healthy, young male human subjects after 4–6 hr of intravenous TNF-α infusion (1 $\mu g/m^2$ body surface area per hr), a dose that produces circulating TNF-α levels of ~30 pg/mL (Lehrskov-Schmidt et al., 2015; Nielsen et al., 2013). In contrast, chronic exposure to TNF-α inhibited GLP-1 secretion from EECs in vitro, whereas administration of the TNF-α blocker, etanercept, to high-fat-diet-fed mice improved the secretion of GLP-1 from primary murine EEC cultures derived from the

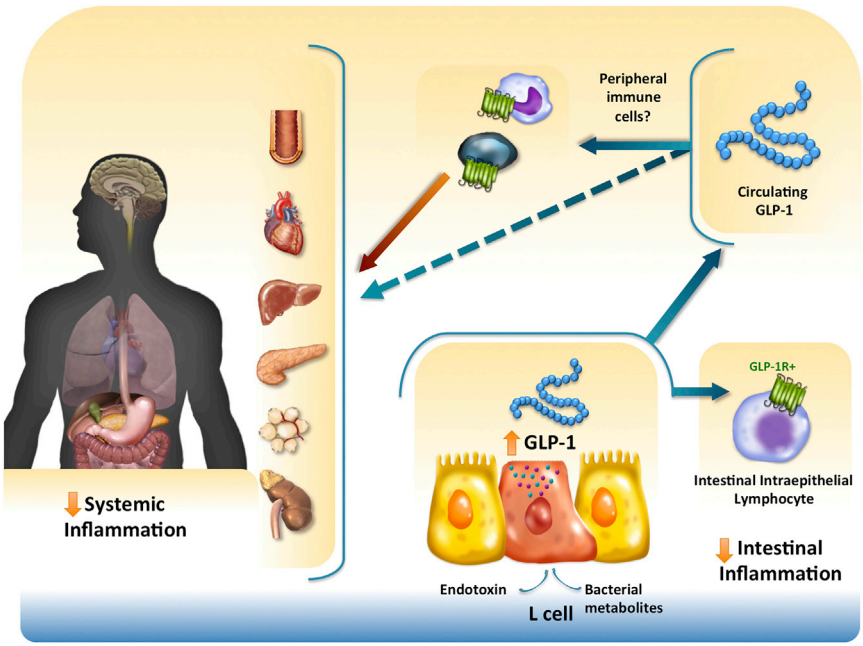

Figure 1. Mechanisms Linking GLP-1 to Modulation of Inflammation

GLP-1 secretion from EECs is alternatively stimulated or inhibited by pro-inflammatory stimuli. GLP-1 in turn may control inflammation locally in the intestine through engagement of GLP-1 receptors on intestinal intraepithelial lymphocytes (IELs). GLP-1 may also reduce inflammation in different peripheral organs indirectly through weight loss or improved glucose control, or by targeting GLP-1Rs expressed on populations of circulating immune cells. Alternatively, GLP-1R activation may directly reduce inflammation in organs and cell types expressing the GLP-1R. The dashed line linking GLP-1 to anti-inflammatory actions in distinct organs reflects current uncertainty as to whether these actions are possibly direct or largely indirect.

etanercept-treated mice ex vivo (Gagnon et al., 2015). Pre-treatment with the cytokine RANTES (regulated on activation; normal T cell expressed and secreted; 10 mM for 2 hr) also directly inhibited glucose-stimulated GLP-1 secretion from NCI-H716 EECs in vitro, and four daily injections of RANTES (10 mg/injection) markedly reduced plasma levels of GLP-1 and the related PGDP, GLP-2, measured following oral glucose administration in 4-month-old C57BL/6 mice. Hence, inflammatory signals regulate L cell secretory activity in multiple species in a context-dependent manner.

A local gut L cell-T cell network has been revealed through studies identifying expression of a functional endogenous GLP-1R expressed within intestinal intraepithelial lymphocytes (IELs) (Yusta et al., 2015). Basal expression of cytoprotective and anti-inflammatory genes was markedly dysregulated in the intestinal mucosa of *Glp1r*$^{-/-}$ mice, and the abnormal gene expression profile was substantially reversed following selective reconstitution of GLP-1R+ intestinal IELs through bone marrow transplantation. The importance of basal intestinal GLP-1R signaling for control of local inflammatory signals was revealed through demonstration that *Glp1r*$^{-/-}$ mice exhibit gut microbial dysbiosis and markedly increased sensitivity to experimental colonic inflammation (Yusta et al., 2015). Whether the gut IEL GLP-1R mediates a component of the systemic anti-inflammatory responses attributed to GLP-1R agonists (Drucker and Rosen, 2011) has not yet been determined.

GLP-1 Exerts Anti-inflammatory Actions in Preclinical Studies of the CV System

Both native GLP-1 and GLP-1R agonists reduce cardiac and vascular inflammation through direct and indirect mechanisms (Figure 1). Multiple studies have demonstrated that GLP-1R agonists, including native GLP-1, exenatide (synthetic exendin-4), and liraglutide, often administered at doses that do not produce weight loss, attenuated the development of atherosclerosis in genetically sensitized murine models (Ussher and Drucker, 2014). These peptides reduced vascular monocyte adhesion and/or macrophage accumulation within blood vessels of several strains of normoglycemic or dysglycemic mice at high risk for the development of experimental atherosclerosis and cardiac dysfunction (Arakawa et al., 2010; Nagashima et al., 2011; Noyan-Ashraf et al., 2013).

The anti-atherogenic and anti-inflammatory actions of native GLP-1 administered for 4 weeks by continuous infusion (2.2 nmol/kg/day) in *Apoe*$^{-/-}$ mice were blocked by co-administration of the GLP-1R antagonist exendin(9-39) (Nagashima et al., 2011) and were not mimicked by GLP-1(9-36)amide, consistent with a role for the canonical GLP-1R in transducing these therapeutic actions. Nevertheless, Burgmaier and colleagues observed anti-inflammatory effects (reduced plaque macrophage and matrix metalloproteinase-9 [MMP-9] accumulation), enhanced plaque stability, and reduced plaque lesion area in *Apoe*$^{-/-}$ mice following 12 weeks of adenoviral expression of either GLP-1(7-37) or its N-terminally truncated cleavage products, GLP-1(9-37) or GLP-1(28-37) (Burgmaier et al., 2013). Hence, non-GLP-1R-dependent mechanisms may underlie some of the anti-inflammatory and anti-atherogenic actions detected using native GLP-1. In contrast, plaque area, macrophage accumulation, or lipid content was not reduced in the aortic arch of high-fat-fed, modestly hyperglycemic *Apoe*$^{-/-}$ mice treated for 12 weeks with a long-acting GLP-1R agonist, taspoglutide, administered continuously at doses sufficient to improve glucose and hepatic lipid metabolism (Panjwani et al., 2013). Moreover, *Glp1r* mRNA transcripts were not detected in RNA from peritoneal or adipose tissue macrophages collected under basal or pro-inflammatory conditions. Hence, experiments examining roles for GLP-1R signaling in the control of atherosclerosis development, macrophage migration, and vascular accumulation yield inconsistent results, perhaps reflecting differences in methodology, mode, and duration of peptide administration, and the age and metabolic status of the experimental models studied.

A short period of liraglutide administration for 7 days reduced cardiac inflammation in C57BL/6 mice fed a high-fat diet for 16 or 32 weeks. These mice exhibited cardiac insulin resistance,

reduced levels of cardiac phosphorylated AMP-activated protein kinase (pAMPK), increased cardiac lipid accumulation, and enhanced TNF-α and NFκB protein expression (Noyan-Ashraf et al., 2013); one week of liraglutide administration, 30 μg/kg twice daily, a dose that did not produce significant weight loss, attenuated inflammation and reduced lipid accumulation in the murine heart. Furthermore, liraglutide reduced monocyte adhesion to TNF-α-activated human umbilical vein endothelial cell (HUVEC) cultures, actions blocked by co-administration of exendin(9-39) (Noyan-Ashraf et al., 2013). Moreover, results of multiple preclinical studies demonstrate that GLP-1R agonists administered prior to or after the induction of ischemic myocardial injury reduced cardiac inflammation after MI. Nevertheless, definitive identification of canonical GLP-1R expression (via demonstration of mRNA transcripts encoding the entire *GLP1R* open reading frame and the use of validated antisera for immunodetection of an authentic GLP-1R protein) in vascular cells, ventricular cardiomyocytes (CMs), or immune cell populations is often lacking. Whether the anti-inflammatory effects described in preclinical studies reflect direct actions of GLP-1R agonists on immune cells, blood vessels, or CMs, or arise secondary to indirect metabolic or weight loss effects, remains controversial (Ussher and Drucker, 2012, 2014).

Human Studies of GLP-1 and Inflammation

The induction of controlled hypoglycemia (2.9 mM glucose) over 2 hr with an insulin infusion in male and female subjects with type 1 diabetes (T1D; mean age 24) was associated with significant increases in plasma levels of IL-6 and intracellular adhesion molecule (ICAM)-1, as well as elevated levels of markers of oxidative stress, nitrotyrosine and 8-iso prostaglandin F 2α (PGF2α) (Ceriello et al., 2013). Co-infusion of native GLP-1 (0.4 pmol/kg/min) significantly reduced plasma levels of these molecules. Similarly, hyperglycemic clamp experiments (15 mM glucose) in a separate cohort of male and female subjects with T1D were also associated with induction of circulating levels of IL-6, ICAM-1, nitrotyrosine, and PGF2α, with co-infusion of GLP-1 markedly attenuating plasma levels of these inflammatory and oxidative stress biomarkers (Ceriello et al., 2013). Complementary studies employed euglycemic hyperinsulinemic or euinsulinemic clamp to examine circulating parameters reflecting oxidative stress and inflammation in non-obese human subjects with T2D. Co-infusion of GLP-1 (0.4 pmol/kg/min) decreased circulating levels of IL-6, sICAM-1, 8-iso-PGF2a, and nitrotyrosine under conditions of normal or increased insulinemia in the presence of euglycemia (Ceriello et al., 2014). Hence, GLP-1 exerts acute anti-inflammatory and antioxidant actions in humans under specific controlled conditions, effects that are not simply mediated by changes in insulin levels.

Administration of exenatide (10 μg twice daily for 12 weeks) in obese (BMI ~39 kg/m^2) subjects with T2D receiving concomitant oral antidiabetic agents and insulin was associated with reduced circulating levels of monocyte chemoattractant protein-1 (MCP-1), serum amyloid A, MMP-9, and IL-6, without any detectable weight loss (Chaudhuri et al., 2012). Nevertheless, significant reductions in HbA1c, free fatty acids (FFAs), fasting glucose, and insulin were demonstrated in these exenatide-treated patients after 12 weeks, precluding ascertainment of whether the anti-inflammatory effects of exenatide reflected direct effects on the immune system or were secondary to metabolic improvement in this clinical setting. A single 5 μg injection of exenatide in the same subjects in the fasting state on the first day of the trial also produced reductions in levels of *TNFα*, *IL1β*, *JNK1*, *SOCS3*, and *TLR4* mRNA transcripts in circulating mononuclear cells over 2–6 hr. Nevertheless, it is similarly challenging to determine whether acute changes in FFAs, glucose, insulin, and other metabolic parameters contributed to the rapid anti-inflammatory effects of exenatide in this setting (Chaudhuri et al., 2012). Anti-inflammatory actions of exendin-4 (50 nM, 24 hr), namely attenuation of cytokine and chemokine production ex vivo, have also been demonstrated in human mononuclear cell cultures propagated from cells isolated from male and female subjects with recent-onset T2D; expression of the *GLP1R* was not examined in these studies (He et al., 2013).

Anti-inflammatory actions of exenatide and liraglutide have also been described in human subjects with diabetes, obesity, and psoriasis, an inflammatory skin disease known to be associated with increased risk of CVD (Drucker and Rosen, 2011). Improvement in psoriasis in three subjects was associated with reduced accumulation of invariant CD1D-restricted natural killer T cells (iNKTCs) in psoriatic plaques. Liraglutide stimulated cyclic AMP accumulation in iNKTCs ex vivo, postulated to be mediated by expression of an endogenous GLP-1R in iNKTC (Hogan et al., 2011). However the patients also experienced rapid weight loss during treatment with exenatide or liraglutide, confounding mechanistic interpretation as to how these drugs decreased inflammation. Anti-inflammatory actions of liraglutide were subsequently reported in ten obese subjects with T2D and psoriasis who experienced a 3 kg weight loss over 8 weeks (Hogan et al., 2014). Circulating levels of soluble cluster of differentiation 163(sCD163) were reduced, and production of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, was decreased in peripheral blood mononuclear cells following liraglutide treatment. The decreased levels of inflammatory markers detected after liraglutide therapy did not correlate with reductions in fructosamine, body weight, or HbA1c; moreover, liraglutide directly modulated cytokine expression in macrophages derived from the human THP-1 monocyte cell line (Hogan et al., 2014). Hence, the authors postulated (despite lack of unequivocal evidence for expression of an endogenous iNKTC GLP-1R) that GLP-1R signaling directly regulates immune cell function.

Direct versus Indirect Mechanisms for the Control of Inflammation

Exendin-4 (10 nM) directly reduced the LPS-stimulated secretion of cytokines (interleukin-1β, TNFα, and interleukin-10) in human peripheral blood mononuclear (monocyte) cells isolated from healthy, non-diabetic male and female subjects, actions that were blocked by co-administration of 50 nM exendin (9-39) (Bułdak et al., 2016). Anti-inflammatory effects of GLP-1R agonists were also demonstrated using human aortic endothelial cells (HAECs) pre-treated with liraglutide (100 nM) for 1 hr, then exposed to TNF-α or LPS plus liraglutide for 3 hr. The induction of vascular cell adhesion molecule-1 (VCAM-1) and E-selectin expression was attenuated, and adhesion of THP-1 monocytes to HAECs was reduced by liraglutide, actions requiring increases in intracellular Ca^{2+}, Ca^{2+}/calmodulin-dependent protein kinase (CAMK)-, and AMPK-dependent signaling (Krasner et al., 2014). The canonical *Glp1r* has been detected in resident and circulating murine immune cells, although levels of mRNA transcripts

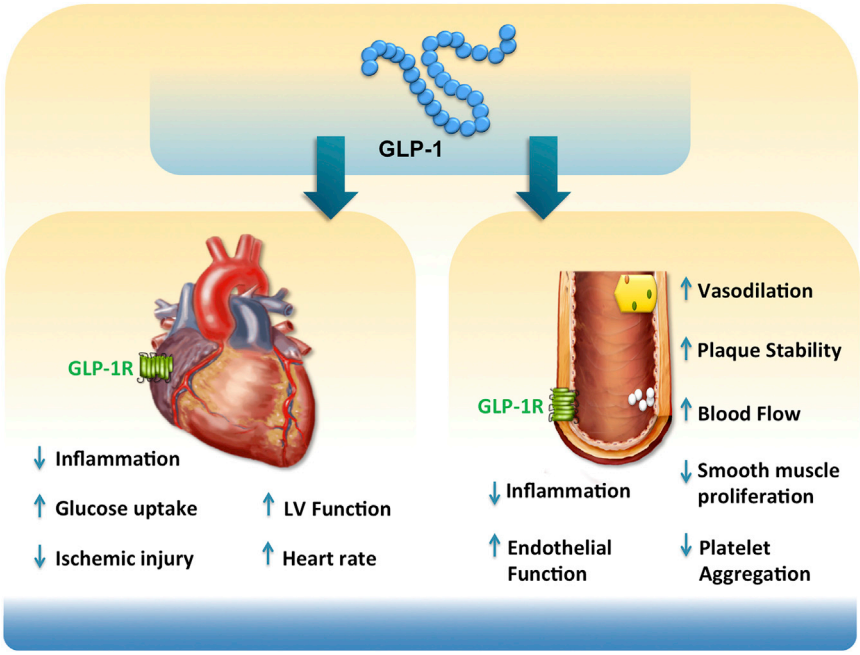

Figure 2. Direct and Indirect Actions of GLP-1 in the Heart and Blood Vessels
The GLP-1R is expressed predominantly in the atrium of the heart. The localization of GLP-1R expression in blood vessels is less well understood. Some blood vessels express the GLP-1R within vascular smooth muscle, whereas potential GLP-1R expression in endothelial cell populations is less completely defined. The actions of GLP-1 on heart and blood vessels are shown, and may be direct or indirect, depending on the species and specific experimental paradigm examined.

corresponding to the full-length open reading frame for the murine *Glp1r* were very low in splenocytes, thymocytes, and bone marrow cells (Hadjiyanni et al., 2010). Similarly, *Glp1r* mRNA transcripts and GLP1R immunoreactive protein(s) have been detected in murine monocytes and macrophages as well as macrophage cell lines by PCR and immunoblotting, respectively; nevertheless, full-length *Glp1r* mRNA transcripts were not consistently detectable in mouse peritoneal or tissue macrophages and the sensitivity and specificity of commonly used GLP1R antisera are suboptimal (Panjwani et al., 2013; Pyke and Knudsen, 2013). Hence, whether GLP1R agonists modify immune cell function through direct or indirect mechanisms (Figure 1), involving signal transduction through the canonical GLP1R requires additional, more precise experimentation.

GLP-1R Signaling and Protection from Ischemic Cardiac Injury

Multiple studies in animals and some clinical trials demonstrate that activation of GLP-1R signaling for even a few hours produces cardioprotective actions (Figure 2) (Lønborg et al., 2012; Ussher and Drucker, 2014). Preclinical studies demonstrate robust cardioprotection following administration of GLP-1R agonists in the setting of permanent or transient left anterior descending coronary artery occlusion, associated with reduced infarct size, improved survival, and preservation of ventricular function (Ussher and Drucker, 2012, 2014). However, the precise mechanisms linking activation of GLP-1R signaling in CM or non-cardiac cell types to attenuation of ischemic cardiac injury remain unclear.

Preclinical Studies of Cardiac Ischemia

Infusion of native GLP-1 for 30 min prior to and 24 hr following ischemia-reperfusion injury in non-diabetic male Sprague-Dawley rats to achieve peak plasma levels of 212 ± 2 pM was associated with reduced infarct size, increased myocardial glucose uptake, as well as increased glucose utilization in the non-ischemic myocardium, during a euglycemic euinsulinemic clamp (Aravindhan et al., 2015). In contrast, no relative substrate switching, determined by assessing the ratio of carbohydrate versus fat oxidation, was detected within the area at risk, despite an increase in cyclic AMP and lactate accumulation. Whether these regional changes in cardiac fuel metabolism were mediated through the GLP-1R and possibly contributed to the reduction in infarct size was not ascertained.

Activation of canonical GLP-1R signaling engages cytoprotective pathways in the rodent heart, leading to reduced infarct size, associated with GLP-1R-dependent induction of a cardioprotective gene and protein expression profile (Noyan-Ashraf et al., 2009). GLP-1R agonists are directly cardioprotective, as exposure of primary CM cultures or CM cell lines to native GLP-1 or degradation-resistant GLP-1R agonists activates signaling pathways, leading to enhanced cell survival and reduced oxidative stress (Ussher and Drucker, 2014). Similarly, infusion of GLP-1 or GLP-1R agonists such as exenatide or lixisenatide in the coronary circulation of isolated perfused hearts, either prior to or concomitant with the induction of ischemia, leads to recovery of ventricular-developed pressure and reduced infarct size (Ban et al., 2008; Ussher and Drucker, 2012, 2014; Wohlfart et al., 2013).

Both native GLP-1 and liraglutide activated AMPK-dependent pathways in normoglycemic and diabetic mouse hearts and rat CMs (Noyan-Ashraf et al., 2013; Ussher and Drucker, 2014); pharmacological blockade of AMPK signaling attenuated the cardioprotective actions of liraglutide in mice with high-fat-diet-fed cardiomyopathy (Noyan-Ashraf et al., 2013). Furthermore, the acute actions of GLP-1 (100 nM, 105 min) to attenuate glucolipotoxicity and reduce generation of reactive oxygen species (ROS) were extinguished in mouse *Prkaa1*$^{-/-}$ CMs ex vivo (Balteau et al., 2014). The cardioprotective actions of native GLP-1 or GLP-1(9-36) were also examined in swine subjected to cardiac ischemia induced by coronary artery ligation (Goodwill et al., 2014). Acute administration of both peptides was commenced 30 min prior to ischemia and continued for an additional 30 min at a dose of 10 pmol/kg/min. Neither peptide affected coronary artery blood flow or myocardial contractility. Native GLP-1, but not GLP-1(9-36), increased cardiac output during ischemia, which correlated with increases in end-diastolic

volume (preload), and which occurred independently of co-administration of hexamethonium to block nicotinic acetylcholine receptors and ganglionic neurotransmission (Goodwill et al., 2014).

Understanding how native GLP-1 and GLP-1R agonists exert cardioprotective actions remains challenging in light of studies re-evaluating cardiac expression of the endogenous primate and rodent GLP-1R. Although immunocytochemical analyses initially localized GLP-1R immunopositivity to ventricular CMs and endothelial cells (Ban et al., 2008; Ussher and Drucker, 2012, 2014), interpretation of these results is challenged by findings that many GLP-1R antisera exhibit suboptimal sensitivity and a lack of specificity (Panjwani et al., 2013; Pyke et al., 2014; Pyke and Knudsen, 2013). Re-analysis of GLP-1R expression in the murine, rat, and human heart has revealed expression of *Glp1r* mRNA transcripts, *GLP1r*-directed reporter proteins, and GLP-1R protein predominantly in atrial cardiomyocytes and the sinoatrial node (Kim et al., 2013; Pyke et al., 2014; Richards et al., 2014; Wohlfart et al., 2013). It seems likely that the cardioprotective actions of native GLP-1 demonstrated in human and animal studies, perfused ischemic hearts, and CM cultures (Ban et al., 2008, 2010; Read et al., 2011, 2012; Ussher and Drucker, 2014) are mediated in part through non-GLP-1R-dependent pathways. Indeed, smaller GLP-1-derived peptides that do not signal through the canonical GLP-1R, including GLP-1(9-36)/(9-37) and the structurally related peptides GLP-1(28-36)/(28-37), attenuated the extent of macrophage plaque infiltration and promoted plaque stability following adenoviral expression (to produce circulating levels of ~200 pmol in *Apoe*$^{-/-}$ mice) independently of changes in glycemia (Burgmaier et al., 2013). Similarly, continuous infusion of GLP-1(9-36), 25 nmol/kg/day for 4 weeks, in female C57BL/6 mice following permanent left anterior descending coronary artery ligation did not modify body weight, plasma glucose, or infarct size. However, the extent of diastolic dysfunction was significantly attenuated, and pro-inflammatory cytokine expression and macrophage infiltration were reduced in hearts of GLP-1(9-36)amide-treated mice (Robinson et al., 2016). Although mechanisms linking GLP-1(9-36) or GLP-1(28-36) to improved ventricular function remain poorly defined, direct cardioprotective actions of GLP-1(9-36) remain evident in hearts or CMs from *Glp1r*$^{-/-}$ mice (Ban et al., 2008, 2010). Nevertheless, multiple preclinical studies also demonstrate direct and rapid cardioprotection ensuing from administration of degradation-resistant GLP-1R agonists in ischemia reperfusion experiments ex vivo (Ussher and Drucker, 2012, 2014). How GLP-1R signaling in atrial CMs, or perhaps in coronary arteries, contributes to the recovery of developed pressure and reduced infarct size is not understood.

Cardioprotection with GLP-1R Agonists in Clinical Studies

Seminal pilot studies commencing native GLP-1 infusion for 72 hr after angioplasty in human subjects with acute MI and impaired (<40%) ejection fraction (EF) revealed significant improvement in ventricular function assessed by echocardiography (Nikolaidis et al., 2004); however, these studies were not randomized or placebo controlled. Rather, the outcomes in subjects treated with GLP-1 were compared to findings in control subjects with similar disease characteristics. A randomized, placebo-controlled study administered daily exenatide (or vehicle) for 3 days in patients with ST-segment elevation myocardial infarction (STEMI) treated with balloon angioplasty and stents; exenatide therapy reduced levels of creatine kinase-MB and troponin I and decreased infarct size assessed by cardiac MRI 38 days after reperfusion (Woo et al., 2013). Furthermore, echocardiographic analysis at 6 months revealed persistent improvement in diastolic function and global longitudinal strain after transient exenatide administration.

A much larger group of subjects (n = 172) with STEMI was randomized to receive intravenous exenatide commencing at 0.12 mg/min (or placebo), started 15 min before their intervention and continued, at a lower infusion rate, for 6 hr, to achieve mean plasma levels of 0.177 nM, after reperfusion via percutaneous coronary intervention (PCI) (Lønborg et al., 2012). A total of 117 patients completed the protocol, including assessment using cardiac MRI after 3 months. Exenatide-treated subjects exhibited a significantly reduced ratio of infarct size to area at risk; however, no changes in peak levels of troponin, EF, or clinical endpoints were detected across groups. Although patients with hyperglycemia (glucose > 8.3 mM, 40% of subjects) presented with larger infarct size, the exenatide-mediated reduction in infarct size was independent of the levels of glycemia (Lønborg et al., 2014).

Cardioprotective actions of exenatide versus placebo have also been examined in human subjects with and without diabetes presenting with STEMI and an EF > 40%; exenatide (n = 18 subjects) or placebo (n = 40) was administered immediately prior to PCI and twice daily (10 μg exenatide) for the next 48 hr (Woo et al., 2013). Subjects treated with exenatide exhibited reduced levels of creatinine kinase MB and troponin-I and reduced infarct size quantified by cardiac MRI ~38 days post-MI. Furthermore, left ventricular EF was higher and strain was lower in exenatide-treated subjects as assessed by echocardiography after 6 months.

Chen and colleagues examined whether liraglutide (or placebo) modified CV outcomes in 92 subjects with STEMI at a single center, with drug administered immediately prior to PCI and continued daily for 7 days (Chen et al., 2015). Liraglutide therapy was associated with lower levels of troponin T assessed at day 5 and a small (4.1%) but significant improvement in left ventricular ejection fraction (LVEF) assessed at 3 months in both non-diabetic and diabetic subjects.

Whether activation of GLP-1R signaling selectively improves ventricular function in human subjects with or without ischemic heart disease or HF remains unclear. Although GLP-1 increases heart rate (HR), which may in turn augment cardiac output, evidence for independent effects of GLP-1 on ventricular function is inconclusive. McCormick and colleagues infused native GLP-1 (1.2 pmol/min) or vehicle alone immediately prior to and after balloon occlusion of the left anterior descending coronary artery in human subjects with single vessel disease and EF > 50% awaiting PCI. GLP-1-treated subjects exhibited reduced systolic and diastolic dysfunction during ischemia and improved recovery of ventricular function, without evidence of changes in myocardial glucose or fatty acid uptake (McCormick et al., 2015). Wallner and colleagues assessed *GLP1R* expression and the direct effects of GLP-1R agonists on right atrial appendages from human hearts. Exenatide (6 nM) augmented developed

force in atrial strips, findings attenuated by co-incubation with exendin(9-39); the effects of exenatide were mimicked by native GLP-1, but not by GLP-1(9-36) (Wallner et al., 2015). Both thapsigargin and the PKA inhibitor H-89 attenuated the contractile effects of exenatide in atrial muscle. In contrast to the response obtained in atrial appendages, exenatide had no effect on developed pressure in the majority of cardiac muscle strips from human ventricular myocardium. However, qPCR analysis detected *GLP1R* expression (full-length *GLP1R* transcript was not assessed) in RNA from both the right atrium and ventricle from seven non-failing hearts and in isolated left ventricular CMs, with relatively higher (3.3-fold) *GLP1R* expression in right atrium compared to right ventricle (Wallner et al., 2015). Similar, transcriptomic analysis of human left ventricular RNA detects the presence of *GLP1R* mRNA transcripts (http://www.gtexportal.org/home/). These findings highlight the importance of determining whether *GLP1R* mRNA transcripts detected in normal or diseased human hearts are translated into a functional GLP-1R protein.

Ventricular Function and HF

A large number of preclinical studies have demonstrated therapeutic benefits of native GLP-1 and degradation-resistant GLP-1R agonists in diverse models of ventricular dysfunction and HF (Ban et al., 2008; Ussher et al., 2014; Ussher and Drucker, 2012). Many of these studies demonstrate striking changes in ventricular genes and proteins following administration of GLP-1R agonists in animal models of HF (Mulvihill et al., 2016; Noyan-Ashraf et al., 2013). Nevertheless, the cellular targets and pathways linking GLP-R signaling to improved cardiac structure and function have not been clearly delineated. Unexpectedly, beneficial actions of native GLP-1 in dogs with pacing-induced dilated cardiomyopathy were mimicked by GLP-1(9-36) (Nikolaidis et al., 2005), suggesting that at least some of the therapeutic actions of native GLP-1 in HF may be mediated by non-GLP-1R-dependent mechanisms. A 5 week continuous subcutaneous infusion of GLP-1 (2.5 pmol/kg/min) in 12 patients with New York Heart Association (NYHA) class III–IV HF improved LVEF, walking time, and quality of life scores (Sokos et al., 2006). However short-term studies of native GLP-1 continuously infused (0.7 pmol/kg/min) over 48 hr in human subjects with stable HF, NYHA class II–III, and EF ~30% failed to show meaningful benefit (Halbirk et al., 2010). Hence, the exciting results of the earlier pilot study, which did not involve a randomized double-blind design or placebo control group (Sokos et al., 2006), have not yet been replicated in larger studies of GLP-1R agonists in HF (Lepore et al., 2016; Ussher and Drucker, 2014).

Lepore and colleagues examined the therapeutic actions of a range of doses of a long-acting high molecular weight GLP-1R agonist, albiglutide, versus placebo over 12 weeks in human, non-diabetic overweight and obese subjects with NYHA class II–III HF and EF < 40%. Subjects treated with albiglutide 30 mg once weekly (n = 27) did not exhibit improvements in LVEF, 6 min walk time, cardiac efficiency, LV structure and function, quality of life scores, or myocardial glucose or oxygen utilization (Lepore et al., 2016). The actions of liraglutide in human subjects with HF have been examined in a randomized, double-blinded, placebo-controlled clinical trial, the Functional Impact of GLP-1 for Heart Failure Treatment (FIGHT) study (Margulies et al., 2014). Study subjects (60% with diabetes, ~80% male, ~8 year history of HF) with LVEF~26%, NYHA class II–III HF (29%/65%), and a history of acute hospitalization for heart failure syndrome (AHFS) within the preceding year (>85%) were treated for 180 days with placebo (n = 155) or liraglutide (1.8 mg daily, n = 146). Liraglutide did not improve time to death, time to HF hospitalization, or time-averaged changes in levels of N-terminal pro-brain natriuretic peptide (BNP). Comparable numbers of patients (12%, liraglutide; 11%, placebo) died, and 34% of patients in the liraglutide treatment group versus 28% of patients in the placebo group were re-hospitalized for HF.

Jorsa and colleagues studied the effects of liraglutide (1.8 mg daily) versus placebo in human subjects (~90% male) with and without diabetes and pre-existing HF, EF < 45%, NYHA class I–III, and eGFR > 30 mL/min/1.73 m^2 over 24 weeks. Subjects were on stable medical regimens for treatment of heart disease, and the pre-defined primary endpoint was change in LVEF from baseline. The majority of subjects (65%) had ischemic heart disease and NYHA class I (38%) or class II (46%) HF, and 35% had T2D (Jorsal et al., 2014). Baseline LVEF was 33.7 versus 35.4 in liraglutide- versus placebo-treated subjects, and no significant treatment-related differences in LVEF were noted after 24 weeks of therapy. However, HR was significantly increased (6.1 BPM) and serious cardiac events, including arrhythmias and acute coronary syndrome, were more common in subjects treated with liraglutide.

Hence, the available evidence does not support the use of GLP-1R agonists for the treatment of subjects with significantly impaired ventricular function and a known history of HF. Nevertheless, there is little evidence that administration of GLP-1R agonists to diabetic subjects with or without ischemic heart disease (unstable angina or MI) will increase the risk of HF, as no differences in rates of hospitalization for HF were observed in several thousand human subjects with acute coronary syndrome (ACS) randomized to receive lixisenatide or placebo in the Evaluation of Lixisenatide in Acute Coronary Syndrome (ELIXA) trial (Pfeffer et al., 2015). Similarly, retrospective analysis of several large cohorts (1,499,650 patients) of subjects with T2D, with and without a pre-existing history of HF, treated with multiple different antidiabetic agents did not reveal any relationship between therapy with GLP-1R agonists and the risk of hospitalization for HF (Filion et al., 2016).

Control of Lipid Synthesis and Secretion

GLP-1 receptor agonists rapidly reduce intestinal chylomicron production and secretion in normoglycemic and diabetic rodents and humans independent of changes in gastric emptying (Hermansen et al., 2013; Hsieh et al., 2010; Xiao et al., 2012). Although these effects are incompletely understood, they require a functional canonical GLP-1R in rodents (Hsieh et al., 2010). Enterocytes, the major cell type responsible for chylomicron synthesis and secretion, do not express the GLP-1R (Richards et al., 2014; Yusta et al., 2015). Hence, the actions of GLP-1R agonists to reduce circulating levels of postprandial triglycerides and apoB48 in normal and diabetic subjects, which are sustained with continuous GLP-1R agonism (Hermansen et al., 2013), are likely indirect. To date, the mediators conveying GLP-1R-dependent signals to reduce chylomicron synthesis

and secretion in enterocytes have not been identified. Similarly, despite multiple animal and human studies demonstrating reduction of liver fat and hepatic inflammation following treatment with GLP-1R agonists (Armstrong et al., 2016; Campbell and Drucker, 2013; Panjwani et al., 2013), hepatocytes do not express the canonical GLP-1R (Landgraf et al., 2015; Panjwani et al., 2013). Hence, actions of GLP-1R agonists to reduce liver fat and decrease hepatic lipoprotein synthesis and secretion are likely indirect, mediated in part by changes in circulating levels of insulin and glucagon, neural inputs, weight loss, enhanced insulin sensitivity, or reduced substrate delivery.

Thrombosis and Platelet Aggregation

Preclinical data demonstrate that GLP-1R agonists such as exenatide directly inhibit mouse and human platelet aggregation and thrombus formation ex vivo; however, exenatide had no effect on bleeding times assessed using a mouse tail vein assay (Cameron-Vendrig et al., 2016). Furthermore, whether adult human platelets express a functional canonical GLP-1R remains uncertain. Exendin-4 treatment of rats (for 16 or 42 days) with experimental chronic kidney disease significantly reduced ADP-stimulated platelet adherence to injured jugular vein endothelium ex vivo, although whether these effects were direct or indirect, and mediated through platelets and/or endothelium, was not ascertained (Chien et al., 2014)

Sechterberger examined multiple parameters of coagulation in 37 overweight or obese non-diabetic male and female subjects treated with once-daily liraglutide or placebo starting 1 day prior to hip surgery, with treatment, including the anticoagulant dabigatran, continuing for the next 3 days (Sechterberger et al., 2015). Circulating levels of molecules important for coagulation, such as D-dimers, were reduced at postoperative day 3 in liraglutide-treated subjects. However, no differences in levels of prothrombin fragment 1+2, thrombin-antithrombin complex, plasmin alpha2-antiplasmin complex, vonWillebrand factor, antithrombin, or plasminogen activator inhibitor-1 were detected following liraglutide administration as assessed in blood samples obtained 2 and 72 hr after surgery (Sechterberger et al., 2015).

Parameters of clotting and platelet function were also evaluated before and after 6 months of liraglutide (1.8 mg daily) therapy in obese, non-diabetic women with and without polycystic ovary syndrome (Kahal et al., 2015). P-selectin expression, fibrinogen, or platelet fibrinogen binding levels and platelet aggregation responses to ADP (0.1–10 μM) or the prostacyclin PGI_2 (1–100 nM) were not different after 6 months of liraglutide administration. Furthermore, there are little other data examining platelet or clotting function in human subjects with diabetes treated acutely or chronically with GLP-1R agonists, in the presence or absence of concomitant therapy with conventional antiplatelet agents. Hence, whether GLP-1R agonists exert clinically relevant effects on platelets and parameters of coagulation on top of conventional anticoagulation therapy is not yet known.

Endothelial Function, Blood Pressure, Blood Flow, and HR

Clinical studies in non-diabetic human subjects, or in individuals with T2D, demonstrate that native GLP-1 infused over several hours improves endothelial function, independent of changes in levels of plasma insulin (Ceriello et al., 2013, 2014; Ussher and Drucker, 2014). Nevertheless, despite reports of GLP-1R expression in endothelial cell cultures ex vivo, it remains uncertain whether endothelial cells within blood vessels express the endogenous GLP-1R in vivo (Ussher and Drucker, 2014). Rather, it seems possible that GLP-1 degradation products, such as GLP-1(9-36) or GLP-1(28-36), partially account for the vasodilatory actions attributed to native GLP-1. Administration of GLP-1 at doses up to 3.6 pmol/min directly into the brachial artery of healthy, non-diabetic subjects, treated with or without sitagliptin to prevent N-terminal cleavage and generation of GLP-1(9-36), resulted in no detectable effect of GLP-1 on forearm blood flow (FBF) (Devin et al., 2014). In contrast, intra-arterial infusion of BNP increased FBF in the same subjects in the presence or absence of sitagliptin. Acute infusion of GLP-1 (0.5 pmol/kg/min) in non-diabetic subjects clamped at euglycemia or hyperglycemia (~5 versus 10 mM glucose, respectively) produced no changes in carotid, brachial, or femoral artery blood flow, despite significant increases in insulin levels during hyperglycemia (Karstoft et al., 2015). In contrast, infusion of GIP (1.5 pmol/kg/min) in the same study subjects increased femoral artery blood flow under hyperglycemic conditions.

Postprandial endothelial function, assessed by measurement of digital hyperemia, was improved in subjects with T2D treated with twice-daily exenatide (versus placebo) for 11 days (Koska et al., 2015). In complementary experiments, intravenous exenatide (50 ng/min) increased endothelial function assessed by peripheral arterial tonometry and digital hyperemia in fasting human subjects with impaired glucose tolerance (IGT), findings abrogated by co-infusion of exendin(9-39). Moreover, exendin-4 directly and dose-dependently increased arteriole vasodilation (in the context of hyperglycemia or exposure to VLDL) in perfused adipose tissue obtained from non-diabetic and diabetic subjects. Exendin-4-mediated vasodilation in adipose tissue ex vivo was attenuated by exendin(9-39) or by co-administration of N (omega)-nitro-L-arginine methyl ester L-NAME or the AMPK inhibitor compound C (Koska et al., 2015). Whether human arterioles in adipose tissue express the endogenous canonical GLP-1R remains uncertain.

Endothelial function was examined in human subjects with abdominal obesity, impaired fasting glucose, or IGT treated with metformin or twice-daily exenatide for 12 weeks (Kelly et al., 2012). Neither therapy improved microvascular endothelial function, assessed by digital reactive hyperemia. Endothelial function was also assessed in human subjects after 12 weeks of therapy with either daily liraglutide (1.8 mg), glimepiride (4 mg), or placebo, administered to subjects with T2D and HbA1c 6.5%–9%. No therapy produced a differential effect on FBF in response to infusion of acetylcholine and sodium nitroprusside (SNP), assessed either before or after infusion of (L)-N(G)-monomethyl arginine, despite weight loss of several kg in subjects treated with liraglutide (Nandy et al., 2014). Consistent with these findings, a randomized controlled trial comparing liraglutide to insulin glargine over 14 weeks in Japanese subjects with T2D (mean age 60, HbA1c 8.6%) on a background of metformin and sulfonylurea did not reveal any preferential effect of liraglutide on endothelial function assessed by analysis of brachial artery vasodilatory reactivity (Nomoto et al., 2015); indeed, FMD declined modestly in both treatment groups over 14 weeks.

A randomized, crossover, placebo-controlled trial also examined the effect of 10 weeks of daily liraglutide (1.2 mg) on coronary artery microflow in subjects with T2D treated with one or more oral anti-diabetic agents without known coronary artery disease. Blood flow was assessed using trans-thoracic Doppler-flow echocardiography during acute dipyridamole-induced stress (Faber et al., 2015). Although liraglutide-treated subjects experienced weight loss (1.9 kg) and a significant reduction in systolic blood pressure (BP; 10 mm Hg), no change in coronary microvascular flow or peripheral blood flow, assessed using changes in fingertip blood flow, was detected in subjects treated with liraglutide (Faber et al., 2015). Hence, although multiple studies demonstrate a role for native GLP-1 to regulate endothelial function, it remains unclear whether activation of the canonical GLP-1R, either indirectly or directly in blood vessels, regulates endothelial function or blood flow independent of changes in glycemia or body weight.

Acute administration of GLP-1 or GLP-1R agonists leads to increases in HR and a transient increase in BP in normotensive and hypertensive subjects (Edwards et al., 1998; Lovshin et al., 2015; von Scholten et al., 2015). More sustained GLP-1R agonism is associated with a persistent increase in HR but a drop in systolic BP in hypertensive individuals (Ferdinand et al., 2014; Meier et al., 2015; Ussher and Drucker, 2014). Patients treated with twice-daily exenatide for 36 months achieved better BP control and required less frequent addition of anti-hypertensive agents to control BP compared to subjects treated with glimepiride (Simó et al., 2015). The increase in HR persists with sustained GLP-1R agonism in subjects with T1D, T2D, or obesity (Dejgaard et al., 2016; Dungan et al., 2014; Meier et al., 2015; Pi-Sunyer et al., 2015). Furthermore, the magnitude of the increase in HR correlates with the dose and pharmacokinetics of the specific drug under examination (Ferdinand et al., 2014; Lovshin et al., 2015; Meier et al., 2015). The relative risk of developing tachyarrhythmias following therapy with GLP-1R agonists is uncertain and is likely drug specific. Analysis of diabetic individuals treated with albiglutide in eight phase 3 and one phase 2B trial revealed numerically more subjects reporting atrial fibrillation or atrial flutter in the albiglutide cohort (Fisher et al., 2015). In contrast, no numerical imbalance in arrhythmias was reported in subjects treated with lixisenatide versus other glucose-lowering agents in the ELIXA trial (Pfeffer et al., 2015). It seems likely that subjects with pre-existing heart disease, including subjects with HF, may be more sensitive to the effect of GLP-1R agonism on HR and rhythm.

Periodic Holter monitoring examined the frequency of arrhythmias in subjects with T2D and pre-existing CVD or at high risk for CVD, randomized to receive mealtime exenatide (up to 20 μg daily) or meal-related bolus insulin on top of once-daily insulin glargine (FLAT-SUGAR Trial Investigators, 2016). Despite a significant reduction in the extent of glucose excursions (quantified as coefficients of glucose variability) detected in the exenatide-treated group, no differences in arrhythmias or rates of tachycardia were detected in the two treatment groups. There is currently insufficient evidence to assess the arrhythmogenic potential of intermittent versus sustained GLP-1R agonism in diabetic subjects with various subtypes of pre-existing CVD. GLP-1 may increase HR through enhanced sympathetic nervous system activation, reduced parasympathetic tone, or direct engagement of GLP-1Rs in the sinoatrial node (Griffioen et al., 2011; Nakatani et al., 2016; Ussher and Drucker, 2014; Yamamoto et al., 2002). Nevertheless, there are little data elucidating the extent to which these or other mechanisms account for increases in HR in humans exposed to sustained GLP-1R agonism.

GLP-1R agonists reduce BP in clinical trials of subjects with T2D and hypertension, and the changes in BP are not strictly dependent on weight loss (Jimenez-Solem et al., 2010; Meier et al., 2015; Simó et al., 2015; Ussher and Drucker, 2014). Treatment of diabetic hypertensive subjects (mean age 64, BMI 31.9, HbA1c 7.7%) for 7 weeks with 1.8 mg liragutide daily reduced both systolic BP (by 7 mm Hg) and extracellular volume (by 2.0 L) (von Scholten et al., 2015). Interrogation of components of the renin-angiotensin system in subjects treated with GLP-1R agonists has not identified consistent changes in plasma levels of aldosterone, angiotensinogen, or renin (Ferdinand et al., 2014; Lovshin et al., 2015), although a modest (21%) yet significant reduction in circulating angiotensin-2 was reported in one small study of 11 subjects with T2D, following acute administration of a single dose of 1.2 mg liraglutide (Skov et al., 2016). Analysis of 24 hr BP profiles in subjects with T2D treated with once-weekly dulaglutide for 16 or 26 weeks revealed reduced systolic BP during both the daytime and nighttime periods, independent of age, baseline BP, concomitant anti-hypertensive therapy, or changes in body weight (Ferdinand et al., 2014). In contrast, a relatively greater increase in HR was observed during the nighttime versus the daytime period after 8 weeks of liraglutide (1.2 or 1.8 mg daily) therapy in subjects with T2D who were also treated with basal insulin glargine. Subjects randomized to once-daily lixisenatide (20 μg) in the same study exhibited a preferential increase in HR during the daytime (Meier et al., 2015), likely reflecting the different pharmacokinetic profiles of these two agents.

The mechanisms linking sustained GLP-1R signaling to weight-loss-independent reduction of BP in hypertensive humans are poorly understood and may include contributions from natriuresis (Lovshin et al., 2015); vasorelaxation, possibly mediated in part by a vascular smooth muscle GLP-1R (Jensen et al., 2015; Pyke et al., 2014); and, potentially, as yet unidentified neurohormonal mechanisms (Figure 3). Although the GLP-1-stimulated natriuresis and diuresis in mice require the canonical GLP-1R (Rieg et al., 2012), the precise cell types and mechanisms conveying GLP-1R-dependent signals that promote natriuresis and urine flow remain obscure. Similarly, the reduction in BP observed in mice (with angiotensin2-induced hypertension) treated with native GLP-1, exendin-4, or liraglutide was mediated through the canonical GLP-1R (Kim et al., 2013); however, the dominant mechanism(s) accounting for GLP-1R-dependent reduction in BP in human subjects are not well defined. How GLP-1R signaling reduces BP in hypertensive subjects, but fails to produce clinically significant hypotension in most normotensive individuals, remains unclear. Furthermore, acute studies examining endothelial function, blood flow, or hemodynamic properties in response to GLP-1R agonists often fail to include active comparators to control for changes in glucose, insulin, free fatty acids, or body weight (Ussher and Drucker, 2014); hence, mechanistic interpretation of much of the existing data remains challenging.

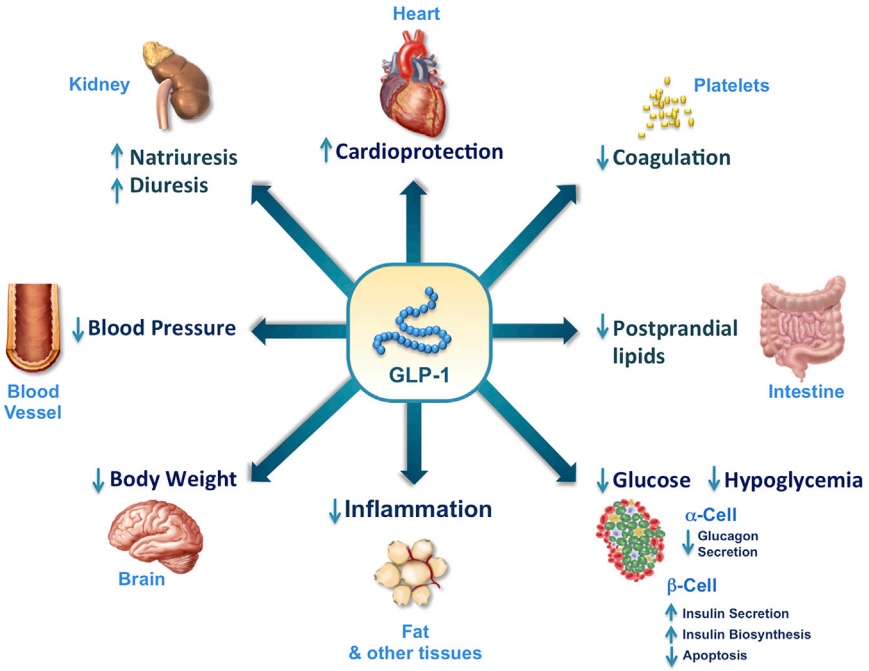

Figure 3. GLP-1 Modifies CV Risk through Direct and Indirect Actions in Multiple Organs

The targets for GLP-1 that may impact the risk of developing CV disease, and the consequences of GLP-1 action in specific tissues and cell types with CV implications, are shown.

CV Outcome Studies

Pursuant to guidance from regulatory agencies, multiple CV outcome studies examining the safety of GLP-1R agonists have been initiated. The ELIXA trial was designed to assess the CV safety of lixisenatide, administered 20 μg once daily, versus placebo and usual care, in 6,068 subjects with T2D who experienced an acute coronary event within 180 days of screening (Pfeffer et al., 2015). No significant differences between treatment groups were observed in rates of major CV events (myocardial infarction, stroke, or CV death), revascularization, or hospitalization for HF. Study subjects (mean age 60, duration of diabetes 9 years, entry HbA1c 7.7%, BMI 30) were followed for ~25 months, although study medication was ultimately discontinued in 27% of lixisenatide-treated and 25% of placebo-treated patients. Systolic BP (0.8 mm Hg), BW (0.6 kg) and mean A1c values were significantly reduced in subjects receiving lixisenatide (Pfeffer et al., 2015), and rates of serious adverse events were similarly distributed across treatment groups. Given the relatively short duration of the trial and the enrollment criteria (pre-existing ACS), no conclusions can be drawn regarding the cardioprotective potential of chronic lixisenatide therapy in other patient populations with T2D.

The CV safety of liraglutide was examined in the Liraglutide Effect and Action in Diabetes: Evaluation of CV Outcome Results (LEADER) trial (Marso et al., 2013). A total of 9,340 subjects with T2D and HbA1c ≥ 7% were enrolled (mean BMI 32.5, mean age 64, predominantly male [64%]), with 81.3% of subjects exhibiting pre-existing CV disease. The mean duration of diabetes at study entry was 12.8 years, and mean HbA1c was 8.7%. Eligibility criteria specified age ≥ 50 with one or more CV co-morbidities, such as cerebrovascular disease, coronary artery disease (CAD), peripheral vascular disease, chronic kidney disease (stage 3 or greater), or chronic HF NYHA class II–III. The remaining subjects without established CVD were older (>60), with one or more CV risk factors. Subjects were enrolled if they were treated with diet alone, one or more oral agents, or pre-specified insulin regimens (Marso et al., 2013) and randomized to treatment with liraglutide (maximum dose 1.8 mg daily) or usual diabetes care.

Liraglutide therapy was associated with a significant reduction in the primary outcome of major CV events (MACE), defined as the first occurrence of CV death, nonfatal MI, or nonfatal stroke (Marso et al., 2016). Liraglutide also reduced CV mortality and all-cause mortality; the frequency of stroke and non-fatal MI also trended lower in the liraglutide treatment group. The number of first microvascular events, largely reflecting a reduction in kidney disease, was also significantly lower in liraglutide-treated subjects (Marso et al., 2016). Consistent with the mechanism of action of GLP-1R agonists, a significant increase in HR (3 BPM) and reduction in systolic BP (1.2 mm Hg) were observed in liraglutide-treated subjects. The numbers of severe and confirmed hypoglycemic events were lower in subjects treated with liraglutide versus placebo and usual diabetes care. Based on results from LEADER, 66 patients would need to be treated for 3 years to prevent one CV event, and 98 patients would need to be treated to prevent one death (Marso et al., 2016).

Safety of GLP-1R Agonists: Mechanistic Insights and Clinical Observations

Preclinical experimentation and clinical investigation have raised important questions concerning the long-term safety of GLP-1R agonists in human subjects with diabetes and/or obesity (Drucker, 2013, 2015; Drucker et al., 2011). Acute administration of GLP-1R agonists increases calcitonin secretion and inhibits bone resorption in mice (Yamada et al., 2008), reflecting expression of a functional GLP-1R in rodent C cells (Bjerre Knudsen et al., 2010; Madsen et al., 2012; Yamada et al., 2008). More sustained GLP-1R signaling promotes C cell hyperplasia and development of medullary thyroid cancer, evident to a greater extent in rats, compared to mice (Bjerre Knudsen et al., 2010). In contrast, monkey and human C cells express much lower levels of the GLP-1R, and calcitonin secretion is not increased following chronic therapy with GLP-1R agonists in subjects with T2D or obesity (Bjerre Knudsen et al., 2010; Pi-Sunyer et al., 2015).

Rates of acute pancreatitis were slightly increased in several phase 3 clinical trials, generally 6–12 months duration, in subjects treated with clinically approved GLP-1R agonists (Meier

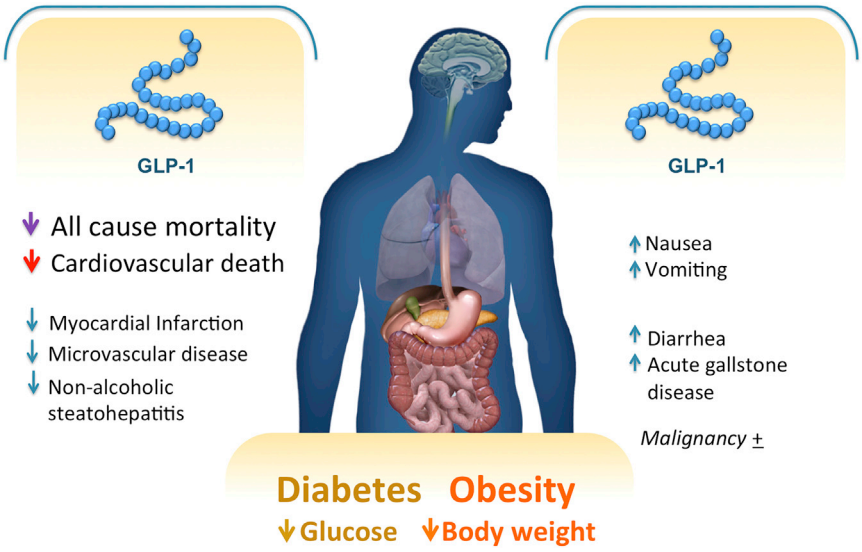

Figure 4. The CV Safety of GLP-1R Agonists
Based on results from the LEADER trial, sustained GLP-1R agonism in subjects with T2D at high risk for CV events produced a reduction in MACE events and CV mortality, balanced by gastrointestinal side effects, and lingering uncertainty about any possible associated increased risk of cancer. The CV benefit of GLP-1R agonists in obese non-diabetic subjects has not been established

and Nauck, 2014); however, numerically fewer reports of pancreatitis were reported in subjects treated with lixisenatide (median follow-up period of 25 months) in the ELIXA trial (Pfeffer et al., 2015). Multiple retrospective database analyses have examined the relationship between GLP-1R agonists and pancreatitis, and reported either no imbalance, or a very slight excess of pancreatitis cases following therapy with GLP-1R agonists. The putative mechanisms linking GLP-1R signaling to pancreatic inflammation remain unclear; GLP-1R agonists reduce experimental pancreatic inflammation and do not increase the susceptibility to drug-induced pancreatic inflammation in preclinical studies (Drucker, 2013; Koehler et al., 2009).

Sporadic concerns that exposure of rats or mice to incretin-based therapies might be associated with histological changes in the exocrine pancreas, summarized in Drucker (2013), have been tempered by more careful systematic and quantitative analyses revealing a very high frequency of spontaneously occurring histological abnormalities, including inflammation, in the pancreata of commonly studied rodent models of T2D (Chadwick et al., 2014). Although modest increases in lipase and amylase have been repeatedly detected in asymptomatic subjects treated with GLP-1R agonists, preclinical studies have demonstrated that GLP-1R agonists increased pancreatic protein synthesis in mice (Koehler et al., 2015a) and directly increase pancreatic enzyme secretion in murine pancreatic slices ex vivo through mechanisms requiring a functional GLP-1R (Hou et al., 2016). Observations demonstrating that acute administration of exenatide to healthy, fasting, non-diabetic subjects inhibited cholecystokinin-stimulated gallbladder emptying (Keller et al., 2012), coupled with the propensity for rapid weight loss to increase the risk of cholelithiasis (Pizzimenti et al., 2016), highlight the small yet emerging risk of developing cholecystitis in subjects treated with GLP-1R agonists. Indeed, acute gallstone disease was more common in liraglutide-treated subjects in the LEADER trial (Marso et al., 2016).

Diabetes and obesity are associated with increased cancer incidence rates; however, whether therapy with GLP-1R agonists modifies the risk of developing specific cancers remains uncertain, due to limitations in existing clinical data (Drucker, 2013, 2015). GLP-1R agonists exert proliferative and anti-apoptotic actions in normal tissues of young, healthy rodents (thyroid C cells, islet β cells, and intestinal mucosa) (Campbell and Drucker, 2013; Koehler et al., 2015b); whether these actions are preserved in corresponding tissues of older diabetic human subjects is challenging to ascertain. Initial concerns surrounding putative expression of the GLP-1R in human pancreatic and thyroid tumors have been modified by the realization that some of these studies employed non-specific GLP-1R antibodies with poor sensitivity (Campbell and Drucker, 2013; Drucker, 2013, 2015; Panjwani et al., 2013; Pyke and Knudsen, 2013). More recent analyses using in situ ligand binding have detected a low level of GLP1R expression and binding sites in many human tumor subtypes, including pancreatic cancer (Waser et al., 2015). A non-significant increase in some cancers, including pancreatic cancer, was reported in LEADER (Marso et al., 2016); however, the confidence intervals were wide, and the number of subjects and duration of follow-up were not sufficient to make clear conclusions about any possible relationship. It seems likely that analysis of large population databases enabling comparative analyses of millions of patient years of otherwise matched subjects with T2D treated with different anti-diabetic agents will be necessary to understand whether GLP-1R agonists differentially impact cancer incidence rates in humans with diabetes or obesity.

Studies of human genetic variation at the *GLP1R* locus have provided useful insights into the link between *GLP1R*, control of glucose, and CV risk. A low-frequency missense *GLP1R* variant, Ala316Thr; rs 10305492, was associated with lower fasting glucose and a decreased risk of developing T2D in human population studies (Wessel et al., 2015). Remarkably, analysis of 61,846 subjects with coronary artery disease versus 163,728 controls revealed that individuals with the rs 10305492 *GLP1R* variant also exhibited a significantly lower risk of coronary artery disease, independent of any changes in BP or BMI (Scott et al., 2016). One limitation of these genetic studies is that the pharmacological signaling properties of the Ala316Thr GLP1R variant have not been extensively characterized in human islets or in animal models of T2D, with or without CVD. Nevertheless, the available evidence from preclinical studies, human pharmacotherapy of subjects with T2D treated with long-acting GLP-1R agonists, as well as human genetic variation, collectively supports a link between enhanced GLP-1R signaling and a reduction in CV risk (Figure 4).

Controversies and Areas of Uncertainty

The exciting demonstration that therapy with liraglutide resulted in reduced MACE events challenges us to refine and extend our understanding of mechanisms linking GLP-1R signaling to improved CV outcomes. Certainly, weight loss, reduction in BP, and improved glycemia are likely to partially contribute to achievement of favorable CV outcomes (Figure 3). The relative importance (for reduction of MACE events) of reduced systemic or tissue inflammation, decreased postprandial lipemia, and direct actions of GLP-1R agonists on platelets, blood vessels, immune cells, or the heart is impossible to ascertain. The reduction of episodes of severe hypoglycemia in liraglutide-treated subjects in LEADER (Marso et al., 2016) is consistent with the known mechanisms of action of all GLP-1R agonists; given the well-established link between hypoglycemia and adverse CV outcomes (Khunti et al., 2015), this finding may be important and requires further analysis of whether subjects in the LEADER trial with MACE also reported more hypoglycemia.

It will be useful to understand whether it is possible to identify subsets of diabetic patients particularly well suited to respond favorably to the cardioprotective actions of GLP-1R agonists. Whether the positive findings of the LEADER study will be consistently reproduced by other ongoing CV outcome trials examining the CV safety of structurally distinct GLP-1R agonists such as once-weekly exenatide, or dulaglutide, is an important question that will soon be answered. Preliminary dissemination of topline results for SUSTAIN-6 indicated that therapy with once-weekly semaglutide for ~2 years was also associated with a significant reduction in MACE events.

Understanding how GLP-1R signaling leads to improved CV outcomes in subjects with T2D is challenging to determine, but even partial answers may enable future targeting of higher risk subpopulations uniquely sensitive to one or more beneficial CV actions of GLP-1. Despite important information gleaned from the LEADER trial, the number of patients studied is too small and duration of exposure is too short to allow definitive conclusions to be made regarding the overall long-term safety and benefit:risk profile of liraglutide and other GLP-1R agonists, particularly in regard to development of rare cancers with long latencies and low incident rates (Figure 4). Even less information is available surrounding the long-term consequences of using 3 mg liraglutide once daily for the treatment of obesity. Nevertheless, after more than 11 years of incretin-based therapies, the value of therapeutically engaging GLP-1R-dependent mechanisms for the treatment of diabetes, most notably in subjects with established CVD or at high risk for development of CVD, is becoming better defined. GLP-1R agonists enable robust control of glucose with a low risk of hypoglycemia, maintenance or reduction of body weight, and a decrease in CV events and rates of CV death (Marso et al., 2016), providing an important and potentially life-saving therapeutic option for many subjects with T2D.

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