

Vascular Biology of Glucagon Receptor Superfamily Peptides: Mechanistic and Clinical Relevance

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Regulatory peptides produced in islet and gut endocrine cells, including glucagon, glucagon-like peptide-1 (GLP-1), GLP-2, and glucose-dependent insulinotropic polypeptide, exert actions with considerable metabolic importance and translational relevance. Although the clinical development of GLP-1 receptor agonists and dipeptidyl peptidase-4 inhibitors has fostered research into how these hormones act on the normal and diseased heart, less is known about the actions of these peptides on blood vessels. Here we review the effects of these peptide hormones on normal blood vessels and highlight their vascular actions in the setting of experimental and clinical vascular injury. The cellular localization and signal transduction properties of the receptors for glucagon, GLP-1, GLP-2, and glucose-dependent insulinotropic polypeptide are discussed, with emphasis on endothelial cells and vascular smooth muscle cells. The actions of these peptides on the control of blood flow, blood pressure, angiogenesis, atherosclerosis, and vascular inflammation are reviewed with a focus on elucidating direct and indirect mechanisms of action. How these peptides traverse the blood-brain barrier is highlighted, with relevance to the use of GLP-1 receptor agonists to treat obesity and neurodegenerative disorders. Wherever possible, we compare actions identified in cell lines and primary cell culture with data from preclinical studies and, when available, results of human investigation, including studies in subjects with diabetes, obesity, and cardiovascular disease. Throughout the review, we discuss pitfalls, limitations, and challenges of the existing literature and highlight areas of controversy and uncertainty. The increasing use of peptide-based therapies for the treatment of diabetes and obesity underscores the importance of understanding the vascular biology of peptide hormone action. (*Endocrine Reviews* 37: 554–583, 2016)

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Abbreviations: AGE, advanced glycosylation end product; AMPK, AMP kinase; AngII, angiotensin-II; ATBF, adipose tissue blood flow; BBB, blood brain barrier; BP, blood pressure; CAD, coronary artery disease; CFR, coronary flow reserve; CNS, central nervous system; CREB, cAMP responsive element binding protein; CSF, cerebrospinal fluid; DPP4, dipeptidyl peptidase-4; EC, endothelial cell; EEC, enteroendocrine cell; EF, ejection fraction; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; Ex-4, Exendin-4; FMD, flow-mediated vasodilatation; GIP, glucose-dependent insulinotropic polypeptide; GIPR, GIP receptor; GLP, glucagon-like peptide; GLP-1R, GLP-1 receptor; GPCR, G-protein coupled receptor; HAEC, human aorta EC; HbA1c, glycosylated hemoglobin; HCAEC, human coronary artery EC; HFD, high-fat diet; HR, heart rate; HUVEC, human umbilical vein EC; ICAM-1, intercellular adhesion molecule-1; icv, intracerebroventricular; IGT, impaired glucose tolerance; IMEC, islet microvascular EC; L-NAME, NG-methyl-L-arginine methyl ester; LV, left ventricular; LVDP, LV developed pressure; LVEF, LV ejection fraction; MBV, microvascular blood volume; MSC, mesenchymal stem cell; NO, nitric oxide; PCI, percutaneous coronary intervention; Pcsk, prohormone convertase; PGDP, proglucagon-derived peptide; PKA, protein kinase A; PKC, protein kinase C; RBF, renal blood flow; RHI, reactive hyperemia index; ROS, reactive oxygen species; SMA, superior mesenteric artery; STZ, streptozotocin; T2D, type 2 diabetes; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cells.

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I. Introduction

The biological products derived from differential post-translational processing of proglucagon, designated the proglucagon-derived peptides (PGDPs), include glucagon, GLP-1 (glucagon-like peptide-1), GLP-2 (glucagon-like peptide-2), glicentin, and oxyntomodulin (Figure 1). These PGDPs exert distinct metabolic actions on multiple target tissues through structurally related yet distinct family B G-protein coupled receptors (GPCRs) (1). The related enteroendocrine peptide, glucose-dependent insulinotropic polypeptide (GIP), secreted from gut K cells, exhibits overlapping incretin actions with GLP-1, secreted from L cells, through its own GPCR highly related in structure and function to other class B family members (2). Although classical concepts of PGDP and GIP action encompass control of nutrient absorption, islet function, appetite, and energy production and storage, the widely distributed expression of the GPCRs for glucagon, GLP-1, GLP-2, and GIP in tissues and cell types not classically associated with energy homeostasis (3) has sparked considerable interest in the nonmetabolic actions of these peptides. Notably, GLP-1 receptor (GLP-1R) agonists exert pleiotropic actions with potential therapeutic benefit in the rodent and human cardiovascular system (4), although much of the focus to date has been on understanding the biology of GLP-1 in the normal, failing, or ischemic heart (4). The development of drugs based on the actions of glucagon, GLP-1, and GLP-2 (5), coupled with ongoing preclinical development of multiple coagonists that transduce their actions through class B GPCRs (3), has fostered great interest in the biology of these peptide hormones. Moreover, guidelines for the development of drugs for the treatment of type 2 diabetes have evolved to encompass large clinical trials examining the cardiovascular safety of new therapeutic agents (6). Here we address the vascular actions of these peptides, linking wherever possible the expression of specific receptors in endothelial cells (ECs), or smooth muscle cells, with reported pharmacological

and physiological activities. As analogs of these peptide hormones and inhibitors of their degradation are increasingly used and new agents are under active investigation for the treatment of metabolic and intestinal disorders (4, 7), understanding the vascular biology of the PGDPs and GIP has potential translational relevance.

II. Synthesis, Secretion, Circulation, and Clearance of PGDPs and GIP

The human glucagon (*GCG*) gene is located on the long arm of chromosome 2; it contains six exons that encode distinct peptide domains; exons 3, 4, and 5 encode glucagon, GLP-1, and GLP-2, respectively (8). Although tissue-specific *Gcg* mRNA splicing has been described in reptiles, fish, and birds (9), the structure of mammalian *GCG* mRNA transcripts is identical in pancreas, intestine, and brain. *GCG* is predominantly expressed in pancreatic α -cells, enteroendocrine L cells in the small and large intestine, and in neurons within the brainstem and to a much lesser extent, the hypothalamus (10, 11). Hence, differential cell-specific posttranslational processing of an identical proglucagon precursor in islet α -cells, enteroendocrine cells (EECs), and central nervous system (CNS) neurons is the dominant mechanism for generation of diversity in PGDP synthesis and secretion in rodents and humans.

Multiple transcription factors contribute to control of *GCG* transcription, and many of these same factors are critical for specification of islet α -cell or EEC L-cell development (12). In the pancreas, inactivation of genes encoding *Pax6*, *Isl1*, *Nkx2.2*, or *Arx* results in impaired formation of α -cells, whereas *Pax6* and *Nkx2.2* are important for specification of *GCG* expression in EECs (13). In contrast, much less is known about the molecular control of *GCG* expression in the brain. Glucagon is liberated from proglucagon in islet α -cells as a result of prohormone convertase (*Pcsk* 2) expression (14), whereas in the gut, *Pcsk*1 cleaves proglucagon to generate two glucagon-like peptides (GLP-1 and GLP-2) as well as glicentin, oxyntomodulin, and two intervening peptides (1) (Figure 1). Studies of proglucagon processing in carboxypeptidase E-deficient fat/fat mice have revealed that carboxypeptidase E is also essential for correct processing of proglucagon to mature PGDPs, notably glucagon and GLP-1, in the pancreas and gut (15). Under some circumstances, such as following complete pancreatectomy or bariatric surgery, a paradoxical increase in plasma glucagon levels after meal ingestion may reflect de novo synthesis of 29-amino acid glucagon in EECs (16). Similarly, under conditions characterized by islet or pancreatic injury or inflammation, islet α -cells may express *Pcsk*1 and liberate bioactive

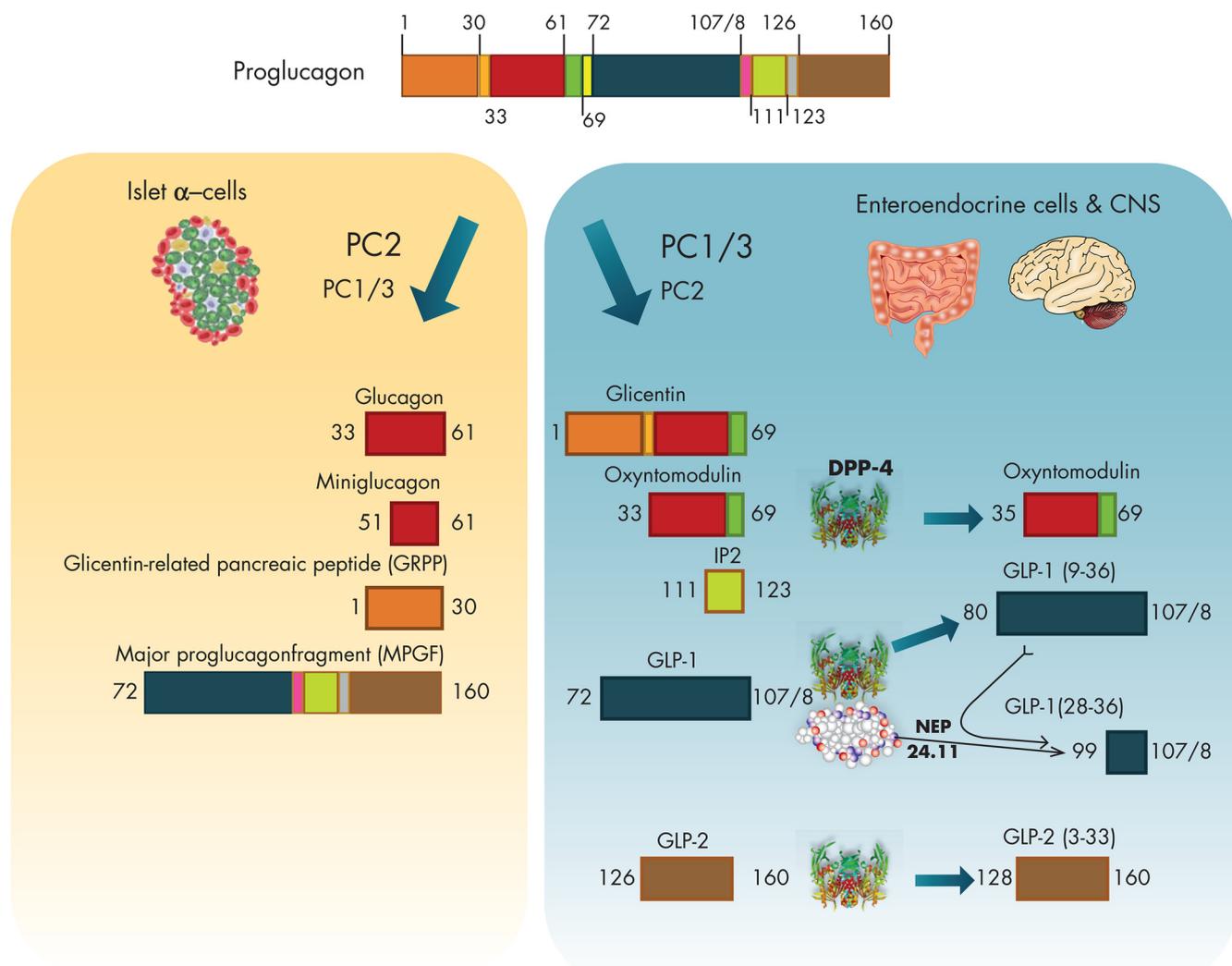
Figure 1.

Figure 1. Posttranslational processing of proglucagon in islet α -cells, enteroendocrine L cells, and the CNS. PC1/3, prohormone convertase1/3; PC2, prohormone convertase 2. Generation of miniglucagon and cleavage by DPP4 and neutral endopeptidase 24.11 (NEP 24.11) takes place largely within the circulation. GLP-1 (72–107) is most commonly known as GLP-1 (7–36) amide.

GLP-1; however, the biological significance of these findings remains uncertain (1, 14).

Activation of cAMP, protein kinase A (PKA), and Epac (exchange protein directly activated by cAMP) signaling pathways up-regulates the synthesis and secretion of PGDPs in gut L cells, islet α -cells, and the brain (17–19). Nutrient ingestion directly and indirectly up-regulates the synthesis and secretion of the intestinal PGDPs; in contrast, meal ingestion may have variable effects on plasma glucagon levels, depending on the precise composition of carbohydrates, proteins, and fats in the meal. Somatostatin, insulin, and other β -cell-derived products generally inhibit secretion of pancreatic glucagon. Moderate to prolonged fasting, amino acids, and hypoglycemia stimulate

glucagon secretion from α -cells, whereas fasting reduces secretion of the intestinal PGDPs.

Glucagon is secreted continuously in the interprandial state to maintain hepatic glucose production; the composition of the meal dictates the pattern of subsequent glucagon secretion in nondiabetic subjects. The advent of impaired glucose tolerance (IGT) or diabetes is often associated with impaired meal- or glucose-related suppression of glucagon secretion and fasting hyperglucagonemia (14). Glucagon exhibits a circulating $t_{1/2}$ of approximately 3 minutes in pigs, is rapidly cleared predominantly by the kidney (20), and also undergoes proteolysis by enzymes present in the pancreas, liver, kidney, and circulation. The circulating $t_{1/2}$ of glucagon is several minutes longer in humans and may be influenced by

the presence or absence of diabetes, obesity, and liver or kidney dysfunction. Nprilysin, also known as neutral endopeptidase, is particularly important for carboxyterminal degradation of glucagon (21). Glucagon (19–29), also known as miniglucagon, is derived from proteolytic cleavage of glucagon and pharmacologically regulates intracellular calcium flux in hepatocytes and cardiomyocytes. Miniglucagon also inhibits insulin secretion (22); however, the physiological relevance of glucagon (19–29) remains challenging to establish, despite several decades of investigation.

The complexity of GLP-1-related peptides derived from intestinal proglucagon processing (Figure 1) is amplified by subsequent cleavage of GLP-1 into smaller N-terminally truncated yet bioactive peptides (23). Carboxyterminal GLP-1 cleavage by neprilysin predominantly in the kidney (24, 25) generates multiple peptide fragments; however, the bioactivity of these cleavage products has not been carefully studied. Several different molecular forms of “intact GLP-1” can be detected in the circulation. The two predominant bioactive species are GLP-1 (7–37) and GLP-1 (7–36)NH₂, the latter representing the most abundant circulating form in humans (26). Fasting levels of active GLP-1 are low, sometimes undetectable, and increase 2- to 3-fold after a meal (27). The circulating half-life of active GLP-1 is very short, around 2 minutes, reflecting avid renal clearance and rapid N-terminal cleavage by the ubiquitous proteolytic enzyme dipeptidyl peptidase-4 (DPP4) (28).

The intestinal PGDPs (Figure 1) are continuously secreted in the basal state, and PGDP secretion and plasma levels increase within minutes of food ingestion. Although GLP-1-immunoreactive L cells are also detected in the proximal gut within the duodenum and jejunum, PGDP content appears more abundant in the distal ileum and colon, classic regions for location of L cells. Hence, the rapid secretion of intestinal PGDPs may reflect direct contact of proximal gut L cells with nutrients and/or activation of a proximal-distal endocrine or neural axis that has been well-described in rodents, but is less well characterized in humans (29). Nutrients, including fat, protein, and carbohydrates, individually or collectively stimulate L-cell secretion after enteral ingestion; many sugars, fatty acids, and some amino acids directly stimulate PGDP secretion from primary intestinal or EEC-enriched cultures or PGDP-secreting cell lines (30). Similarly, microbial metabolites, and neurotransmitters also engage GPCRs expressed on EECs to stimulate PGDP secretion (30).

Although GLP-1 is the L-cell peptide that has received the greatest scientific analysis, GLP-2 (33 amino acids), oxyntomodulin (37 amino acids), and glicentin (69 amino acids) are cosynthesized and secreted together with GLP-1 from EECs and exhibit unique yet overlapping biological activities. Both GLP-2 and oxyntomodulin are also rap-

idly cleared and degraded, yet exhibit somewhat longer circulating half-lives, relative to GLP-1 (1). The larger glicentin protein is even more stable in the circulation, yet it is more difficult to study and has received less experimental scrutiny relative to other PGDPs.

GIP is a 42-amino acid peptide encoded by a single gene that contains six exons and is localized to human chromosome 17q21.32 (31). GIP is derived from a 153-amino acid proGIP precursor and is predominantly synthesized in and secreted from enteroendocrine K cells within the duodenum and proximal jejunum in a nutrient-sensitive manner. Single-cell transcriptomics of individual GIP+ or GLP-1+ EECs has confirmed the plurihormonal complexity of most K- and L-EECs (32); it seems evident that the historical concept of predominantly unihormonal EECs is outdated (33). Low levels of *Gip* mRNA transcripts have also been reported in the brain, and *Gip* mRNA transcripts and a truncated GIP-immunoreactive protein have been localized to rodent and human α -cells (34). Nevertheless, transcriptomic analysis of normal murine and human α -cells does not always corroborate evidence for α -cell *Gip* expression. Hence, the putative importance of GIP expression in and secretion from islet cells under different conditions requires further investigation.

Consistent with the importance of *Pcsk1* for processing of proglucagon in the gut, mature GIP also requires *Pcsk1* activity for its liberation from proGIP in EECs (35). Within subsets of islet α -cells, a truncated but bioactive GIP (1–30) peptide may be produced via *Pcsk2* activity and contribute to intra-islet control of insulin secretion (34). *Pcsk2* may, under some circumstances, also cleave proGIP in the gut; however, the biological importance of this cleavage has not been established. Amidated GIP (1–30), generated via *Pcsk2*-mediated cleavage, has been detected in a subset of EECs in the murine stomach and small bowel (36). The molecular control of *Gip* gene expression is poorly understood; however, inactivating mutations in transcription factors such as *Pax4*, *Pax6*, and *Pdx1* that impair specification of EEC development also reduce the numbers of GIP+ EECs (37, 38).

Fat is the most potent stimulator of GIP secretion in humans, whereas carbohydrates are more effective in rodents and pigs (2). Basal circulating levels of GIP in humans range between 60 and 100 pmol/L, increasing to 200–500 pmol/L after meal ingestion. GIP is also highly susceptible to DPP4 degradation; hence, bioactive intact GIP (1–42) exhibits a half-life of <2 minutes in rodents and 5–7 minutes in human subjects (2). As is the case with GLP-1, the kidney is the major organ responsible for GIP clearance (39), and DPP4 is the dominant enzyme responsible for N-terminal inactivation of intact bioactive GIP (40, 41).

DPP4 is a serine protease that preferentially cleaves peptides, including GLP-1, GLP-2, oxyntomodulin, and GIP, classically at penultimate position 2 alanine or proline residues (40, 42). Although glucagon is a pharmacological substrate for DPP4, there is little evidence supporting a role for DPP4 in the physiological degradation of circulating bioactive glucagon (20, 28). In contrast, oxyntomodulin, which contains the identical 29-amino-acid sequence of glucagon, including the position 2 serine, is cleaved more efficiently, relative to glucagon, by DPP4 (43). DPP4-generated peptides such as GLP-1 (9–37)/(9–36)NH₂, GLP-2 (3–33), GIP (3–42), or N-terminally truncated oxyntomodulin do not bind with high affinity to their cognate GPCRs. Some of these peptides may exhibit weak antagonist activity at high concentrations, but it seems unlikely that they act as endogenous circulating antagonists *in vivo*.

III. Blood Brain Barrier (BBB), Peptide Transport, and Vascular Permeability

The *Gcg* and *Gip* genes and their receptors are expressed in the CNS constituting local CNS peptidergic networks. There is also considerable interest in the extent to which PGDPs (and GIP) or their peptide agonists enter the CNS after endogenous secretion or peripheral pharmacological administration and directly engage their cognate CNS receptors. Many small peptides, including glucagon and GLP-1, are detected within the brain after systemic administration (44). Although some sites in the CNS such as the area postrema are readily accessible and outside the BBB, GLP-1 and small peptide GLP-1R agonists such as Ex-4 (synthetic exenatide is the structurally identical pharmaceutical form of Ex-4) and liraglutide appear to rapidly access brain regions classically viewed as beyond the BBB (45, 46).

Considerable evidence supports a role for peripherally administered PGDPs, acting directly or indirectly through the CNS, in the control of appetite, glucose metabolism, gastrointestinal motility, and neuroprotection (1, 47). However, whether these signals are initiated peripherally or arise after vascular peptide transport into the brain and direct engagement of CNS receptors remains incompletely understood. Alternatively, many CNS regions important for the control of metabolic or vascular function may be directly accessible via circumventricular organs, independent of the BBB. Intravenous injection of ¹³¹I-GLP-1 in rats resulted in approximately 9% (relative to radioactivity remaining in blood) of the tracer localized to the brain 10 minutes after injection (44); however, whether this brain radioactivity reflected receptor-dependent uptake of

intact peptide or accumulation of radiolabeled peptide fragments was not determined. Kastin et al (46) assessed the interaction of ¹²⁵I-[Ser⁸]-GLP-1 with the BBB of adult male albino ICR mice. Ten minutes after injection of peptide into the jugular vein, 60% of the radioactivity in the brain corresponded to intact ¹²⁵I-[Ser⁸]-GLP-1. The Ki for brain ¹²⁵I-[Ser⁸]-GLP-1 localization was not diminished by coadministration of unlabeled peptide, the GLP-1R antagonist, exendin (9–39), insulin, glucagon, neuropeptide Y, or melanin-concentrating hormone. Considerable reversible binding of the tracer within brain capillaries was observed, and about 50% of the total CNS ¹²⁵I-[Ser⁸]-GLP-1 radioactivity was localized to the brain parenchyma (46). In similar experiments, 30 minutes after intrajugular injection of adult male CD1 mice with ¹²⁵I-Exendin-4 (Ex-4), 60% of the radiolabel detected in the brain represented intact nondegraded ¹²⁵I-Ex-4, with the majority (~90%) of peptide detected within brain parenchyma (45). In contrast to the findings with GLP-1, excess unlabeled Ex-4 modestly decreased BBB transport of coadministered ¹²⁵I-Ex-4.

Intraperitoneal injection of smaller GLP-1R agonists such as lixisenatide or liraglutide in mice was followed by detection of both peptides in the brain within 30 minutes (48); nevertheless, the mechanisms mediating brain uptake were not addressed, and the extent to which the measurement of levels of these peptides within the brain using ELISAs reflected detection of intact nondegraded peptide remains unclear. Secher et al (49) localized peripherally injected fluorescently labeled bioactive VivoTag-S 750 (liraglutide750) in multiple brain regions in wild-type C57BL/6 but not in the CNS of *Glp1r*^{-/-} mice. Specifically, liraglutide 750 (400 μg/kg) was administered by peripheral injection, and 6 hours later, fluorescent peptide was observed in regions both outside and protected by the BBB, including the arcuate, supraoptic, and paraventricular nuclei and the supraoptic decussation, the latter regions normally protected by the BBB (49). Similar CNS localization was reported using other peptide probes with affinity for the GLP-1R, including exendin (9–39) (49). Although the mechanisms through which GLP-1-related peptides gain access to the brain have not been extensively studied, very low but detectable (pM) levels of liraglutide were reported in the cerebrospinal fluid (CSF) after peripheral injection in mice (49). Consistent with these findings in rodents, extremely low levels (~6.5 pM) of liraglutide were detected in the CSF of human subjects with type 2 diabetes (T2D) chronically treated with peripheral daily injections of liraglutide (1.8 mg); hence, the appearance of liraglutide in the CSF appears negligible (50).

Studies in rats with experimental brain injury induced by trauma or associated with streptozotocin (STZ)-in-

duced diabetes have demonstrated that treatment with GLP-1R agonists may attenuate brain injury in association with functional preservation of the BBB (51, 52). Administration of liraglutide (200 $\mu\text{g}/\text{kg}$ twice daily) after controlled cortical injury in rats led to a reduction in cerebral edema in the injured brain, associated with a reduction in BBB permeability assessed by exudation of Evans Blue dye as a marker for albumin extravasation (52). Similarly, Wistar Kyoto rats with STZ-induced diabetes exhibited marked impairment of the BBB as assessed by Evans Blue extravasation and the ratio of CSF:serum albumin, and functional integrity of the BBB was improved in animals treated with Ex-4 (10 mg/kg) for 28 days (51). Liraglutide also improved cerebral and microvascular integrity in 7-month-old APP/PS1 transgenic mice. Treatment with once daily liraglutide (25 nmol/kg) for 8 weeks attenuated the reduction in splenic microvascular density and reduced extravascular extravasation evident in cerebral vascular casts (53). Extravascular extravasation was also reduced, and vascular ultrastructure was preserved in the renal glomeruli of liraglutide-treated APP/PS1 mice. The mechanisms and cellular targets through which GLP-1R signaling reduces vascular damage and decreases permeability remain unknown.

In contrast to the ability of small peptides to access the CNS, larger GLP-1R agonists are able to rapidly activate CNS signaling pathways without detectable CNS penetration. Intraperitoneal injection of a recombinant human albumin-GLP-1 protein (albiglutide) in wild-type mice acutely inhibited food intake and gastric emptying while stimulating c-Fos expression in multiple brain regions (assessed 60 minutes after albiglutide injection) (54); these actions of albiglutide were not detected in *Glp1r*^{-/-} mice and, in control studies, no CNS penetration of peripherally injected human serum albumin was detected by immunocytochemistry. Moreover, iv injection of CNTO736, a GLP-1 analog-Ig hybrid protein, in fasted Sprague Dawley rats, resulted in detection of the protein (within hours) by immunocytochemistry only in the median eminence, the area postrema, and neurons within the nucleus of the solitary tract, circumventricular organs accessible to the circulation (55). Similar results were obtained in the analysis of a GLP-1-human transferrin fusion protein, which rapidly increased c-Fos expression in multiple CNS regions of Sprague Dawley rats 90 minutes after ip administration (56); however, the protein was not detected by ELISA or Western blotting in CSF or brain homogenates assessed at 2 and 24 hours after protein injection. Hence, although large GLP-1R agonists clearly communicate with the CNS, small peptide GLP-1R agonists may gain preferential access to more distal regions of the CNS beyond the BBB through

incompletely understood mechanisms involving peptide uptake and transport.

A. Controversies and limitations of the data

Interpretation of data assessing tissue and circulating levels of PGDPs and GIP requires a comprehensive understanding of the sensitivity and specificity of the assays used to detect these peptides. Several limitations of many assays have now become apparent, including their incomplete characterization, insufficient sensitivity, and lack of specificity. Hence, it is frequently difficult to precisely interpret experimental findings with incompletely characterized assays, and comparison of data sets generated across independent studies that employ different assay techniques is both quantitatively and qualitatively challenging.

The observations that PGDPs may be localized to brain parenchyma after peripheral administration raises important questions about the BBB and transport mechanisms that enable some peptides to traverse the vasculature and interact with membrane GPCRs classically localized to CNS nuclei protected by the BBB. Many of the studies assessing peptide transport across the BBB do not actually report the detection of intact peptide; rather, they quantify radioactivity or fluorescence, which may represent intact or partially cleaved peptide. It seems likely that molecular structures, relative hydrophobicity and hydrophilicity, and interaction with peptide transporters represent important yet poorly understood determinants of CNS peptide penetration. The realization that GLP-1R agonists may be successfully used to reduce food intake and treat obesity, coupled with numerous preclinical studies highlighting the neuroprotective actions of PGDPs, has increased interest in how peripherally administered peptides traverse the vasculature and enter the brain. Moreover, evidence for the therapeutic efficacy of peripherally administered exenatide in human subjects with Parkinson's disease (57) highlights the compelling translational rationale for better understanding how PGDPs interact with the BBB and access the CNS.

IV. Receptor Expression in Heart and Blood Vessels

Interpretation and assessment of much of the published GLP-1R expression and localization literature is difficult because many antisera widely used for detection of the GLP-1R exhibit poor specificity and sensitivity (58, 59). Wei and Mojsov (60) detected *GLP1R* mRNA transcripts in RNA prepared from whole human heart using both ribonuclease protection and RT-PCR (60). Within the mouse heart, full-length *Glp1r* mRNA transcripts were

detected in RNA from atrial but not ventricular cardiomyocytes (61). Consistent with these findings, transgenic reporter mice expressing a fluorescent protein contingent upon endogenous *Glp1r* promoter activity revealed scattered fluorescent cells within the atria, but not the ventricular myocardium (62). Putative GLP-1R+ labeled cells were also detected in the aorta, as well as arteries and arterioles of the heart, kidney, pancreas, and intestine, colocalizing with smooth muscle α -actin and the pericyte marker neural/glial antigen 2. Reporter protein expression under the control of the *Glp1r* promoter was largely restricted to the arterial circulation (62). Evidence for a translated GLP-1R protein within the atrium was obtained by Pyke et al (63), who used a monoclonal GLP-1R antibody (MAB3F52) to detect expression of a GLP-1R-immunoreactive protein, complemented by radioligand binding to the same myocytes, within the sinoatrial node of monkeys and humans.

Glp1r mRNA transcripts were detected in kidney vasculature of 8-week-old C57BL/6 male mice by both in situ hybridization and RT-PCR, predominantly in renal arteries (64). Independent evidence for functional GLP-1R binding sites in afferent renal arterioles was obtained using radiolabeled ^{125}I -labeled GLP-1, ^{125}I -labeled Ex-4, and ^{125}I -labeled exendin (9–39) (65). Ligand binding was observed in the kidney cortex and in blood vessels adjacent to glomeruli as well as in interlobular and arcuate arteries, with localization of grains over vascular smooth muscle. Immunocytochemistry using a monoclonal antibody for the mouse GLP-1R, clone 7F38A2, localized GLP-1R+ cells within the renal vasculature in a pattern consistent with that described above for ligand binding (65). Notably, no GLP-1R-immunopositive cells were detected in kidneys from *Glp1r*^{-/-} mice. Vascular structures, predominantly smooth muscle cells within the monkey kidney, also contained GLP-1R-immunopositive cells, specifically within afferent arterioles, interlobular arteries, and arcuate arteries (63). No GLP-1R-immunoreactive ECs in the kidney were observed; however, weak GLP-1R immunopositivity was detected in smooth muscle cells within the monkey pulmonary artery (63).

Glp2r mRNA and GLP-2R protein expression was reported in ventricular cardiac tissue from adult male Wistar rat hearts by quantitative real time-PCR and by Western blot analysis using a GLP-2R antibody (66). In contrast, *Glp2r* mRNA transcripts were not detected in RNA from adult Sprague Dawley rat heart by RT-PCR analysis of the full-length *Glp2r* mRNA transcript (67). Moreover, *Glp2r* mRNA transcripts were not detected in RNA from right or left atria or ventricle of the adult C57BL/6 mouse heart (68). As is the case for immunodetection of the GLP-1R, commer-

cial antisera commonly used for detection of the GLP-2R exhibit major problems with sensitivity and specificity (7).

Usdin et al (69) detected *Gipr* expression by RT-PCR and in situ hybridization within the fetal and adult rat heart, predominantly in the cardiac endothelium, as well as in the endothelium of major blood vessels. *Gipr* mRNA transcripts corresponding to the entire GIP receptor (GIPR) open reading frame were also detected by PCR in RNA from the atrium and ventricle of the adult C57BL/6 mouse heart (68). Berglund et al (70) detected *Gipr* mRNA by PCR in RNA from mouse aorta and localized GIPR protein to ECs and a few smooth muscle cells using a combination of Western blotting and immunohistochemistry. A relative increase in *GIPR* expression was reported in carotid endarterectomy sections procured from individuals with symptoms of cerebral ischemia, relative to biopsy sections analyzed from asymptomatic subjects (70). The GIPR was also localized by immunohistochemistry to the endothelium of healthy but not atherosclerotic blood vessels. Nevertheless, the GIPR antibody used in a subset of these studies also detected immunoreactive GIPR protein in muscle, a tissue not known to express *Gipr* mRNA transcripts (71).

Gcgr mRNA transcripts were detected in rat heart using a combination of ribonuclease protection and RT-PCR (72). *Gcgr* mRNA transcripts were also detected by RT-PCR in RNA isolated from both right and left atria and ventricle of the adult mouse heart (68). Although glucagon has pleiotropic actions in the cardiovascular system, the localization of GCGR expression in the human vasculature has not been carefully studied.

V. Receptor Expression in Vascular Cell Lines

A partial *Glp1r* cDNA product was detected by RT-PCR in MS-1 cells (73), a mouse islet EC line; however, ascertainment of the relative abundance of *Glp1r* mRNA in this experiment and whether MS-1 and other EC lines robustly express the full-length *Glp1r* mRNA and translated GLP-1R protein requires more careful experimentation. Zhong et al (74) reported the presence of *GIPR* mRNA transcripts and protein in the ECV-304 cell line, as well as in ECs isolated from the human umbilical vein (HUVEC), human pulmonary artery, and human aorta (HAEC). Using primer pairs designed to generate a single 383 base pair cDNA fragment, multiple distinct PCR products were detected in the EC RNA preparations, attributed to differentially spliced *GIPR* mRNA transcripts (74); however, only a single 50-kDa immunoreactive GIPR protein was detected using an affinity-purified polyclonal antibody against the N-terminal domain of the receptor by Western

blot analysis of extracts from the human EC lines and mouse heart. The expression of a 50-kDa immunoreactive GIPR protein was also detected in HUVEC extracts by Western blot analysis (75). Alternative RNA splicing of *GIPR* mRNA transcripts has also been described in RNA from mouse islets (76) and human adipose tissues, yet most RNA transcripts detected were not capable of encoding a full-length functional mature GIPR protein (71). Taken together, these findings highlight the challenges of interpreting data from cells and tissues expressing *GIPR* mRNAs based on detection of incomplete small PCR products.

VI. Signal Transduction Pathways Linked to GPCR Signaling in Vasculature

In rat aortic smooth muscle cells isolated from the thoracic aorta of 8-week-old Sprague Dawley rats, Ex-4 (10 nM) inhibited angiotensin-II (AngII)-stimulated cell proliferation and migration. Preincubation of cells with Ex-4 also attenuated AngII-induced ERK1/2 and JNK phosphorylation; however, expression of the native *Glp1r* in rat aortic smooth muscle cells was not examined (77). Ex-4 administration to adult male nondiabetic Sprague Dawley rats (1 nmol/d by continuous infusion) for 4 weeks after carotid artery balloon injury reduced intimal hyperplasia and decreased proliferation of rat aortic vascular smooth muscle cells (VSMCs) *ex vivo* (78). Ex-4 also reduced the number of proliferating cell nuclear antigen+ smooth muscle cells within the injured carotid artery and reduced the proliferation of VSMCs *ex vivo* through mechanisms sensitive to inhibition of PKA and endothelial nitric oxide synthase (eNOS). Unexpectedly, Ex-4 (48-hour treatment; 10 nM) also increased apoptosis in VSMCs through PKA- and p38 MAPK-dependent mechanisms (78), and both the proliferative and apoptotic actions of Ex-4 were blocked by exendin (9–39).

Ex-4 (continuously infused at 24 nmol/kg/d for 4 weeks) reduced neointima formation in the denuded femoral artery of C57BL/6 mice; Ex-4 (10 nM during 12 hours) also produced a dose-dependent reduction of platelet-derived growth factor-stimulated mouse coronary artery smooth muscle proliferation *ex vivo* (79). Although the effects of Ex-4 were thought to be mediated through the GLP-1R expressed in smooth muscle cells, Ex-4 did not activate canonical GLP-1R signaling pathways, such as cAMP accumulation, activation of p44/42 MAPK, Akt, cAMP responsive element binding protein (CREB), p70 S6 kinase, or cyclin expression in murine VSMCs (79). Similarly, Shi et al (80) reported that the GLP-1R agonist liraglutide (100 nmol/L) reduced the glucose-stimulated proliferation and migration of rat thoracic aorta smooth

muscle cells *in vitro* and augmented the extent of high glucose (25 mM)-induced smooth muscle cell apoptosis. The actions of liraglutide were attenuated by the GLP-1R antagonist exendin (9–39); however, expression of the endogenous GLP-1R was not examined in the aortic smooth muscle cells. Liraglutide also inhibited the expression of osteoblastic differentiation markers and reduced osteoblastic differentiation and calcification in human VSMCs through mechanisms associated with activation of PI3K/Akt/mTOR/S6K1 signaling (81). However, whether these cells expressed the full-length authentic human GLP-1R remains uncertain.

GIP (at concentrations ranging from 10^{-10} to 10^{-7} M) increased intracellular calcium levels detected by Fura-2 imaging in HUVEC and human pulmonary vein EC cells in a manner consistent with mobilization of intracellular stores. In contrast, the smaller rise and pattern of elevation of $[Ca^{2+}]_i$ was more consistent with mobilization of extracellular calcium stores in ECV-304 cells (74). GIP treatment (50 pM for 4 hours) reduced advanced glycosylation end product (AGE) (100 μ g/mL for 24 hours)-induced reactive oxygen species (ROS) production. At the same time, GIP decreased levels of mRNA transcripts for: 1) the receptor for AGEs; 2) vascular cell adhesion molecule 1 (VCAM-1); and 3) plasminogen activator inhibitor-1, in HUVECs, findings mimicked by the cAMP analog 8-Bromoadenosine 3',5'-cyclic monophosphate (75). These actions of GIP were evident in HUVECs cultured in 5.5 mM but not 22 mM glucose. GIP also exerted mitogenic actions in ECs, reportedly at doses as low as 10^{-13} M, stimulating cell proliferation in both HUVEC and ECV-304 cells (82). The proliferative actions of GIP in HUVEC cells were associated with the induction of endothelin-1 (ET-1) secretion (EC_{50} of 4.6 nM) and attenuated by co-incubation with the endothelin B receptor subtype inhibitor BQ-788 (82). The proliferative effects of combining ET-1 (1 nM) and GIP (10 nM) were additive in ECV-304 but not in HUVECs (82).

VII. Human Genetic Variation in Class B GPCRs and Risk of Vascular Disease

As noted, the *GIPR* mRNA transcript undergoes considerable mRNA splicing, most extensively described in human adipose tissue, giving rise to 64 distinct mRNA transcripts (detected using PCR conditions encompassing more than 50 cycles), of which only two are predicted to encode for a biologically active functional receptor (71). The genetic variant rs10423928 is an A/T single-nucleotide substitution within the *GIPR* gene associated with increased 2-hour glucose and significantly reduced insulin

levels after an oral glucose challenge in several large human population analyses (83). Subjects with T2D carrying the A allele of the single nucleotide polymorphism rs10423928 exhibit an increased associated risk of stroke in a meta-analysis of seven independent studies (without any genotype-dependent change in blood pressure [BP]), but this association was not observed in nondiabetic control subjects or in studies of individuals with type 1 diabetes (70). The precise molecular mechanism(s) linking genetic alteration at rs10423928 to functional alteration of GIPR signaling and increased risk for stroke in diabetic subjects remains uncertain.

Genetic variation in the *GLP1R* has also been described, and the minor variant Ala316Thr, rs10305492, was associated with a lower fasting glucose, a reduced risk of T2D, and lower rates of cardiovascular disease (84). No specific information was provided regarding the variant and the development of vascular disease or stroke, and no association with BP, Alzheimer's disease, Parkinson's disease, or specific cancers was reported. Furthermore, the limited information to date describing the functional properties of this receptor variant precludes definitive understanding of whether this variant receptor is constitutively active in some cell types or confers an enhanced response to endogenous GLP-1.

A. Controversies and limitations of existing data

Ascertainment of the precise tissue and cellular distribution of GPCR expression is challenging, due to the paucity of available antibodies that provide a high degree of sensitivity and specificity. Notably, Class B GPCRs are often expressed at low levels, exhibit a high degree of sequence homology, and are susceptible to proteolytic degradation or alterations in conformation arising from procedures associated with tissue dissociation and cell isolation. Furthermore, RNA splicing and posttranslational modification may produce multiple receptor isoforms that are incompletely characterized and that may result in masking of epitopes recognized by various antisera. Collectively, these issues complicate immunodetection of individual receptor proteins, ideally using highly specific antisera. Our own experience mirrors that of others in finding substantial problems with the sensitivity and specificity of antisera widely used to detect the GLP-1R (58, 59) and GLP-2R (7). GLP-1R-immunoreactive protein has been repeatedly reported in EC lines and primary EC cultures; however, the sensitivity and specificity of most of the GLP-1R antisera used in these studies are frequently unknown or demonstrably suboptimal (58, 59). Remarkably, despite multiple published reports highlighting limitations of available GLP-1R antisera, recurring use of poorly validated GLP-1R antisera in the published lit-

erature remains widespread. Although reporter mice have been developed enabling detection of fluorescent proteins under the control of the endogenous *Glp1r* transcriptional locus (62), these mice do not enable direct detection of the endogenous translated GLP-1R protein. Furthermore, analysis of reporter mice, although potentially valuable, does not solve the problem of assessing GLP-1R expression in cells and tissues from other species, including humans. Moreover, even GIPR antisera with good sensitivity may inadvertently result in the detection of putative GIPR immunoreactivity in human and rodent skeletal muscle (71), a tissue that does not express an endogenous *GIPR* mRNA transcript. Hence, a high degree of vigilance and caution is required in regard to immunodetection of class B GPCR proteins and interpretation of the relevant literature. Moreover, most studies reporting detection of GPCR mRNA transcripts employ quantitative PCR or regular PCR to detect small (100–200 base pair) amplicons that do not encode the full-length receptor, often using PCR conditions (35–40 cycles or more) that favor detection of extremely low levels of mRNA transcripts. Hence, these reports ideally require confirmation using independent techniques that the cell type under study does indeed express a full-length mRNA transcript capable of encoding a mature functional receptor protein. Reports linking human genetic variation within GPCR genes to the risk of metabolic or vascular disorders are intriguing, yet the mechanisms through which changes in genetic sequences alter the expression or activity of the GPCRs frequently remain poorly understood.

Similar caveats also apply to the use of peptide antagonists for GLP-1, GIP, and GLP-2, which often exhibit only partial selectivity and are also generally weak or partial agonists at their cognate receptors. Although exendin (9–39), Pro(3)-GIP, and GLP-2 (3–33) are widely used as receptor antagonists, caution should be exercised in regard to the concentrations of these reagents used and their potential for partial agonism and generation of “off target” effects in cell and animal experiments.

VIII. Direct Actions of Peptide Hormones on Vascular Cells and Blood Vessels

A. GLP-1 and GLP-1R agonists

A large number of studies of EC lines have described actions of native GLP-1 and degradation-resistant GLP-1R agonists; however, few reports have included rigorous analysis of whether these actions are transduced by the endogenous canonical GLP-1R. GLP-1 (7–36) (0.03 nmol/L for 4 hours in the presence of bovine serum albumin reduced levels of the receptor of AGE mRNA tran-

scripts and protein in HUVECs, findings mimicked by 8-Bromoadenosine 3',5'-cyclic monophosphate and attenuated by small interfering RNA-mediated knockdown of a putative endogenous GLP-1R (85). Furthermore, AGE-induced ROS generation and subsequent up-regulation of VCAM-1 mRNA levels was attenuated by coinubation with GLP-1 (0.03–3 nM). Similarly, both Ex-4 and native GLP-1 (10 nmol/L, 48 hours) increased cell proliferation in human coronary artery ECs (HCAECs) through the activation of a PKA-eNOS-PI3K/Akt signaling pathway, actions attenuated by exendin (9–39). Nevertheless, GLP-1 (9–36) also increased DNA replication, and both eNOS and Akt phosphorylation in the same experiments with HCAECs, raising uncertainty as to the mechanisms transducing the actions of these peptides (86). Similarly, both Ex-4 and GLP-1 reduced palmitate (125 μ mol/L, 24 hours)-induced lipoapoptosis in HCAECs through activation of PKA-PI3K/Akt-eNOS-p38 MAPK-JNK-pathways, actions blocked by exendin (9–39) and Rp-cAMP[S], a competitive PKA antagonist. However, the changes in phosphoproteins detected in these experiments in response to Ex-4 or GLP-1 were modest, and GLP-1 (9–36) did not attenuate the cytotoxic actions of palmitate in these experiments (87). As noted above, most antibodies used to detect immunoreactive GLP-1R proteins in HUVECs or HAECs may not actually detect the authentic endogenous GLP-1R (58).

TNF α -induced nuclear factor- κ B activation in HUVECs was significantly blunted by treatment with liraglutide (30–300 nM); however, the actions of liraglutide were not inhibited by coinubation with exendin (9–39), and *GLP1R* expression was not evaluated (88). Pretreatment of HAECs with liraglutide (100 nmol/L, 1 hour) followed by an additional 3 hours of liraglutide exposure attenuated the induction of VCAM-1 and E-selectin by TNF α (10 ng/mL) or lipopolysaccharide (2 μ g/mL) (89). Liraglutide also decreased THP-1 monocyte adhesion to HAECs, actions attenuated by inhibition of the Ca²⁺/calmodulin-dependent protein kinase kinase with STO-609 and by lentivirus-mediated RNA knockdown of 5'-activated AMP protein kinase (AMPK) expression. Whether the actions of liraglutide in HAECs were mediated through the GLP-1R was not examined.

Culture of HUVECs in high glucose (12 hours at 25 mM) induced the expression of multiple proteins active in the endoplasmic reticulum stress response, including Bip1/Grp78, protein disulfide isomerase (*PDI*), endoplasmic reticulum oxidoreductin-1 α (*Ero1a*), calnexin, inositol requiring enzyme 1a (*IRE1a*), phospho-eukaryotic initiation factor 2 (*pEIF2a*), and p53-up-regulated modulator of apoptosis (*PUMA*) (90). Coinubation of HUVEC cultures with 100 nM liraglutide prevented the high glucose-mediated ER stress response; however, the mechanisms linking

liraglutide to attenuation of ER stress were not identified. Liraglutide-mediated attenuation of glucose-induced oxidative stress was demonstrated in HAECs incubated in 25 mM glucose (91). Although liraglutide, with or without metformin, modestly inhibited the extent of p47phox and PKC β translocation, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation, and phosphorylation of 5' AMPK, it was not determined whether any of these pathways or proteins transduced the inhibitory actions of liraglutide on glucose-generated oxidative stress.

Native GLP-1 (10 nM) directly suppressed ROS production and NADPH oxidase activity and reduced the expression of NADPH oxidase subunit proteins (p47phox, gp91phox, p22phox, and p40phox) in cardiac microvascular ECs isolated from the left ventricle of adult male Sprague Dawley rats and cultured under 25 mM glucose (92). GLP-1 also attenuated features of high glucose-induced apoptosis (Terminal deoxynucleotidyl transferase dUTP nick end labeling-positivity and levels of cleaved caspase-3), and a subset of GLP-1 actions (high glucose-induced Rho activation) was diminished by coinubation with the PKA-inhibitor H89. Whether the antioxidant actions of GLP-1 in cardiac microvascular ECs were mediated by the GLP-1R remains uncertain (92).

The expression and functional activity of the GLP-1R was analyzed in human islet microvascular ECs (IMECs). Robust GLP-1R protein expression was detected in human islets, including a subpopulation of ECs that exhibited immunopositivity for both CD31 and the GLP-1R (93). RT-PCR also detected *GLP1R* expression in RNA isolated from IMECs, surprisingly however, at levels much lower than those detected in RNA from white adipose tissue, a tissue itself not known to consistently express the human *GLP1R* at meaningful levels (1). Treatment of IMEC cultures chronically exposed to hyperglycemia (28 mM) with Ex-4 (10 mmol/L, 2 or 14 days) significantly increased cell viability and cell proliferation; Ex-4 also increased the levels of pAkt, pERK, and cAMP, whereas the cytoprotective and proliferative actions of Ex-4 were completely abrogated by coinubation with the signal transduction inhibitors LY294002, PD98059, MDL12330A, or KT5720. High glucose also increased the accumulation of vascular endothelial growth factor (VEGF)-1 (VEGF-A) in culture supernatants, an effect that was inhibited by coinubation with Ex-4. Unexpectedly, the GLP-1R antagonist exendin (9–39) inhibited not only the actions of Ex-4, but also the cytoprotective effects of obestatin, a structurally distinct peptide with actions overlapping those of Ex-4 in IMECs under high glucose culture conditions (93). The extent to which findings in this study, predominantly obtained using simian virus 40 T antigen-immortalized IMECs, might be replicated in pri-

mary cultures of nonimmortalized IMECs requires further experimentation.

Arakawa et al (94) continuously infused Ex-4 (300 pmol or 24 nmol/kg/d) into adult male C57BL/6 wild-type or apoE^{-/-} mice for 28 days and detected reduced numbers of Mac-2⁺ mononuclear cells adhering to the aortic endothelium and decreased aortic levels of VCAM-1 and intercellular adhesion molecule-1 (ICAM-1) mRNA transcripts. Ex-4 also directly reduced lipopolysaccharide-induced inflammatory gene and protein expression in peritoneal macrophages from wild-type mice. GLP-1R protein expression was detected by immunoblotting using extracts from murine macrophages, cultured murine ECs, HUVECs, and human THP-1 cells. Nevertheless, the antibody used to detect GLP-1R protein expression was subsequently shown to lack sensitivity and specificity (58, 59), raising questions about mechanistic interpretation of these findings.

The GLP-1R agonist liraglutide reduced the expression of inflammation-related genes via a direct effect in immortalized C11-STH EC cultures *ex vivo*, actions that were insensitive to the PKA antagonist H89 (95). Moreover, endothelium-dependent vasorelaxation was enhanced in isolated abdominal aortic segments from liraglutide-treated ApoE^{-/-} maintained on a high-fat diet (HFD; 22% fat and 0.15% cholesterol) for 12 weeks, whereas coadministration of continuously infused exendin (9–39) (150 pmol/kg/min) completely eliminated the vasodilatory actions of liraglutide (80 nmol/kg twice daily) (95). Liraglutide-treated mice also exhibited reduced expression of ICAM-1 and increased levels of eNOS protein, assessed by immunohistochemistry, in the aortic endothelium of ApoE^{-/-} mice, but these changes were eliminated in aortas from ApoE^{-/-} mice treated with both liraglutide and exendin (9–39). Whether C11-STH ECs or the abdominal aortic ECs from ApoE^{-/-} mice expressed the endogenous GLP-1R was not determined. Furthermore, liraglutide did not improve endothelium-dependent vasorelaxation in aortic rings from ApoE^{-/-} mice with early-onset low-burden atherosclerotic disease (only 4 weeks of high-fat feeding) (96).

Some studies have also examined direct effects of native GLP-1 or GLP-1R agonists on vascular smooth muscle cells. Ex-4 directly reduced AngII-mediated cell senescence in aortic smooth muscle cell cultures prepared from Sprague Dawley rats (97), findings associated with reduced superoxidant production, nuclear translocation, acetylation, and phosphorylation of Nrf2 and increased phosphorylation of PKA and CREB. The expression or functional importance of the canonical *Glp1r* within VSMCs was not examined in these experiments (97).

B. GIP Actions

Direct mitogenic actions of GIP have been demonstrated in ECs at doses as low as 10⁻¹³ M in HUVECs, although higher doses of GIP were required for stimulation of proliferation in the immortalized HUVEC line ECV-304 (10⁻⁸ M) (82). GIP selectively and dose-dependently increased ET-1 secretion (EC₅₀ of 4.6 nM) from HUVEC but not ECV-304 cells, and the proliferative actions of GIP were attenuated by coinubation of HUVECs with the endothelin B receptor blocker BQ-788. Although multiple *GIPR* mRNA transcripts were detected in ECV-304 cells using PCR, whether this immortalized cell line expresses a full-length canonical GIPR remains uncertain (98). Vascular bed-specific actions of GIP have also been reported in experiments using ECs isolated from the canine hepatic artery or portal vein (99). Partial cDNA PCR products were generated from both hepatic artery and portal vein EC cultures; however, whether one or both of these ECs express a full-length functional canine GIPR was not demonstrated. GIP (10⁻¹⁰ to 10⁻⁷ M) increased intracellular calcium levels detected by Fura-2 imaging and increased ET-1 secretion in canine hepatic artery ECs, but not in portal vein ECs; no effect on cAMP accumulation was detected in either EC culture. In contrast, GIP increased nitric oxide (NO) production only from portal vein ECs. The molecular basis for these divergent GIP responses favoring vasoconstriction vs vasodilation in canine ECs from different vascular beds was not determined (99).

Berglund et al (70) delineated an ET-1-osteopontin axis in intact mouse aortas cultured for several days *ex vivo*. GIP (1.0 nmol/L for 24–72 hours) increased levels of secreted ET-1 in aortic cultures, which in turn induced osteopontin expression (detected by immunohistochemistry) in aortic smooth muscle cells of the same arteries; these actions of GIP were eliminated by coinubation of cultures with BQ788 and BQ123 to block ET-1 receptor signaling. No direct effect of GIP on osteopontin expression was detected in isolated VSMCs. Both the GIP-mediated stimulation of ET-1 secretion and the ET-1-dependent induction of osteopontin expression were blocked by KG-501, a small molecule CREB antagonist. Hence, the authors envisioned a role for the transcription factor CREB in both the secretion of ET-1 from ECs and the synthesis and secretion of osteopontin from VSMCs. Remarkably however, GIP-stimulated ET-1 release was not blocked by the PKA inhibitors Rp-cAMPS or H-89. Hence, the precise pathway(s) linking GIPR signaling in ECs to CREB phosphorylation was not delineated (70).

IX. Vascular Bioactivity of Cleaved Peptide Fragments

GLP-1 metabolites such as GLP-1 (9–37) or GLP-1 (9–36) amide circulate at levels accounting for approximately 80% of total circulating GLP-1 immunoreactivity (100); however, pharmacological studies have demonstrated actions of these peptides or their C-terminal fragments such as GLP-1 (28–37) or GLP-1 (28–36) amide in liver, cardiomyocytes, blood vessels, and ECs through incompletely understood mechanisms (23). The physiological significance of these peptide cleavage products is unclear because they normally circulate at lower levels than those associated with their bioactivity in pharmacological studies, and normally these metabolites are rapidly cleared from the circulation. Under circumstances characterized by hypersecretion of native GLP-1, such as after some forms of bariatric surgery, antagonism of the GCGR, or in the context of a PGDP-secreting tumor, circulating levels of these GLP-1 metabolites may be quite elevated (1). As outlined below, it seems highly likely that at least some of the vascular effects of GLP-1 on endothelial function that have been demonstrated after infusion of the intact peptide likely reflect the actions of one or more GLP-1 metabolites. There is much less information on the putative endogenous physiological or pharmacological vascular activity of GIP (3–42) or GLP-2 (3–33).

A. The heart and coronary arteries

A large number of studies describe actions of GLP-1 degradation products on ventricular function and cardiomyocyte survival (4, 101). However, the translated functional GLP-1R protein corresponding to the canonical GLP-1R is largely expressed in the atrium of rodents and primates, focusing attention on the possibility that at least some of the cardiac actions attributed to these peptides may be indirect, perhaps mediated by alterations in blood flow to the ischemic or failing heart. Nevertheless, *GLP1R* mRNA transcripts have also been detected in the human ventricle, including in RNA prepared from isolated ventricular cardiomyocytes (102, 103), and ascertainment of whether the rodent or human ventricle expresses a canonical functional GLP-1R protein under some circumstances requires further experimentation. Continuous infusion (1.5 pmol/kg/min) of native GLP-1 or GLP-1 (9–36) for 48 hours in dogs with pacing-induced heart failure and dilated cardiomyopathy (104) improved ventricular function (left ventricular [LV] systolic, improved LV developed pressure [LVDP], LV contractility, blood flow, and myocardial glucose uptake). Both GLP-1 and Ex-4 increased LVDP after ischemia-reperfusion injury in WT mouse hearts; however, only GLP-1, likely through one or more

of its metabolites, improved LVDP in isolated *Glp1r*^{-/-} mouse hearts. GLP-1 (9–36) amide (0.3 nmol/L) also exerted direct cardioprotective actions *ex vivo* in isolated perfused 12-week-old mouse hearts subjected to ischemia-reperfusion injury (105). Notably, although both GLP-1 (9–36) amide and Ex-4 increased cAMP accumulation and reduced cell death in WT mouse cardiomyocytes treated with hydrogen peroxide, GLP-1 (9–36) amide (but not Ex-4) enhanced cAMP generation in cardiomyocytes from *Glp1r*^{-/-} mice (106).

Studies with isolated ischemic rat hearts demonstrated that when peptides were administered via intracoronary artery perfusion transiently during the first 15 minutes postischemia, Ex-4 (0.03 or 0.3 nmol/L), but not GLP-1 (9–36) amide (0.03 to 3 nmol/L), reduced infarct size, actions blocked by coadministration of the GLP-1R antagonist exendin (9–39) (107). In contrast, both Ex-4 and GLP-1 (9–36) amide augmented LV performance (LVDP and rate-pressure product) when administered from 60–120 minutes of the reperfusion period. These divergent results highlight how even modest differences in experimental protocols and timing of peptide administration can lead to different interpretations and conclusions. The biology of these peptide hormones in the normal and diseased heart, including actions arising independently from direct effects on the vasculature, has been extensively reviewed (4, 101).

B. Blood vessels

Numerous reports describe how native GLP-1 and GLP-1 (9–36) exert their effects on blood vessels within diverse vascular beds (Figure 2), many of which yield conflicting results and interpretations. Both peptides produced vasoconstriction in isolated segments of endothelium-intact mouse aorta *ex vivo* (146). In contrast, GLP-1 (7–36) amide, GLP-1 (9–36), Ex-4, and exendin (9–39) all produced vasodilation in isolated segments of thoracic aortas from adult male Sprague Dawley rats (146). The vasodilatory actions of GLP-1 were not abrogated by endothelial denudation, the NO synthase inhibitor, NG-methyl-L-arginine methyl ester (L-NAME), the cyclooxygenase inhibitor, indomethacin, or the hydrogen peroxide scavenger, catalase. In contrast, coincubation of GLP-1 with 30 mM KCl, the cAMP antagonist Rp-cAMPs, or 10 μ M glibenclamide significantly diminished the vasodilatory actions of GLP-1. Native GLP-1 also stimulated cAMP accumulation in isolated segments of thoracic aorta, consistent with the putative detection of *Glp1r* mRNA transcripts in aortic RNA by real-time PCR. In contrast, although both GLP-1 and GLP-1 (9–36) amide exerted direct vasodilatory actions in isolated rat femoral arterial rings *ex vivo*, Ex-4 was unable to produce signif-

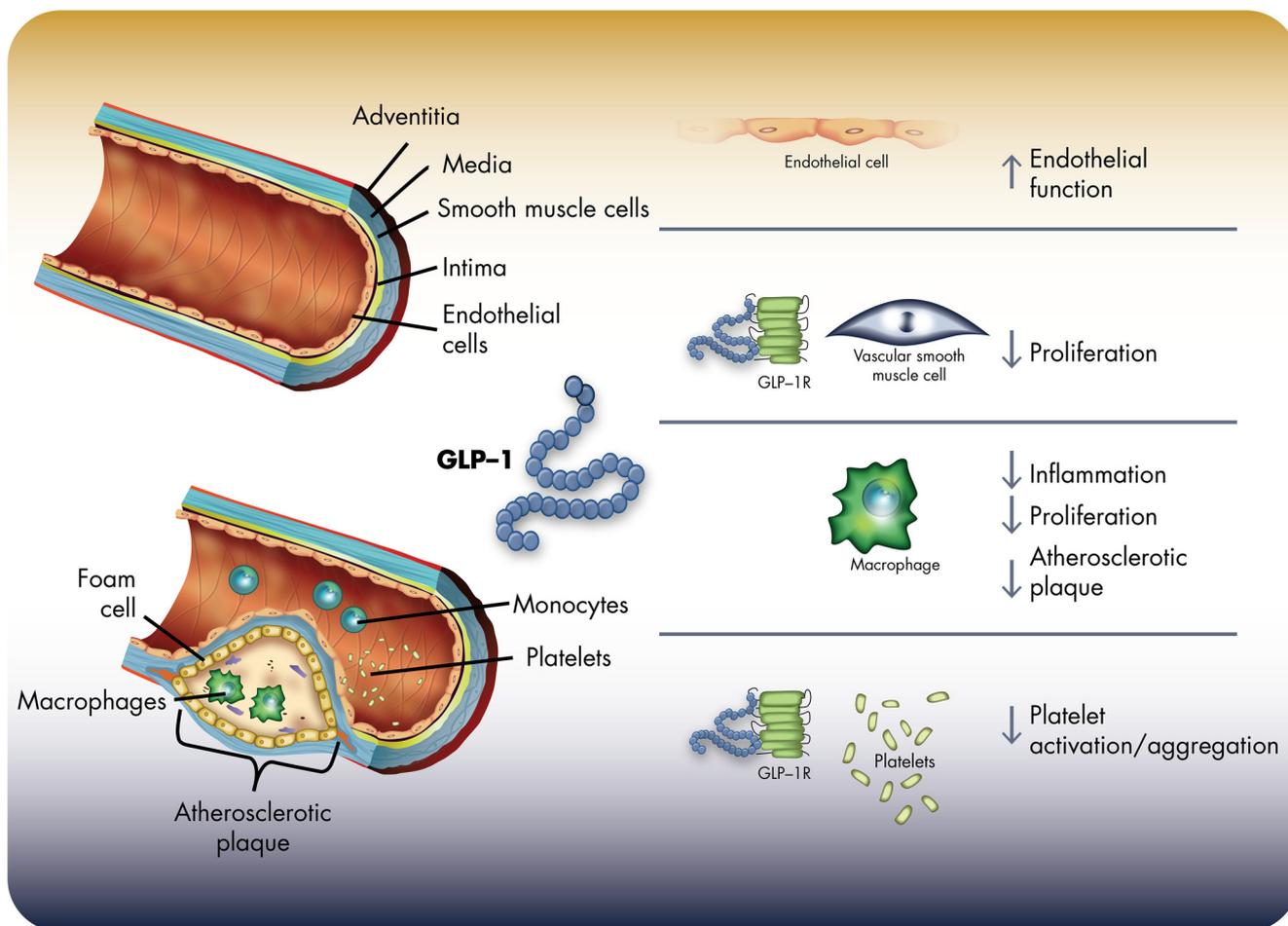
Figure 2.

Figure 2. The vascular biology of GLP-1 action. The precise cellular localization of the GLP-1R within blood vessels in different vascular beds remains incompletely understood. The vascular actions of GLP-1 in normal or diseased blood vessels may be direct or indirect, and clarification of the precise cellular sites of GLP-1R expression in blood vessels, immune cells, platelets, and hematopoietic cells requires further investigation.

icant vasodilation, either alone or after induction of lipid-mediated endothelial dysfunction, in the same experiments. Hence, the direct vasodilatory actions of native GLP-1 may be mediated by GLP-1R-independent mechanisms (108). The precise mechanisms through which GLP-1 (9–36) acts on blood vessels and ECs remain poorly understood but may include activation of cAMP- or cyclic GMP-dependent pathways (105, 106) and suppression of ROS through pathways associated with phosphorylation of voltage-dependent ion channel protein and activation of glycogen synthase kinase 3 and Akt phosphorylation (109).

C. Vascular inflammation

In studies using HAECs cultured *in vitro*, GLP-1 (9–36) amide directly attenuated glucose (12 mM)-stimulated generation of ROS production by reducing glycogen synthase kinase-3 β and increasing Akt activity. GLP-1 (9–36)

amide simultaneously reduced voltage-dependent anion channel phosphorylation, allowing the formation of a voltage-dependent anion channel/hexokinase-II complex that prevents mitochondrial ROS formation. Moreover, mice exposed to sequential ip glucose injections (3 g/kg) at 2-hour intervals and treated with a continuous infusion of GLP-1 (9–36) amide (300 mg/mL) for 24 hours exhibited a recovery of aortic prostaglandin I₂ activity that was otherwise persistently suppressed for days in mice treated with glucose alone. These findings suggest that recovery of prostaglandin I₂ activity reflects a reduction in endothelial ROS production mediated by GLP-1 (9–36) amide (109).

X. Actions of GLP-1 and GLP-1R Agonists on Endothelial Function in Human Studies

Ceriello et al (110) studied the effect of GLP-1 infusion (0.4 pmol/kg/min) on endothelial function, comparing in-

dividuals with T2D (glycosylated hemoglobin [HbA1c], 8.4%) and nondiabetic controls after a standard meal, oral glucose tolerance test, and hyperglycemic clamp. Diabetic subjects exhibited impaired flow-mediated vasodilatation, in parallel with an increase in oxidative stress detected by increased levels of nitrotyrosine and 8-iso-PGF2a (110). Flow-mediated vasodilatation was reduced, and markers of oxidative stress were similarly increased after a test meal, oral glucose tolerance test, or hyperglycemic clamp, and coadministration of GLP-1 for 2 hours reversed these abnormalities. After 2 months of insulin treatment to improve glycemic control (final HbA1c, 7.2%), plasma levels of nitrotyrosine and 8-iso-PGF2a were reduced, and FMD was significantly improved (110).

Similar beneficial effects of short-term GLP-1 infusion (0.4 pmol/kg/min for 2 hours) were observed in 15 human subjects with type 1 diabetes subjected to hypo- or hyperglycemic clamps. The metabolic stress of hypoglycemia (nadir 2.9 mM glucose) or hyperglycemia (15 mM) decreased endothelial function (FMD) and increased markers of oxidative stress (plasma levels of nitrotyrosine and 8-iso-PGF2a plasma) and inflammation (soluble ICAM-1 and IL-6) (111). The detrimental effects of hypo- or hyperglycemia on endothelial function were attenuated by coinfusion of GLP-1.

Nystrom et al (112) studied endothelial function in subjects with T2D (n = 12) and coronary artery disease (CAD) as well as healthy younger nondiabetic controls (n = 10). Infusion of recombinant GLP-1 (2 pmol/kg/min) during a hyperinsulinemic isoglycemic clamp improved endothelial function measured by FMD in diabetic subjects but had no effect in nondiabetic control subjects. GLP-1R protein was detected by Western blotting of extracts from HCAECs (112). Koska et al (113) examined endothelial function in subjects with T2D treated twice daily with exenatide (maximum tolerated dose, 10 μ g twice daily) or placebo for 11 days in a crossover design study. A separate group of 32 subjects with IGT or diet-controlled T2D was also treated with iv exenatide (50 ng/min), in the presence or absence of exendin (9–39). Endothelial function, reported as the reactive hyperemia index (RHI), was assessed before and 2 and 4 hours after a standard breakfast and again after lunchtime meals. Acute infusion of exenatide improved RHI in both experimental groups, and the acute effects of exenatide were blunted by coinfusion of exendin (9–39). Exenatide also directly improved arterial vasodilation in small arterioles within human adipose tissue biopsies from subjects with or without T2D and directly increased AMPK α and eNOS phosphorylation in HAECs, actions blocked by exendin (9–39) (113). The expression of the endogenous GLP-1R within arteries or HAECs was not examined in these studies.

Although several uncontrolled studies demonstrated favorable effects of GLP-1 on FMD, intima media thickness, or markers of oxidative stress, several randomized controlled trials failed to demonstrate preferential effects of GLP-1R agonists on endothelial function. Liraglutide was administered (1.2 mg/d) to a group of subjects (n = 10) with T2D and no history of CAD for 10 weeks, followed by a 2-week washout and another 10-week treatment period, with patients following their usual antidiabetic regimen (114). A second group of patients (n = 10) was given their usual antidiabetic agents first for 10 weeks, then treated with liraglutide for 10 weeks in a single-blind crossover design study with each subject serving as their own control. Ten weeks of liraglutide had no effect on peripheral endothelial function (RHI) or on coronary flow reserve (CFR), an assessment of coronary microcirculation, in either treatment group (114).

Microvascular endothelial function was assessed in human subjects treated with exenatide (10 μ g twice daily) or metformin (1000 mg/d) for 12 weeks. Studies were carried out in nondiabetic obese subjects with prediabetes (IGT, impaired fasting glucose, or mild elevation in HbA1c), and circulating markers of oxidative stress and inflammation were measured at baseline and after the 12-week treatment period (115). No treatment-specific differences in RHI, circulating C-reactive protein, oxidized low-density lipoprotein, or VCAM-1 were detected. Nandy et al (116) assessed the effects of liraglutide (n = 16), glimepiride (n = 17), or placebo (n = 16) administered over 12 weeks in subjects with T2D with no known history of CAD. Endothelial function was assessed at baseline and after 12 weeks, at ambient glycemia and euglycemia, by measurement of forearm blood flow in response to acetylcholine and sodium nitroprusside before and after L-NG-monomethyl arginine infusion. No treatment-specific differences in endothelial function were detected.

The effect on nailfold skin capillary perfusion of either acute iv exenatide (50 ng/min for 30 minutes, followed by a continuous infusion rate of 25 ng/min) or chronic administration of liraglutide (1.8 mg once daily for 12 weeks) was examined in human subjects with T2D, baseline HbA1c 6.5–9% (117). Neither acute nor chronic GLP-1R agonism significantly changed skin capillary perfusion. Hence, taken together, the existing literature does not favor a consistent improvement in endothelial function or microvascular perfusion after sustained administration of clinically utilized GLP-1R agonists in subjects with T2D.

A. Controversies and limitations of existing data

Although a considerable literature supports a link between administration of native GLP-1 and subsequent improvement in endothelial function in human subjects,

these actions of GLP-1 may be mediated by GLP-1 (9–36), a peptide with potent vascular activity (23, 105, 106, 109). Furthermore, many of these studies do not include active controls for the potent metabolic actions of GLP-1 (increased insulin, reduced glucagon, decreased glucose, changes in free fatty acids, reduced inflammation), which may be rapid in onset and indirectly modulate vasorelaxation and endothelial function. The challenges surrounding detection of authentic full-length functional GLP-1Rs in blood vessels, as well as ECs and smooth muscle cells (Figure 2), raise further uncertainty about the mechanisms linking pharmacological administration of GLP-1R agonists to changes in the biology of blood vessels and their constituent cell types.

Most data generated examining the bioactivity of peptide fragments, exemplified by studies using GLP-1 (9–36), GLP-1 (28–36), or GLP-1 (32–36), have been generated using doses of these peptides that result in circulating levels and cellular exposure often 10 to 1000 times higher than would be evident under normal physiological circumstances (1, 23). Furthermore, the mechanisms by which these peptide fragments exert their actions are poorly understood, membrane receptors for these peptides have not been identified, specific immunoneutralizing antisera or antagonists for peptide fragments have not been developed, and it is challenging to deploy mouse genetics to identify the importance of these smaller peptides. Hence, whereas the pharmacological actions of these GLP-1-derived peptides may have potential therapeutic relevance, the physiological importance of these peptide degradation products remains questionable.

XI. Physiological and Pharmacological Actions of Glucagon-Related Peptides on Blood Vessels

A. Angiogenesis

Aronis et al (118) used a three-dimensional scaffold of HUVEC spheroids to examine the direct effects of GLP-1 on angiogenesis. After 48 hours, GLP-1 (200–1000 nM) increased sprout length; lower doses of GLP-1 (50–100 nM) had no significant effect. Whether these actions reflect expression and activation of an endogenous canonical GLP-1R in EC spheroids or a receptor-independent pathway remains unclear. Similar effects were observed in studies of the angiogenic effects of Ex-4 using an in vitro fibrin gel bead assay populated with HUVECs. A significant increase in the number of sprouts and sprout length was detected after incubation with 100 nM Ex-4 for 5 days, findings attenuated by coincubation with 200 nM exendin (9–39) (119). Ex-4 (1 $\mu\text{g}/\text{kg}/\text{d}$ for 7 days) also enhanced

erythrocyte recruitment to a Matrigel plug microvessel model injected sc in 7-week-old C57BL/6 mice and increased CD31 immunopositivity within capillaries in vivo; the expression of the native GLP-1R within these angiogenic models was not examined (119).

Daily administration of Ex-4 (0.1 mg/kg) for a period of 9 weeks, commenced immediately after left anterior descending (LAD) coronary artery ligation in nondiabetic adult C57BL/6 mice, was associated with increased capillary density in the peri-infarct zone, assessed by quantification of EC CD31 and vascular smooth muscle cell α -actin+ immunoreactive blood vessels (120). In contrast, Ex-4 treatment did not increase capillary density after myocardial infarction in similarly treated *Mkk3*^{-/-} or *Akt*^{-/-} mice. Proangiogenic actions of Ex-4, delivered via either twice daily injections or administration of a pegylated-Ex-4 conjugate every 3 days, have been demonstrated in the mouse heart after experimental myocardial infarction (121).

Angiogenic properties of GLP-1R agonists have also been observed in studies of transplanted islets. Remarkably, islets pretreated with liraglutide (24 hours; 1 μM) before intraportal transplantation exhibited a greater number of CD31-immunopositive cells within days of transplantation, findings maintained upon analysis of islets 21 days after transplantation; treatment of islets in vitro with liraglutide (12–48 hours; 10 μM) increased expression of HIF-1 α , platelet-derived growth factor- α , VEGF (*Vegf*), and VEGF release into cultured medium (122). GLP-1R agonists, specifically Ex-4, have also exhibited angiogenic properties in murine models of hindlimb ischemia. Administration of daily injections of Ex-4 (1 $\mu\text{g}/\text{kg}$) for 3 consecutive days increased the CD31-immunopositive capillary area within the hypoperfused gastrocnemius muscle of 8-week-old male Balb/c mice, assessed 7 days after femoral artery ligation (123). Similarly, Ex-4 administration (10 $\mu\text{g}/\text{kg}$ once daily) partially reversed the decreased numbers of CD31+ cells and increased VEGF protein expression (detected by Western blotting) in ischemic mouse quadriceps muscle of lean or obese mice 14 days after initiation of critical limb ischemia, induced using a combination of vessel ligation and ambient hypoxia (124).

Cell therapy approaches have also been utilized to examine the angiogenic actions of GLP-1 in the context of hindlimb ischemia in CD1 mice. Application of encapsulated human mesenchymal stem cells (MSCs) transduced with a vector expressing two tandemly linked copies of GLP-1, onto the perivascular fascia, significantly increased capillary and small arteriolar density along the occluded artery assessed 21 days after induction of femoral artery ischemia (125). Although MSC application

was associated with detection of human angiogenic proteins in MSCs and modulation of both pro- and antiangiogenic factors in murine muscle, no significant differences in levels of gene or protein expression were observed when comparing data obtained in mice treated with control MSCs vs mice treated with GLP-1-secreting MSCs (125). Hence, the precise mechanisms through which GLP-1-transduced MSCs produced benefit in this model remain unexplained.

B. GLP-1 and blood flow

The effects of GLP-1 on the control of pancreatic and intestinal blood flow were examined in acute studies of normoglycemic Wistar rats and diabetic Goto Kakizaki (GK) rats. GLP-1 had no significant effect on pancreatic islet blood flow in Wistar rats but decreased blood flow in diabetic GK rats (126). Whether these actions were direct or indirect, reflecting changes in islet hormones or glycemia, was not determined. Similar results were obtained in studies of pancreatic and islet blood flow in Wistar rats. Administration of either GLP-1 or Ex-4 prevented the glucose-induced increase in pancreatic islet blood flow, independent of simultaneous NO blockade using L-NAME (127). Central infusion of Ex-4 (0.5 pmol/kg/min) for 3 hours into the lateral ventricle of awake freely mobile mice clamped under conditions of hyperinsulinemia and hyperglycemia decreased femoral arterial blood flow (128). Conversely, blockade of central GLP-1R signaling with exendin (9–39) or analysis of *Glp1r*^{-/-} mice revealed an increase in femoral artery blood flow under the same clamp conditions. These central actions of Ex-4 to control peripheral blood flow were associated with translocation of protein kinase C (PKC), most notably PKC δ , to the plasma membrane and were reversed by inhibitors of PKC activity (129). In contrast, central Ex-4 administration had no effect on femoral artery blood flow in the fasting normoglycemic state (128).

GLP-1 (10 pmol/kg/min for 4 hours) acutely increased coronary artery flow reserve, assessed at 1 and 4 hours by intracoronary artery Doppler in resuscitated domestic swine with electrically induced ventricular fibrillation (130). Nevertheless, these findings were not associated with any changes in ventricular function in the different treatment groups. In contrast, infusion of GLP-1 (at doses sufficient to obtain coronary perfusate GLP-1 concentrations from 10 pmol/L to 1 nmol/L) directly into the coronary arteries of open chest anesthetized dogs had no acute effect on coronary blood flow studied under a range of different (40 or 100 mm Hg) coronary perfusion pressures (131). In parallel studies *ex vivo*, GLP-1 had no effect on the developed isometric tension of intact or endothelium-denuded precontracted epicardial coronary artery

rings (131). Hence, under these specific conditions, no direct effect of GLP-1 on vasodilation or blood flow was detected in dog coronary arteries.

Several studies have examined the effects of peripheral GLP-1 administration on muscle blood flow. Infusion of GLP-1 (30 pmol/kg/min) into anesthetized fasted normoglycemic adult male Sprague Dawley rats resulted in a rapid increase (2.5-fold) in muscle microvascular blood volume (MBV), without changes in muscle microvascular blood flow velocity and without simultaneous changes in femoral artery blood flow assessed by contrast-enhanced ultrasound (132). The GLP-1-induced increase in skeletal muscle blood flow remained evident during coinfusion of somatostatin to eliminate the confounding effects of increased insulin secretion. Plasma levels of NO increased 3-fold during the GLP-1 infusion; however, total muscle eNOS (Ser1177) phosphorylation was not increased in skeletal muscle, but infusion of L-NAME starting 30 minutes before GLP-1 completely eliminated the GLP-1-mediated induction of MBV and prevented the increase in plasma NO (132). In separate experiments, GLP-1 infusion increased the muscle uptake and clearance of ¹²⁵I-insulin, actions also attenuated by L-NAME.

In related studies, initiation of a 60-minute GLP-1 infusion (30 pmol/kg/min) after 60 minutes of an initial euglycemic insulin clamp in normal rats resulted in further augmentation of muscle MBV and MBF, associated with a 40% increase in muscle uptake of ¹²⁵I-insulin without changes in plasma glucose or insulin levels (133). Moreover, the actions of GLP-1 to acutely increase muscle MBF were preserved in rats with experimental insulin resistance induced by 4 weeks of high-fat feeding or via an acute intralipid plus heparin infusion. Remarkably, GLP-1 infusion reversed the decrements in MBV, MBF, plasma NO, and muscle oxygen saturation (induced by acute intralipid infusion or chronic high-fat feeding) to control levels in rats given insulin alone (133). Furthermore, levels of phosphorylated PKA were enhanced in the aortas of insulin-resistant rats after GLP-1 infusion.

Very similar results were reported in studies of male Sprague Dawley rats with HFD-induced insulin resistance. Insulin-stimulated muscle microvascular recruitment was rapidly attenuated in rats with insulin resistance, whereas coinfusion of GLP-1 (100 pmol/kg/min) under conditions of a euglycemic hyperinsulinemic clamp increased microvascular recruitment by 27 and 35% after 5 days and 8 weeks of high-fat feeding, respectively (134).

Although much less data are available on whether GLP-1 regulates renal blood flow (RBF), Jensen et al (65) administered GLP-1 through the left renal artery directly into the rat kidney and observed a 13% increase in RBF after 5 minutes, findings attenuated by coinfusion of ex-

endin (9–39). Moreover, these findings were mimicked by peripheral administration of GLP-1 into the jugular vein, whereas intrajugular coadministration of exendin (9–39) blocked the GLP-1-dependent induction of RBF. In contrast, intrarenal infusion of GLP-1 (9–36) amide had no effect on RBF. Simultaneous administration of L-NAME and indomethacin together did not attenuate the actions of intrarenal GLP-1 to increase RBF (65).

Fewer studies examining how GLP-1R agonists regulate blood flow in humans are currently available. The acute effects of placebo vs exenatide (0.066 pmol/kg/min to achieve steady-state plasma levels of 170 pg/mL) on myocardial blood flow were examined in a randomized, double-blinded, placebo-controlled crossover study of eight obese male subjects with T2D. Myocardial blood flow, quantified using $^{13}\text{N-NH}_3$ as a perfusion tracer, was significantly increased by 24% during the exenatide infusion, without any associated changes in myocardial glucose uptake assessed during a pancreatic pituitary clamp (glucagon plus somatostatin infusion) maintaining a glucose level of 9 mM (135). In contrast, analysis of cardiac blood flow in fasted lean normoglycemic vs obese human subjects with T2D, after acute saline or GLP-1 infusion (1.5 pmol/kg/min started at 10 PM the evening before analysis of cardiac hemodynamics), revealed no augmentation of myocardial blood flow in GLP-1-treated subjects (136). In contrast, GLP-1 stimulated myocardial glucose uptake in lean subjects, but not in obese subjects with T2D.

Using a crossover study design, Faber et al (114) examined the effects of a 10-week course of liraglutide (1.2 mg/d), in addition to usual diabetes care, on CFR in 18 subjects with T2D without a prior history of cardiovascular disease. Although liraglutide therapy was associated with a reduction in HbA1c, systolic BP, and weight, no significant differences in CFR, ascertained after dipyridamole infusion, were detected after 10 weeks of liraglutide administration, as assessed by transthoracic Doppler flow echocardiography.

Subaran and colleagues examined the acute effects of a GLP-1 infusion (1.2 pmol/kg/min over 150 minutes) on muscle and cardiac microvascular blood flow in fasted healthy young human subjects (137). Both skeletal and cardiac muscle MBV and MBF and brachial artery blood flow were increased in subjects infused with GLP-1, compared to baseline values. However, plasma glucose and glucagon were reduced and insulin was increased, complicating interpretation of the data.

C. GLP-1 and BP

Studies in normotensive adult male Sprague Dawley rats using different doses of glucagon, GLP-1 (1–37), GLP-1 (7–36) amide, and GLP-2 administered via acute iv

injection demonstrated that only GLP-1 (7–36) amide increased systolic and diastolic BP, as well as heart rate (HR), in a dose-dependent manner (0.1, 10, 100, or 1000 ng) (138). Pretreatment with the vesicular monoamine transport inhibitor reserpine (10 mg/kg) or the adrenergic receptor antagonists phentolamine (0.1 mg/kg) and propranolol (1 mg/kg) did not prevent the GLP-1-stimulated increase in BP or HR. The importance of CNS GLP-1Rs for transduction of the peripheral cardiovascular effects of GLP-1 was revealed by demonstration that intracerebroventricular (icv) injection of Ex (9–39) (25-fold excess concentration relative to GLP-1) blocked the increase in BP and HR induced by either central or peripheral GLP-1 administration (139). Pretreatment with iv exendin (9–39) also blocked the increase in HR and BP induced by peripheral GLP-1 infusion. Nevertheless, it remains uncertain as to whether the peptides administered centrally also reached the peripheral circulation, and vice versa.

The importance of central autonomic circuits for transduction of GLP-1R-dependent induction of HR and BP was revealed in studies of adult male Sprague Dawley rats injected with Ex-4 either iv (30, 300, or 3000 ng/rat) or icv (3, 30, or 300 ng/rat) (140). A dose-dependent increase in HR and BP was observed by telemetric monitoring, associated with increased c-Fos expression in catecholaminergic neurons as well as in the adrenal medulla. Increased HR and BP levels were detected after icv or iv administration of even 30 ng of Ex-4, a dose that did not induce hypoglycemia. Furthermore, both iv and icv administration of Ex-4 rapidly induced tyrosine hydroxylation gene expression in medullary catecholaminergic neurons (140). Hence, peripheral and central administration of GLP-1R agonists engages CNS circuits, leading to a rapid but transient increase of HR and BP in normotensive rats and mice.

Studies in adult male Wistar albino rats, in which different doses of GLP-1 (100, 500, 1000 ng/10 μL) were administered via icv injection, implicated a role for cholinergic signaling in the transduction of GLP-1 effects on BP (141). Pretreatment with mecamylamine (25 $\mu\text{g}/10 \mu\text{L}$ icv), a nonselective, noncompetitive antagonist of nicotinic cholinergic receptors; or atropine (5 μg icv); or a vasopressin V1 receptor antagonist (intra-arterial injection, 10 $\mu\text{g}/\text{kg}$) blunted the GLP-1-induced increase in BP. In contrast, mecamylamine only partially attenuated the GLP-1 induction of HR, whereas vasopressin receptor 1 blockade with (B-mercapto B, B-cyclopentamethylene-propionyl, O-Me-Tyr,Arg)-vasopressin did not prevent the increase in HR (141).

D. GLP-1 and control of BP in preclinical models of hypertension

Hirata et al (142) used two experimental mouse models to assess the antihypertensive effects of GLP-1: 1) high-salt

diet-induced hypertension in obese diabetic (db/db) mice; and 2) AngII-induced hypertension in lean nondiabetic C57BLK/6J mice. Twice daily Ex-4 (20 mg/kg for 12 weeks) prevented the development of hypertension in male db/db mice and increased urine volume and sodium excretion after 2 weeks in separate groups of mice treated with 2.0% saline (NaCl) (142). A single injection of Ex-4 rapidly increased urinary cAMP levels, consistent with detection of *Glp1r* mRNA transcripts in the mouse kidney by PCR (142). Similarly, Ex-4 blunted the AngII (1 $\mu\text{g}/\text{kg}/\text{min}$)-induced rise in BP over 19 days in 8-week-old C57BLK/6J mice. Nevertheless, the potential indirect contribution of Ex-4-induced weight loss to the BP reduction observed in these studies cannot be ascertained.

Liraglutide administration (30 $\mu\text{g}/\text{kg}$, ip injection twice daily for 3 weeks) to wild-type C57BL/6 mice with AngII (490 ng/kg)-induced hypertension reduced both systolic and diastolic BP and increased urine sodium excretion through mechanisms independent of weight loss; these actions were completely absent in *Glp1r*^{-/-} mice (61). The reduction in BP after administration of GLP-1R agonists was associated with increased secretion of atrial natriuretic factor and abolished in *Nppa*^{-/-} mice. Although GLP-1R agonists failed to directly promote vascular cyclic GMP release or relaxation of precontracted blood vessels *ex vivo*, conditioned medium from liraglutide-treated hearts produced endothelium-independent vasorelaxation. Continuous administration of recombinant GLP-1 (7–36) amide (1 $\mu\text{g}/\text{kg}/\text{min}$) for 14 days also reduced BP and increased urine sodium excretion in Dahl salt-sensitive (DSS) rats fed a saline diet (8% NaCl) (143). Rats treated with GLP-1 had a reduction in mean arterial BP, an attenuated rise in creatinine, decreased urine protein excretion, increased urine flow and sodium excretion, and enhanced acetylcholine-induced vasorelaxation in isolated aortic rings (143). Nevertheless, whether the weight loss evident in the GLP-1-treated rats contributed to the reduction in BP and attenuation of renal injury was not ascertained.

Chronic administration of GLP-1 (25 pmol/kg/d for 4 weeks by continuous sc infusion) or the exenatide analog AC3174 (1.7 pmol/kg/d) in low salt (0.3% NaCl) vs high salt (8% NaCl)-fed DSS rats reduced systolic BP in hypertensive rats, independent of any changes in body weight (144). Rats treated with AC3174 alone or in combination with captopril exhibited increased survival, lower serum creatinine, less histological evidence of kidney damage, reduced levels of fasting insulin, and lower homeostasis model of assessment for insulin resistance. The mechanisms contributing to the reduction in BP were not addressed.

Although the available data are limited, a single study has demonstrated that chronic therapy with liraglutide twice daily (200 $\mu\text{g}/\text{kg}$) for several weeks attenuated pulmonary artery pressure, decreased vascular thickness, and reduced right ventricular hypertrophy in Wistar rats with monocrotaline-induced pulmonary artery hypertension (145). These therapeutic effects were accompanied by reductions in mean arterial pressure, decreased circulating levels of ET-1, and weight loss. Levels of cGMP in lung tissue were reduced in control rats and restored toward normal after liraglutide administration. Moreover, liraglutide directly reduced pulmonary artery smooth muscle cell migration and increased eNOS and soluble guanylyl cyclase expression in these cells *ex vivo* (145). Whether these actions were mediated by the canonical GLP-1R expressed in pulmonary artery smooth muscle cells was not determined.

E. GLP-1, atherosclerosis, and vascular inflammation

Both native GLP-1 and GLP-1R (as well as GIPR agonists) reduced the development of atherosclerosis and vascular inflammation in sensitized genetic mouse models (4, 103). Twenty-one-week-old *Apoe*^{-/-} mice fed an atherogenic diet and treated with either continuous sc infusions of GLP-1 (7–36) amide (2.2 nmol/kg/d) or GIP (1–42) (25 nmol/kg/d) for 4 weeks exhibited a reduction in atherosclerotic lesion score, atheromatous plaque formation, and aortic macrophage infiltration in the aortic root and abdominal aorta compared to control animals. Coinfusion of either exendin (9–39) or the non-selective GIPR antagonist and partial agonist (Pro3)GIP attenuated the therapeutic actions of GLP-1 (7–36) amide and GIP (1–42), respectively (147). Similar results were obtained in studies of *Apoe*^{-/-} mice infused with liraglutide (107 nmol/kg/min) via mini-osmotic pumps for 4 weeks in conjunction with the administration of an atherogenic diet. Liraglutide had no effect on BP, triglycerides, or levels of total cholesterol, but the atherosclerotic lesion area, plaque size, and monocyte/macrophage accumulation were significantly reduced in the aortic arch (148). Notably, body weight was reduced by 21% in liraglutide-treated mice over 4 weeks. Similarly, a 28-day infusion of Ex-4 at low (300 pmol/kg/d) or high (24 nmol/kg/d) doses in 8-week-old *Apoe*^{-/-} mice reduced the density of monocyte adhesion in the aorta, and the higher dose of Ex-4 reduced the atherosclerotic lesion area in the aortic sinus (94). These actions of Ex-4, particularly in mice infused with the lower dose, were not associated with significant weight loss or changes in insulin sensitivity.

Some studies have failed to show a reduction of experimental atherosclerosis after administration of GLP-1R agonists. *Apoe*^{-/-} male mice fed a HFD and treated with

STZ to induce dysglycemia were randomly assigned at 12 weeks of age to receive the long-acting GLP-1R agonist (tasoglutide, 4 mg sc), placebo, or metformin (400 mg/kg/d). Tasoglutide failed to reduce the extent of atherosclerotic plaque development in the abdominal aorta or aortic arch of older diabetic *Apoe*^{-/-} mice (58), despite a marked reduction in the accumulation of liver fat in the same animals. Contrasting effects of liraglutide on the development of atherosclerosis were noted in *Apoe*^{-/-} mice, depending on the age of the mice and the duration of exposure to a HFD (96). Liraglutide administered (300 µg/kg twice daily) alone or in combination with exendin (9–39) (150 pmol/kg/min sc via mini-osmotic pump) reduced the atherosclerotic plaque area, as well as the intima:media ratio in mice with a lower burden of atherosclerosis, findings partially attenuated by coadministration of exendin (9–39). In contrast *Apoe*^{-/-} mice fed a HFD for 12 weeks exhibited minimal reduction in atherosclerotic lesion area after 4 weeks of twice daily liraglutide administration (96). Hence, the actions of GLP-1R agonists to attenuate the development of experimental atherosclerosis appear highly model- and context-dependent (103) (Figure 2).

F. GLP-1R agonists regulate HR and BP in clinical studies

The effects of native GLP-1 or GLP-1R agonists have been studied after both short-term and sustained administration, in healthy normotensive subjects as well as in hypertensive subjects with diabetes and/or obesity. Acute administration of native GLP-1 or GLP-1R agonists increased HR and BP in normal healthy subjects, obese individuals, or subjects with T2D (4). The increase in HR (and cardiac output) in adult healthy fasting individuals given GLP-1 (iv infusion at 1.5 pmol/kg/min for 180 minutes) occurred independent of changes in blood flow assessed by ⁵¹chromium-labeled EDTA infusion and was associated with a small drop in plasma renin activity (149). A short 3-week crossover design involving administration of liraglutide or placebo to 20 hypertensive overweight or obese patients with T2D demonstrated increases in HR with liraglutide, no change in systolic BP, and a small increase in diastolic BP, although increases in urine sodium excretion were detected at the end of the 3-week study intervention (150). No significant changes in levels of cardiac natriuretic hormones or angiotensin 2 were detected after liraglutide administration in human subjects.

More sustained administration of GLP-1R agonists in clinical trials of subjects with T2D or obesity generally demonstrates a small reduction in systolic BP, often independent of changes in body weight; however, the mechanisms for BP reduction remain incompletely defined (103). The magnitude of BP reduction may be related in part to

the reduction of excess fluid retention in some subjects because relatively greater BP reduction has been detected in some clinical trials of GLP-1R agonists in subjects treated with pioglitazone or rosiglitazone (151). In the largest randomized placebo-controlled cardiovascular outcome studies reported to date that examined the cardiometabolic actions of GLP-1R agonists in subjects with T2D, the reduction in systolic BP reported was 0.8 mm Hg with lixisenatide 20 µg once daily (152) and 1.2 mm Hg with liraglutide 1.8 mg daily (153). In contrast, in a smaller outcome study examining the cardiovascular safety of once-weekly semaglutide, systolic BP reductions of 3.4–5.4 mm Hg were observed in subjects receiving 0.5 or 1.0 mg of semaglutide at week 104 (154). Hence, the incremental reduction of BP observed in diabetic hypertensive human subjects concomitantly treated with GLP-1R agonists and conventional antihypertensive agents ranges from modest to potentially meaningful and may depend on the specific GLP-1R agonist and the extent of treatment-associated weight loss (103, 154).

G. GLP-2 and blood flow

Multiple preclinical studies demonstrated that GLP-2 infusion rapidly increases intestinal blood flow. Neonatal pigs administered total parenteral nutrition and coin fused with GLP-2 (500 pmol/kg/h iv for 4 hours) to achieve circulating levels approximately 3-fold higher than normal postprandial levels of GLP-2 exhibited a rapid increase (within 10 minutes) in superior mesenteric artery (SMA) blood flow and intestinal blood volume, without changes in arterial BP (155). The GLP-2-stimulated increase in portal blood flow was NO-dependent and was abolished by coadministration of L-NAME (50 µmol/kg/h). Regional analysis of changes in blood flow using fluorescent microspheres demonstrated that GLP-2 increased blood flow in total parenteral nutrition-fed neonatal piglets predominantly in the proximal and distal jejunum, but not in the ileum or colon (156). GLP-2 infusion (4.6 nmol/kg) also increased intestinal SMA blood flow within 10 minutes (without changes in arterial BP) in anesthetized adult male Sprague Dawley rats, actions attenuated by coadministration of L-NAME (72 µmol/kg) or lidocaine (74 µmol/kg) (157).

Administration of GLP-2 by acute infusion on day 0 and day 10 (1000 pmol/kg/h) in Holstein calves also treated with GLP-2 (50 mg/kg twice daily for 10 days) produced an increase in SMA blood flow (175% increase after one injection, 137% increase after 10 days of treatment), findings associated with increased small bowel epithelial mass (158). The attenuation of the GLP-2-stimulated changes in blood flow suggest that this action of

GLP-2 is subject to tachyphylaxis through mechanisms that remain unexplored.

GLP-2 also rapidly increases blood flow in humans. Intravenous infusion of GLP-2 (from 0.5–2.0 pmol/kg/min), resulting in peak plasma GLP-2 levels of just over 300 pmol/L, increased SMA blood flow in healthy fasted human subjects. Acute sc injection of a larger dose of GLP-2 (450 nmol) which produced peak plasma GLP-2 levels of 1725 pmol/L (80-fold higher than normal circulating levels) increased HR, cardiac output, and SMA blood flow without changes in other vascular beds (159). Acute sc GLP-2 injection (1600 μ g) also increased intestinal blood flow in humans with short bowel syndrome and end-jejunosomy with <200 cm of remnant small intestine (160), with the increase in blood flow correlating with the length of the remnant intestine.

H. Glucagon, BP, and blood flow

Acute administration of glucagon increases HR and reduces systemic vascular resistance in preclinical studies and in human subjects (14, 161). Despite vasodilatory activity in many vascular beds, glucagon robustly increases cardiac output in the setting of hypovolemia or shock and is occasionally used to treat human subjects with refractory hypotension and cardiogenic shock (14); however, the efficacy of glucagon in this setting has not been rigorously assessed in clinical trials.

In dogs with hypovolemic shock, acute glucagon infusion increased blood flow to the intestine (162) with a relative preservation of blood flow in the small and large bowel, relative to other organs. Analysis of regional gut blood flow by video microscopy in anesthetized rats demonstrated that glucagon (1 μ g/kg/min) had no effect on SMA blood flow, but markedly increased flow within villous arcade blood vessels (163). In contrast, both systemic iv infusion and selective intra-arterial (SMA) administration of glucagon (1 mg/kg/min) rapidly and robustly increased (2-fold) blood flow within the SMA of dogs, associated with a reduction in systemic vascular resistance (164). The physiological importance of endogenous glucagon in the control of blood flow was examined using radioactive microspheres in rats with splanchnic hyperemia secondary to experimental venous occlusion and portal hypertension. Immunoneutralization of glucagon reduced blood flow to the stomach and small and large bowels and decreased hepatic arterial blood flow (165). Similar studies using radioactive microspheres examined the importance of endogenous glucagon for intestinal blood flow in rats with STZ-induced diabetes. The onset of experimental diabetes was associated with a 60% increase in blood flow to the gastrointestinal tract (166). Immunoneutralization of glucagon reduced blood flow to

the kidney, stomach, and proximal small bowel without affecting blood flow to the ileum and colon in diabetic rats. Hence, under some experimental conditions, endogenous glucagon may contribute to the control of regional blood flow within the gastrointestinal tract. Pharmacological intraportal glucagon administration (5 ng/kg/min) also increases RBF and glomerular filtration rate, independent of increases in blood glucose (167); however, whether these effects of elevated plasma glucagon levels on RBF are sustained with chronic administration is less certain.

I. Glucagon and blood flow in humans

Analysis of changes in mesenteric vessel flow velocity using duplex ultrasound scanning demonstrated that both meal ingestion and glucagon infusion (40 μ g/min) increased peak systolic velocity in the SMA and celiac artery, without any associated changes in vessel diameter (168). Consistent with the preclinical data, glucagon infusion (10 ng/kg/min for 20 minutes, then 20 ng/kg/min) acutely and selectively increased blood flow within the azygous vein of patients with good liver function (Pugh Class A) and well compensated cirrhosis (169). In contrast, blood flow was not increased by glucagon in more severely ill patients (Pugh Class B). Moreover, glucagon infusion had no effect on HR, mean arterial pressure, systemic vascular resistance, or hepatic blood flow. Hence, the actions of glucagon on intestinal blood flow are not uniformly conserved in different pathophysiological conditions.

Acute iv administration of 1 mg glucagon in normal subjects as well as in subjects with cirrhosis produced rapid increases in HR, cardiac index, and BP; however, changes in these parameters were quantitatively greater in normal control subjects (170). In some subjects, glucagon administration may be associated with nausea and gastrointestinal side effects, indirectly contributing to an increase in HR. Glucagon also increased azygos blood flow in subjects with cirrhosis. Overlapping yet distinct results were obtained by Nakahara et al (171), who administered 1 mg of glucagon via im injection in normal healthy control subjects and in patients with cirrhosis. Glucagon did not increase HR, mean arterial pressure, or femoral artery velocity, but it did increase SMA velocity to a greater extent in control subjects than in those with cirrhosis (171). Similarly, human subjects with chronic hepatic C infection exhibit an acute increase in portal blood flow velocity as assessed by Doppler ultrasound after 1 mg iv glucagon infusion, with the magnitude of the change in blood flow attenuated with increasing severity of coexistent liver disease (172). Whether the attenuated effects of glucagon on hemodynamic parameters in subjects with liver injury and cirrhosis reflect down-regulation of GCGR

sensitivity due to increased circulating glucagon levels remains speculative.

J. GIP, experimental vascular injury, and blood flow

GIP reduced the development of experimental atherosclerosis in genetically sensitized mice prone to accelerated lesion formation. Fifteen-week-old *ApoE*^{-/-} mice were administered STZ injections (50 mg/kg for 5 days) and treated with saline or GIP (25 nmol/kg/d) for 4 consecutive weeks. GIP did not affect BP or HR; however, atherosclerotic lesion progression in the abdominal aorta and aortic root as well as macrophage accumulation in the aortic wall were attenuated by GIP infusion in diabetic *ApoE*^{-/-} mice (173). GIP also reduced accumulation of cholesterol ester accumulation, an index of foam cell formation, in peritoneal exudate macrophages from diabetic *ApoE*^{-/-} mice.

The actions of GIP on pancreatic and intestinal blood flow were analyzed in adult Sprague Dawley rats after acute GIP administration (5 or 15 μ g/kg) in the presence or absence of an acute bolus injection of iv glucose. In normoglycemic rats, the lower dose of GIP did not increase organ blood flow, whereas the higher dose of GIP decreased pancreatic and duodenal blood flow (174). However, after acute glucose administration in rats, both doses of GIP further increased islet blood flow without affecting total pancreatic or duodenal blood flow. Administration of GIP at different doses (3, 50, and 800 pmol/kg) selectively increased superior mesenteric but not celiac artery blood flow, whereas pancreatic blood flow was decreased in the conscious dog (175). Although celiac artery blood flow was not affected by any of the GIP doses administered, SMA blood flow was increased in a dose-dependent manner, and pancreatic flow was decreased by the highest doses of GIP (175). Acute administration of GIP also increased portal vein blood flow in dogs (176). GIP (iv bolus of 1, 100, or 500 pmol/kg) simultaneously increased portal vein blood flow while decreasing hepatic artery blood flow at all doses tested. Whether these actions of GIP reflected direct actions on blood vessels or indirect effects secondary to changes in metabolism was not ascertained.

Systemic infusion of GIP (1.5 pmol/kg/min) to achieve circulating levels of approximately 68 pmol/L also increased sc adipose tissue blood flow (ATBF), assessed by the ¹³³Xenon washout technique, in healthy lean human subjects studied under conditions of a hyperinsulinemic hyperglycemic clamp (177). These actions of GIP were associated with increased adipose tissue glucose and free fatty acid uptake, favoring lipid accumulation. In contrast, infusion of GIP alone, in the absence of simultaneous hyperglycemia and hyperinsulinemia, had no effect on ATBF (177). Subsequent studies in lean healthy subjects under conditions of normal or increased insulin infusions, with

and without hyperglycemia, demonstrated that hyperinsulinemia is essential for revealing the effects of GIP to increase ATBF and triglyceride clearance (178). Similar studies examined the effects of GIP, infused at a rate of 1.5 pmol/kg/min over 3 hours to achieve plasma levels of 36.7–39.5 pM, on ATBF in obese subjects with normal or impaired glucose tolerance (179). A modest but significant 2-fold increase in ATBF was detected in obese subjects with normal glucose tolerance. Obese subjects with IGT failed to increase ATBF in response to combined GIP, hyperinsulinemic hyperglycemic clamp conditions. Triacylglycerol, glucose, and fatty acid uptake in adipose tissue was not altered during the GIP clamp study (179). The actions of GIP were not examined independently of concomitant hyperglycemia and hyperinsulinemia. Hence, obesity is associated with a relative resistance to the combined actions of GIP, insulin, and glucose to promote fatty acid uptake and enhanced ATBF in human subjects.

In a separate study of five obese male subjects studied before and after a 12-week weight loss program followed by 4 weeks of eucaloric weight maintenance (mean weight loss, 7.5 kg), basal ATBF trended higher after weight loss. The combined infusion of GIP together with a hyperglycemic hyperinsulinemic clamp had no effect on ATBF in obese subjects before weight loss, but ATBF increased significantly after GIP infusion during the clamp after weight loss (180). Modest increases in glucose uptake and no changes in fatty acid flux were detected in adipose tissue during the GIP hyperglycemic hyperinsulinemic clamp after weight loss.

Although much less information is available surrounding the possible effects of GIP on systemic blood flow, acute infusion of GIP (1.5 pmol/kg/min) in normal healthy subjects during a two-step euglycemic hyperglycemic 3-hour clamp demonstrated no effect of GIP on carotid or brachial artery blood flow; however, GIP significantly increased blood flow in the femoral artery during hyperglycemia, but not euglycemia (181). Notably, incomplete somatostatin-mediated suppression of insulin secretion during the hyperglycemic clamp resulted in significant increases in plasma insulin levels during GIP infusion, complicating mechanistic interpretation of the increased femoral artery blood flow.

K. Controversies, species specificity, and limitations of existing data

The effects of gut hormones, including GLP-1, on BP vary depending on the duration of exposure and the baseline BP. Although GLP-1R agonists reduce BP in hypertensive subjects, the mechanisms through which GLP-1R signaling lowers BP are not well understood. Robust changes in blood flow have been observed with GLP-1,

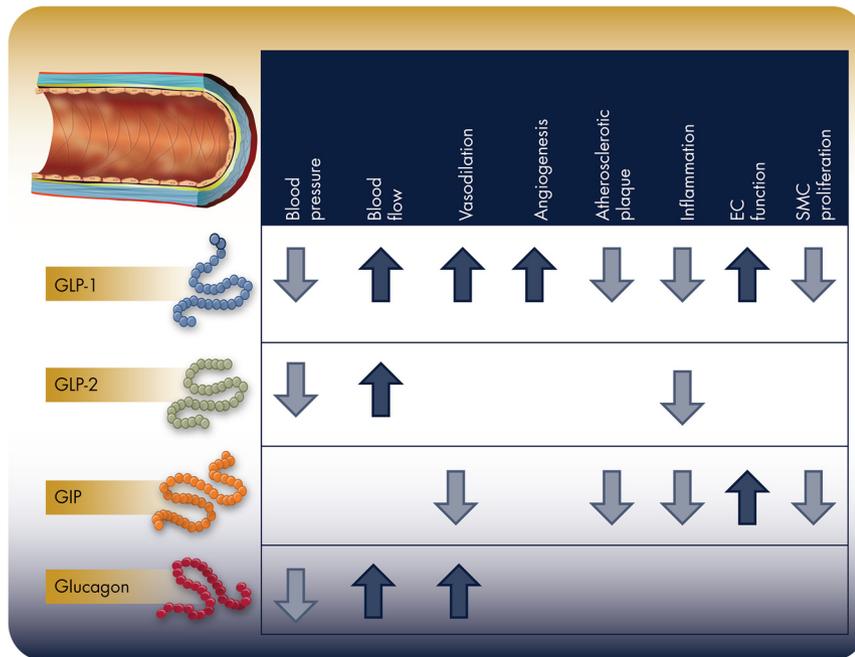
Figure 3.

Figure 3. The major direct and indirect actions of glucagon, GLP-1, GLP-2, and GIP that converge on the function of blood vessels.

GLP-2, and GIP (Figure 3). Whether these findings reflect primary actions of blood vessels or secondary responses to increased metabolic activity of downstream target organs is difficult to ascertain. The expression and cellular distribution of receptors for glucagon, GLP-1, GLP-2, and GIP in different arterial or venous vascular beds have not been carefully studied in rodents or humans. Although some of these receptors are expressed in EC lines, the extent to which normal ECs in situ express functional receptors for these peptides is unknown. Administration of glucagon, GLP-1, and GIP to animals and humans may be associated with profound metabolic changes, including induction of insulin, lowering of glucose, and changes in levels of free fatty acids. Many studies reporting actions of these peptides on vascular function do not control for the putative indirect activity of these peptides on blood vessels that may be mediated by acute changes in the metabolic milieu. Moreover, a considerable number of experiments localizing receptor expression within blood vessels employ a PCR to detect small partial cDNA products or antisera to detect receptor proteins that are often incompletely validated and prone to generation of spurious results (7, 58, 59). Whether the action of these peptides on blood flow is further modified by the development of localized or selective vascular disease has not been extensively studied. Moreover, the extent to which the acute actions on induction of blood flow are subject to tachy-

phylaxis with sustained receptor agonism requires further investigation. At present, most studies in the literature examining vascular actions of these peptides are pharmacological, reflecting in part the inadequacy of available antagonists and insufficient data from studies of mice with loss of function mutations in the genes encoding the peptide hormone themselves or their receptors. Nevertheless, the pharmacological actions of these peptides have considerable relevance for understanding the clinical effects of these peptides and their analogs in subjects with diabetes, obesity, or gastrointestinal disorders.

XII. Therapeutic Relevance of Glucagon-Related Peptides in Humans With Vascular Injury or Dysfunction

In a small nonrandomized study, nondiabetic individuals with acute myocardial infarction and reduced LV ejection fraction (LVEF) (<40%) were infused with GLP-1 (1.5 pmol/kg/min for 72 hours) after reestablishment of blood flow via primary percutaneous coronary intervention (PCI) (182). LVEF and both global and regional wall motion were improved after 72 hours of GLP-1 administration, relative to baseline findings immediately after successful angioplasty; a randomly and retrospectively selected control group not treated with GLP-1 did not exhibit comparable improvement (182). Read et al (183) examined the impact of GLP-1 infusion in human subjects with normal LVEF but single vessel CAD in the context of elective PCI. After two separate transient balloon occlusions of the diseased artery, LV function was studied in subjects infused with GLP-1 (1.2 pmol/kg/min) or saline; LVEF was preserved to a much greater extent after ischemia in subjects treated with GLP-1 for just over 30 minutes (183).

Protective effects of short-term GLP-1 infusion against ischemia-induced LV dysfunction were also reported in a similar study design in 20 predominantly male patients approximately 58 years of age with normal LVEF but single-vessel CAD (184). GLP-1 (1.2 pmol/kg/min) or saline was infused commencing immediately before 1 minute of low-pressure balloon occlusion of the coronary artery, and LV function was assessed at baseline, during the oc-

clusion, and 30 minutes after at the end of the recovery period. Plasma GLP-1 levels rose 40-fold in GLP-1-infused subjects, associated with a significant reduction in plasma glucose and free fatty acids. No differences in transmyocardial glucose concentrations were detected between treatment groups. Balloon occlusion significantly impaired systolic and diastolic ventricular function and ejection fraction (EF) in saline-treated subjects. In contrast, subjects treated with GLP-1 were largely protected against the deleterious effects of ischemia and exhibited preservation and more rapid recovery of LV function without demonstrable changes in myocardial energy metabolism (184).

The acute cardioprotective effects of exenatide vs placebo were examined in a study of 172 patients (90% nondiabetic) with ST-segment elevation myocardial infarction treated with PCI. Exenatide (0.12 $\mu\text{g}/\text{min}$) or placebo was administered 15 minutes before reperfusion and for 6 hours (0.043 $\mu\text{g}/\text{min}$) afterward. Three months later, assessment by magnetic resonance imaging demonstrated an increased myocardial salvage index corresponding to a reduced infarct size/area at risk ratio in subjects treated with exenatide (185). However, no differences in peak levels of troponin T, EF at 3 months, or rates of complications or hospitalization were observed between groups.

In a smaller study, 58 patients with ST-segment elevation myocardial infarction were assigned to receive placebo or exenatide (10 μg sc plus an iv bolus of 10 μg 5 minutes before the onset of PCI, followed by 10 μg sc injection twice daily for the following 2 days). About 5 weeks after reperfusion, infarct size assessed by cardiac magnetic resonance imaging was reduced in the exenatide-treated group, and 6 months after the intervention LV EF, as assessed by echocardiography, was also modestly enhanced (186).

The largest study to date of GLP-1R agonists in the setting of acute coronary syndrome was the Evaluation of Lixisenatide in Acute Coronary Syndrome (ELIXA) trial (187). Subjects with T2D and an acute coronary syndrome event within the prior 6 months were eligible for enrollment to receive either placebo or once daily lixisenatide (20 μg), in addition to usual diabetes care, with the primary composite endpoint including time to cardiovascular death, nonfatal myocardial infarction, nonfatal stroke, or hospitalization for unstable angina. After a median follow-up period of 25 months, lixisenatide therapy was noninferior to placebo with regard to the number of accrued events (152).

A reduction in Major Cardiovascular Events (MACE) was reported in subjects with T2D randomized to receive liraglutide (vs placebo) in the Liraglutide Effect and Action

in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) trial (153). Study subjects included patients with T2D who were at least 50 years of age with a documented history of cardiovascular or cerebrovascular disease, or at least 60 years of age with at least one cardiovascular risk factor. After a median time of exposure to liraglutide of 3.5 years and a median follow-up period of 3.8 years, significantly fewer MACE events were reported in subjects randomized to liraglutide (13%) compared to placebo (14.9%) (153). Liraglutide-treated subjects had fewer myocardial infarctions, although information about separate ACS events or outcomes after revascularization was not reported. No significant difference in the number of nonfatal strokes was detected between groups. Subjects treated with liraglutide also developed fewer microvascular events, largely due to a lower rate of nephropathy, primarily reflecting reduced numbers of subjects with macroalbuminuria (153). No significant difference in the number of retinopathy events was reported. Whether the benefits of GLP-1R agonist therapy in the LEADER trial reflect contributions from one or more direct vascular actions of liraglutide cannot be ascertained. A smaller study of the cardiovascular safety of once-weekly semaglutide over 104 weeks in subjects with T2D (83% of subjects had pre-existing cardiovascular disease or chronic kidney disease) revealed a significant reduction in MACE events, driven by reduced reports of nonfatal stroke in semaglutide-treated subjects (154). Hence, the cardioprotection and reduced incidence of MACE observed in some human subjects with T2D treated with GLP-1R agonists likely reflects a class effect and a GLP-1R-dependent mechanism, rather than a finding restricted to a single GLP-1R agonist.

A. Summary, controversies, and future research directions

Despite a considerable body of literature highlighting actions of PGDPs and GIP on normal and diseased blood vessels (summarized in Figure 3), numerous questions abound surrounding the mechanisms underlying these effects and their putative physiological translational relevance. Most experiments employ pharmacological doses of peptides, producing circulating levels of active hormone or agonist well in excess of normal circulating levels of the endogenous hormone. Conversely, the available antagonists are often suboptimal, exert partial agonist activities, and may not be suitable for long-term use in animals or approved for use in mechanistic human studies. Very few reports to date have described the use of tissue-specific knockouts for interrogation of the vascular actions of these peptides and their receptors in endothelial or smooth muscle cells. Hence, although the pharmacological data

are reasonably robust, the corresponding physiological data are considerably weaker.

Moreover, hormones such as GLP-1 and GIP exert rapid and potent actions on metabolism, confounding interpretation of whether changes in vascular function are likely to be direct or indirect. Although specific receptors for glucagon, GLP-1, GLP-2, and GIP have been isolated and extensively characterized, much of the published data on the vascular expression of these receptors is confounded by failure to detect full-length functional receptor protein using highly sensitive and specific, well-validated antisera. Furthermore, most RNA expression data are generated using PCR to generate small PCR products that may not reflect the expression of the full-length receptor.

Although dozens of papers had identified actions of these peptides on endothelial function in animals and humans, and multiple studies have identified signal transduction pathways activated by these same peptides in EC lines, it remains uncertain whether ECs within their native vascular context also express these receptors. Furthermore, similar uncertainty surrounds the extent to which smooth muscle cells within different vascular beds express one or more class B GPCRs transducing the effects of PGDPs and GIP. It is likely that within some vascular beds, receptor expression and activity may vary, depending on the anatomical location of the receptor, disruption of normal vascular flow, abnormalities in vessel shear stress, and development of vascular injury secondary to inflammation, atherosclerosis, or hypertension.

Despite these uncertainties, the translational relevance and importance of understanding the vascular biology of these peptides has never been greater (Figure 3). Glucagon is approved for the treatment of hypoglycemia, and both glucagon receptor antagonists and glucagon-containing coagonists remain under active investigation for the treatment of metabolic disorders (14, 47). GLP-1R agonists are approved for the treatment of T2D and obesity and are used to treat a spectrum of patients, many with cerebrovascular, cardiovascular, or peripheral vascular disorders. A GLP-2 analog, teduglutide, is approved for the treatment of short bowel syndrome, and many of the candidates for teduglutide therapy have concomitant vascular disease (7). Moreover, GIP analogs and coagonists are under investigation for the therapy of patients with diabetes and obesity (188). Hence, understanding the mechanisms through which these peptides exert their pleiotropic actions in the normal and diseased vasculature has considerable immediate translational relevance.

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