Glucagon's Metabolic Action in Health and Disease

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▶ ABSTRACT

Discovered almost simultaneously with insulin, glucagon is a pleiotropic hormone with metabolic action that goes far beyond its classical role to increase blood glucose. Albeit best known for its ability to directly act on the liver to increase *de novo* glucose production and to inhibit glycogen breakdown, glucagon lowers body weight by decreasing food intake and by increasing metabolic rate. Glucagon further promotes lipolysis and lipid oxidation and has positive chronotropic and inotropic effects in the heart. Interestingly, recent decades have witnessed a remarkable renaissance of glucagon's biology with the acknowledgment that glucagon has pharmacological value beyond its classical use as rescue medication to treat severe hypoglycemia. In this article, we summarize the multifaceted nature of glucagon with a special focus on its hepatic action and discuss the pharmacological potential of either agonizing or antagonizing the glucagon receptor for health and disease. © 2021 American Physiological Society. *Compr Physiol* vol number: page range, year.

Introduction

Seeking to develop a rapid and inexpensive method to purify insulin from pancreatic homogenates, Charles Kimball and John Murlin in 1923 identified a pancreatic factor that opposes the hypoglycemic effect of insulin (203). Due to its ability to increase blood glucose, the factor was named "the glucose agonist," or shortly glucagon. Subsequent studies by Earl Sutherland and Christian deDuve then identified the pancreatic α-cells as the origin of glucagon (101, 396). The hyperglycemic effect of glucagon resides in its ability to directly act on the liver where it stimulates *de novo* glucose production and glycogen breakdown (36–38, 91, 115, 357, 382). Studies by Roger Unger then showed in 1970 that glucose inhibition of glucagon secretion is diminished in patients with type-2 diabetes, suggesting that postprandial hyperglucagonemia plays a causal role in the development of type-2 diabetes (271, 386). Several clinical studies subsequently assessed the pharmacological potential of suppressing glucagon action for the treatment of type-2 diabetes, revealing that postprandial levels of glucagon are increased in patients with type-2 diabetes (5, 6, 39, 93, 122, 192, 261, 271, 281, 282, 313, 371) and that blocking of glucagon action improves glucose handling in patients with type-2 diabetes (5, 6, 189, 193). For decades, these liver-mediated hyperglycemic effects of glucagon overshadowed that glucagon is a pleiotropic hormone with metabolic effects beyond its role to buffer against hypoglycemia. In line with this notion, glucagon stimulates insulin secretion (329), lowers body weight by decreasing food intake and by enhancing energy expenditure (23, 68, 326), stimulates lipolysis, while inhibiting lipid synthesis (4, 43, 71, 86,

286, 326), slows down gastric emptying (262, 345, 401), increases cardiac output (131, 188, 224, 241, 413), and stimulates autophagy and renal glomerular filtration (270). Recent years have witnessed a remarkable renaissance of glucagon's multifaceted biology (as reviewed elsewhere (95, 270)) with therapeutic implications not only as a life-saving rescue medication to treat severe hypoglycemia but also when combined with glucagon-like peptide-1 (GLP-1) to treat obesity and type-2 diabetes (5, 6, 53, 66, 159, 299, 377) and nonalcoholic steatohepatitis (NASH) (27). In this article, we summarize glucagon's role in regulating systemic energy balance with a special focus on its hepatic action and

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highlight its multifaceted nature that led to its use to develop drugs to treat obesity and type-2 diabetes.

Transcriptional and Translational Control of Glucagon

In rodents, glucagon is the first hormone found in the developing endocrine pancreas (135, 179, 294), with detectable levels as early as embryonic (E) day E9.5. In contrast, in the human pancreas, detection of insulin-expressing cells by week 8 of gestational age precedes the detection of glucagon-positive cells by approximately one week (175). Glucagon is derived from the cleavage of proglucagon, a 160-amino acid (AA) precursor protein originating from the preproglucagon (Gcg) gene. Proglucagon gives in a tissue-selective manner rise to several other peptides, including glicentin, glicentin-related pancreatic polypeptide (GRPP), oxyntomodulin (OXM), GLP-1 and -2 (GLP-2), and the major proglucagon fragment (MGPF) (18, 82, 265). Proglucagon processing into these smaller peptide fragments is cell-type specific. While glucagon, MPGF, and GRPP are mainly produced in the pancreatic α-cells, GLP-1, GLP-2, OXM, and glicentin are the main proglucagon cleavage products of the enteroendocrine L-cells, which are predominantly located in the large intestine. Tissue specificity in preproglucagon expression is achieved by binding of specific transcription factors (TFs) to distinct DNA control elements in the preproglucagon promoter region to initiate or inhibit preproglucagon expression (135, 179) (Figure 1). The rat preproglucagon promoter includes at least six DNA control elements positioned within a 0.3 kb region upstream of the ATG start codon of Gcg (179, 294). The control elements can be separated into a critical promoter, encompassing the TATA box and the G1 and G4

elements. These are pivotal for α-cell-specific expression of preproglucagon (127, 162, 179, 296) (Figure 1).

The preproglucagon DNA control elements are targeted by several homeodomain proteins, which either activate or repress preproglucagon expression (179, 180, 206). In α-cells, Pax6 heterodimerizes with cMaf or MafB and induces preproglucagon expression through binding to the G1 element (117, 134). In β-cells, Pdx1, Pax4, and Nkx6.1 bind to G1 and competitively inhibit preproglucagon expression through blocking the binding of the preproglucagon activating Pax6/Maf heterodimer to the G1 element (116, 135, 316) (Figure 1). Adenoviral overexpression of Pdx1 alone, however, is not sufficient to suppress *Gcg* expression in α-cells (100). Pax6 stimulates preproglucagon expression through binding of Pax6 to the G3 element of the preproglucagon promoter (135). Mice devoid of Pax6 have markedly reduced levels of preproglucagon mRNA (356). In addition, Foxa1 (HNF-3α) and Foxa2 (HNF-3β) stimulate *Gcg* expression through binding to the G1 and G2 elements of the preproglucagon promoter (135). Mice lacking either Foxa1 or Foxa2 have a 70–90% reduction in preproglucagon mRNA levels and are hypoglycemic (85, 185).

In addition to the cell-type-specific expression of preproglucagon through direct interactions of selective TFs in the preproglucagon promotor region, preproglucagon expression is also controlled by increased levels of cAMP via the cAMP-response element (CRE) and the respective CRE-binding protein (CREB) (229), as well as the exchange protein activated by cAMP signaling pathways (Epac) (84, 127, 179, 206). Insulin inhibits preproglucagon expression in α-cells (293–295), while stimulating preproglucagon mRNA levels in the intestine (427). Finally, specific effectors of the Wnt signaling pathway have been shown to promote preproglucagon expression in the intestine but not the pancreas (275, 426, 427).
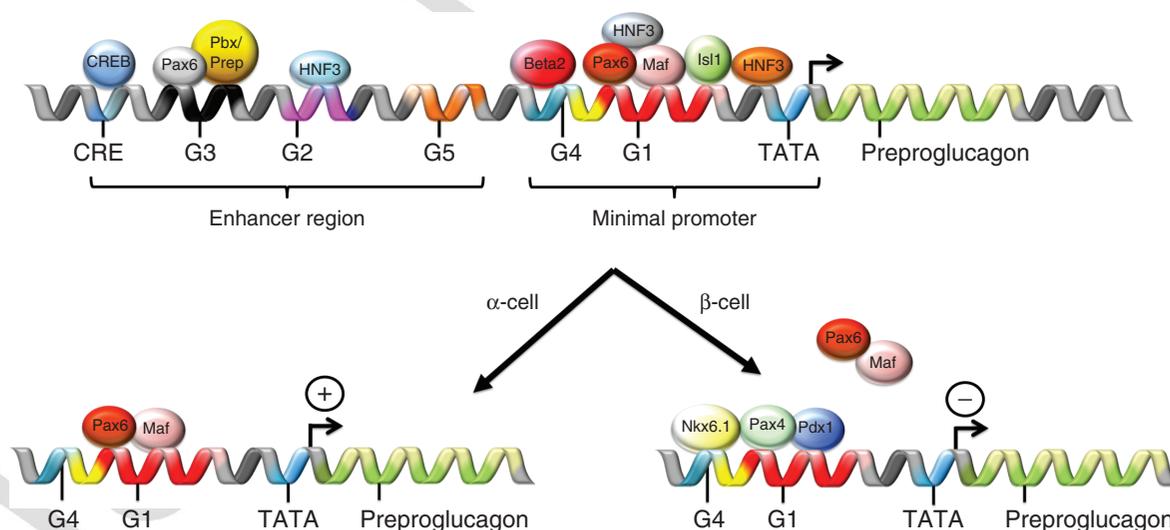

Figure 1   Schematic on the transcriptional regulation of preproglucagon in the pancreatic α- and β-cells. The expression of preproglucagon is regulated through interaction of home domain proteins that bind to the preproglucagon promoter region, which comprises a minimal promoter region and an enhancer region. For further explanations please see text.

The majority of glucagon is produced in the pancreatic α-cells, with small amounts also being synthesized in a subset of neurons in the brain stem (83, 148, 178) and seemingly also in the gut (242). The latter has been subject of debate for several decades since the measurement of glucagon is challenging due to its low abundance in the circulation and cross-reactivity of glucagon detecting antibodies with oxm and glicentin, which both contain the full AA sequence of glucagon. However, more recently developed enzyme-linked immunosorbent assays (ELISAs) show reduced cross-reactivity to oxm (<5%) and glicentin (<2%) (407). Their use in combination with mass-spectrometry-based proteomics revealed that a 29 AA molecule indistinguishable from glucagon is detectable in the circulation of pancreatectomized patients and circulating levels of this molecule increase in response to oral but not intravenous administration of glucose (242). These data collectively suggest that extrahepatic glucagon secretion can, at least under conditions of α-cell dysfunction, contribute to postprandial hyperglucagonemia. Future studies need to clarify if and to which extent extrapancreatic glucagon is also produced in humans without disturbed α-cell function.

Specific prohormone convertase (PC) enzymes are responsible for tissue-specific proglucagon cleavage. In α-cells, the prohormone convertase 2 (PC2; also called PCSK2) cleaves the proglucagon protein to produce glucagon, GRPP, and MPGF. In contrast, prohormone convertase 1 (PC1; also called PCSK1)-mediated cleavage of proglucagon yields GLP-1, GLP-2, OXM, and glicentin in the brain and the intestine (12, 225, 379, 395). Consistent with the crucial role of PC2 in proglucagon cleavage, PC2 knockout (KO) mice have lower circulating glucagon levels, are hypoglycemic and display signs of α-cell hyperplasia. The latter can however be corrected by continuous intraperitoneal supplementation of glucagon (108, 402). The chaperone protein 7B2 is responsible for the maturation of PC2 as well as its enzymatic activity and thus helps to facilitate the αcell-specific processing of proglucagon to glucagon (319). While cell-specific expression of PC2 ensures that glucagon is the main proglucagon cleavage product in the α-cells, STZ-induced β-cell destruction increases PC1 expression in rat α-cells, resulting in concomitant production of Glp-1 in the islets, and plausibly in the α-cells itself (276). In line with these data, overexpression of PC1/3 in α-cells increases islet Glp-1 secretion and leads to improved glucose-stimulated insulin secretion (414). Collectively, these data suggest a potential role of the α-cells to produce Glp-1 under conditions of impaired β-cell function. The PC enzymes might thus play an important, yet underappreciated role in regulating this plasticity in islet function.

Regulation of Glucagon Secretion

Glucagon secretion is similar to insulin secretion intimately tied to circulating levels of blood glucose (318). In the β-cell,

high levels of blood glucose increase the ATP over ADP ratio with the result that ATP-sensitive potassium (K_{ATP}) channels close and depolarize the cell membrane. This leads to opening of voltage-dependent Ca^{2+} channels (VDCC), influx of Ca^{2+}, and exocytosis of the insulin granules (136). In the α-cells, low glucose levels lead via moderate activation of the K_{ATP} channels to a membrane potential of about ~60 mV, which entails opening of T-type Ca^{2+} channels, followed by depolarization of the cell membrane and opening of voltage-dependent Ca^{2+} and Na^+ channels. The influx of Ca^{2+} and Na^+ then triggers release of glucagon into the circulation (305). An increase in extracellular glucose increases the cytosolic ATP over ADP ratio with the result that K_{ATP} channels close and depolarize the cell membrane to level where the voltage-dependent Ca^{2+} and Na^+ channels are inactive. The resulting lack of Ca^{2+} and Na^+ influx then shuts down glucagon secretion (305). In support of this model, sulfonylurea-induced blockage of K_{ATP} channels mimics high glucose-mediated inhibition of glucagon secretion in isolated α-cells (137) and islets independent of changes in insulin secretion (245). In addition to glucose-dependent mechanisms, AAs and free fatty acids (FFAs) also regulate glucagon secretion. Individual intravenous administration of 20 natural AAs in dogs identified that 17 out of 20 natural AAs increase glucagon secretion (317). The branched-chain AA's were the only ones that failed to stimulate glucagon secretion, while arginine produced the greatest stimulation (317). Consistently, high protein meals (113, 236, 248) also stimulate glucagon secretion in humans. However, hyperglycemia attenuates (309, 386) or abolishes (309) the increase in plasma glucagon following intravenous arginine or a high protein meal, suggesting AA-mediated regulation of glucagon secretion is dependent on glycemic status.

Early studies in dogs (246, 338) and humans (123) revealed that FFA inhibit glucagon secretion; however, more recent *in vitro* studies in isolated rodent islets suggest that palmitate increases glucagon secretion in euglycemic but not hyperglycemic conditions (28, 167). These seemingly contradictory findings may depend on the type of FFA administered, or whether exogenous or endogenous FFAs were studied. Hong et al. (167) observed that FA chain length, spatial configuration, and degree of saturation influence glucagon secretion independent of glucose concentration. These data suggest that FFA may affect glucagon secretion differently depending on the source (exogenous vs. endogenous) and the FFA characteristics. Another recent study suggests that glucagon secretion is also triggered by enhanced fatty acid oxidation since loss of CPT1a lowers glucagon secretion by decreasing the pool of ATP supply for the Na^+/K^+ ATPase (32).

Also, paracrine factors affect glucagon secretion. Insulin receptors are present on α-cells (74) and insulin inhibits glucagon secretion under hypoglycemic conditions (58) through modulating K_{ATP} channel activity (102) in a phosphoinositide 3 kinase-dependent manner (186). Additionally, insulin may indirectly suppress glucagon secretion through increasing translocation of α-cell GABA-A receptors (420).

Inhibition of GABA receptors increases glucagon secretion (404) and GABA released from β-cells (103, 367) is postulated to mediate glucose-facilitated inhibition of glucagon secretion (404). Further, zinc (Zn^{2+}) is co-secreted with insulin (102, 231) and inhibits glucagon secretion (102). Also, somatostatin, which is secreted from δ-cells, inhibits both insulin and glucagon secretion (107, 204, 322, 353), suggesting that glucagon is tightly controlled by pancreatic factors. However, glucose is still sufficient to suppress glucagon secretion independently of insulin (143, 310, 397), Zn^{2+96}, GABA (245), or somatostatin (393), indicating a dominant regulatory function of glucose on glucagon action, most likely via its ability to modulate K_{ATP} channel activity. Nonetheless, GABA- or somatostatin-receptor antagonism at low glucose levels increased basal glucagon secretion, suggesting a paracrine role for GABA in the regulation of glucagon release independent of glucose levels (245). These observations collectively highlight the complex interaction of glycemia and paracrine signaling in regulating glucagon secretion. It is likely that all factors play a complementary role in inhibiting glucagon secretion, thereby ensuring compensation across multiple physiological conditions.

Also, gut hormones regulate glucagon secretion. GLP-1 (60, 153, 184) and glucose-dependent insulinotropic polypeptide (GIP) (49, 96) both indirectly inhibit glucagon secretion, presumably via their ability to stimulate the secretion of insulin and Zn^{2+}. Importantly, while GIP stimulates insulin secretion under hyperglycemic conditions, it stimulates glucagon secretion in hypoglycemic or euglycemic states (51, 254, 288), suggesting a bi-functional role to maintain euglycemia. In line with these data, GIP inhibition of glucagon secretion seems to be mediated indirectly rather than directly, since GIP treatment of αTC1 cells does not decrease (but rather increases) glucagon secretion (49). In the isolated perfused rat pancreas, GIP affects glucagon (and insulin) secretion in a glucose-dependent manner with stimulation of insulin secretion under glucose concentrations >5.5 mM and stimulating of glucagon secretion at glucose concentrations <5.5 mM (288). These data align with studies in humans in which GIP increases postprandial glucagon levels (49, 243) and ameliorates insulin-induced hypoglycemia (52).

The pancreas is highly innervated by both the sympathetic (splanchnic) and parasympathetic (vagus) nervous system (283). Vagal stimulation increases insulin secretion (106), whereas splanchnic stimulation decreases insulin and increases glucagon secretion (26, 214, 283, 364). While central regulation of glucose homeostasis has been appreciated since the mid-1800s (298, 380), it was not until 1971 that the ventromedial hypothalamus (VMH) was implicated in regulating glucagon secretion (105) and that neuronal activation of glucagon correlates with rises in blood glucose levels (249). Further, glucagon secretion has been implicated in the cephalic phase (335, 369) of feeding. Intriguingly, this regulation is observed in healthy controls but not individuals with a kidney and pancreas transplant (335), suggesting functional pancreatic innervation is necessary to mediate cephalic-induced glucagon secretion. The relative contributions of direct and/or indirect neuronal efferents to glucagon secretion, however, remain unclear. Centrally regulated glucagon secretion could be mediated via direct sympathetic innervation on the α-cell, indirectly via the sympathetic tone and signaling through the hypothalamic-adrenal-pancreas signaling axis, and/or potential indirect parasympathetic signaling (283, 363). Altogether, glucagon secretion is a complex process regulated by multiple interactions between glycemic, paracrine, endocrine, and neural factors.

α-Cell Regulation of β-Cell Function

While PCSK2 is under nonpathological conditions the predominant PC in the α-cells, PCSK1/3 expression/activity increases in α-cells under pregnancy and under conditions of metabolic stress such as insulin resistance and diabetes (198, 276, 372, 415). Increased PCSK1/3 expression with concomitant GLP-1 production has also been demonstrated in α-cell lines and in isolated islets that have been cultured at conditions of high glucose (251, 412). The production of GLP-1 in the α-cells has been linked to the action of interleukin 6 (IL-6). The IL-6 receptor is highly expressed in murine α-cells (88) and administration of IL-6 increases expression of preproglucagon and of PCSK1/3 and accelerates the production of GLP-1 in the intestinal L-cells and the α-cells (89). Consistent with these data, adenoviral overexpression of PCSK1/3 in the α-cells enhances GLP-1 production and improves glucose-stimulation of insulin secretion and islet survival in mice (414). Collectively, there is growing evidence indicating that α-cells produce GLP-1 under conditions of higher β-cell demand to improve islet function in a paracrine fashion (47, 234, 264, 374, 391). Notably, intraislet paracrine signaling also plays a role in β-cell function under nonpathological conditions. In line with this notion, glucagon stimulation of insulin secretion was already described by Ellis Samols and Vincent Marks in 1965 (329) and was later confirmed in numerous other studies (263, 269, 270). β-Cells with contact to α-cells also secrete more insulin when challenged with glucose relative to β-cells without α-cell contact (416). Glucagon amplifies glucose-stimulated insulin secretion through direct action (169) and the receptors for glucagon and insulin are expressed on both α- and β-cells (190, 196). Glucagon was also recently shown to cross-react with GLP-1R in the β-cells and interaction of glucagon with GLP-1R has been demonstrated to enhance insulin secretion (360). Other factors potentially playing a paracrine role in α-cell regulation of β-cell function include glutamate and acetylcholine (263).

Glucagon Receptor Signaling

Once glucagon is secreted into the circulation, it elicits its function intracellularly by binding to its cell surface receptor, a seven-transmembrane protein belonging to the large

superfamily of G protein-coupled receptors (GPCRs) (69). The glucagon receptor (Gcgr) belongs to the class B family of GPCRs, which are peptide hormone receptors of the secretin family that are widely used drug targets for many human diseases, including diabetes, cancer, neurodegeneration, cardiovascular diseases, and others (285). Gcgr is mainly expressed in the liver. Only traces of Gcgr are found outside the liver such as in the kidney, adipose tissue, pancreas, spleen, lymphoblasts, brain, the gastrointestinal tract, and the adrenal gland (361). In the liver, Gcgr expression is zonated and occurs only at the periportal area, where also the metabolic effects of glucagon occur (213).

In liver cells, the Gcgr as a dimer induces the activation of two signaling cascades mediated by two classes of G proteins, a cAMP stimulatory G protein (G_s) and a G_q protein that signals via Ca^{2+} through the inositol 1,4,5-trisphosphate (IP_3) pathway (174, 260). Production of IP_3 is mediated by G_q-dependent activation of phospholipase C (PLC) and concomitant Ca^{2+} release from the endoplasmic reticulum (ER) to the cytosol and into the mitochondria. Increase of cellular calcium activates downstream signaling cascades and contributes to enhanced mitochondrial respiration observed under elevated glucagon levels (15, 34, 94). Interestingly, recent data highlight the role of the mitochondrial IP_3R1 receptor in Ca^{2+} dependent activation of mitochondrial β-oxidation (422). The interaction between the mitochondria and the ER has received a lot of attention due to membrane contact site formation and their function in calcium flux and signaling (315). However, the effects of glucagon on this cellular interaction have not been elucidated and might represent an underappreciated site of glucagon action.

Glucagon signaling via G_s represents the canonical Gcgr signaling pathway. Here, glucagon-induced G_s activation leads to the dissociation of the $G_{s\alpha}$ subunit from the G protein α/β/γ heterotrimer and its subsequent interaction with adenylate cyclase. Activated adenylate cyclase enhances its production of cAMP and consequently activates protein kinase A (PKA), enhances signaling via Rap guanine nucleotide exchange factor 3 (RAPGEF3, also known as Epac1) and the cAMP response element-binding protein (Creb)-regulated transcription coactivator 2 (Crtc2, also called Torc2) (144, 173). Stimulated PKA translocates to the nucleus, where it initiates the nuclear localization and phosphorylation of Creb at the serine-133 residue (Ser133) (174, 208). Once phosphorylated, Creb binds to the CRE elements located in the promoter region of downstream target genes and induces their transcription. This signaling cascade causes the expression of gluconeogenic and glycogenolytic genes, such as glucose-6-phosphatase (*G6Pase*), phosphoenolpyruvate kinase (*Pepck*), *Pc*, and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc-1α*) (3, 163, 208, 306, 418). The activation of the transcriptional co-activator Crtc2 is regulated by fasting-feeding cycles and changes in ATP levels. Underfeeding conditions, when ATP is high, salt-inducible kinase 2 (Sik2) and AMP-activated protein kinase (AMPK) phosphorylate Crtc2 on Ser171 and Ser307,

respectively, causing its localization in the cytosol (3, 208, 381). Upon fasting, Sik2 is inhibited causing Crtc2 dephosphorylation by calcineurin in response to elevated cAMP and calcium levels, leading to its nuclear translocation (3, 30). In the nucleus, Crtc2 binds along with Creb to the CRE element in the promoter region of target genes and thereby, for example, enhances the expression of gluconeogenic and glycogenolytic gene programs in the liver (3, 208, 323).

Glucagon Receptor Trafficking

GPCR signaling is regulated by endosomal membrane trafficking, where rapid internalization of the ligand-receptor complex contributes to signal termination and receptor desensitization (150). GPCRs are mainly internalized via clathrin-mediated endocytosis involving the β-arrestin family (14, 266). Here, arrestins are recruited to the activated GPCR, upon phosphorylation via the G protein-coupled receptor kinase (GRK) family, which results in uncoupling the GPCR from its corresponding G protein (244). Arrestins then connect the GPCR to the clathrin coat due to its dual binding function and facilitate internalization (244, 297). Importantly, GPCRs differ substantially in their way in which they engage with the GRK/arrestin/clathrin machinery. This helps to provide GPCR diversity in signaling, as only limited amounts of G protein pathways exist. In fact, class B receptors have been shown to recruit both β-arrestin-1 and 2 equally well and co-internalize with them, whereas class A receptors (e.g. β2-adrenergic receptors, β2AR) preferentially recruit arrestin-2 and co-localize only transiently on the plasma membrane with clathrin and arrestin (150). An alternative way of internalization involves caveolin-mediated endocytosis, where mainly GPCRs with primary signaling pathways via G_q are internalized (50, 279). In fact, GPCRs, G proteins, as well as arrestins, have been shown to sequester in caveolae, mediated through direct interaction of Caveolin 1 (Cav1) with G_q leading to internalization and initiation of Ca^{2+} signaling (50, 279, 337). These data suggest that G_q signaling is mainly mediated via caveolin-mediated endocytosis.

The Gcgr, a prototypical class B receptor, has been shown selectively interact with β-arrestin-2, and not β-arrestin-1, as only the knock-down of β-arrestin-2 lead to an impaired glucose tolerance as consequence to enhanced GPCR cell surface signaling (433). However, other *in vitro* overexpression studies reported the importance of both β-arrestins for Gcgr trafficking, emphasizing the differences between endogenous and exogenously overexpressed receptors (211) and the requirement for research in appropriate cell types. Gcgr has been shown to be internalized into endosomal fractions within 30 min after glucagon stimulation *in vitro* (Figure 1) (33, 212) and *in vivo* (255), causing a relatively mild decrease in membrane-localized Gcgr (8, 255, 387). Short-term activation leads to its phosphorylation through GRKs, both at the cell surface and after internalization into endosomes (255), highlighting the importance of phosphorylation for
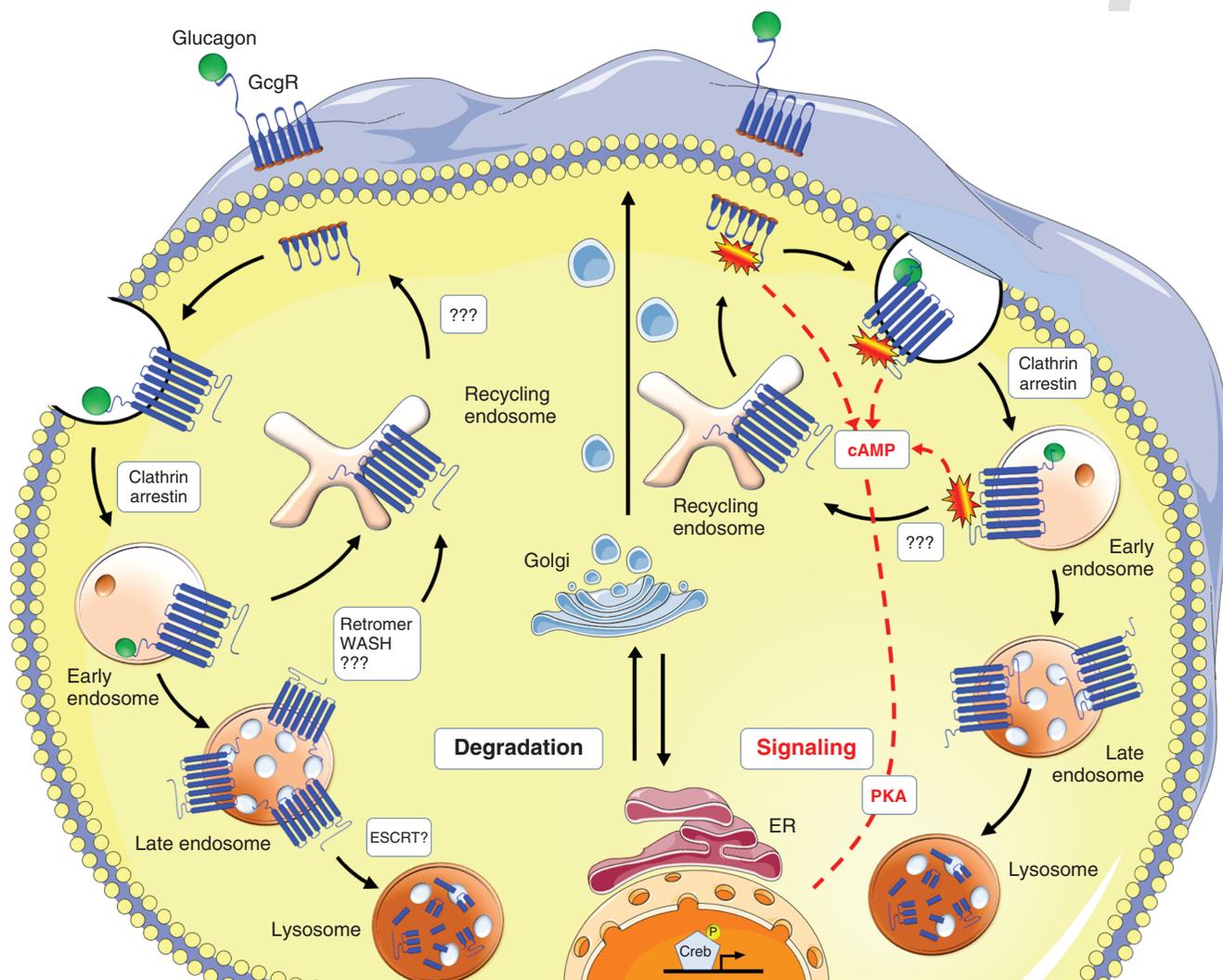
Figure 2   Proposed model of Gcgr trafficking and signaling. Stimulations with glucagon induce glucagon receptor recruitment into clathrin-coated vesicles on the plasma membrane through the interaction of β-arrestins with the cytoplasmic tail of the receptor and subsequent interaction with the clathrin coat. Short-term stimulations with glucagon increase glucagon receptor presence in early endosomes and enhanced signaling, followed by receptor recycling. Upon long-term treatments, reoccurrence of the receptor on the membrane is reduced, and its lysosomal degradation increases. Regulators of these sorting mechanisms on early and late endosomes, such as retromer and WASH complex for recycling and ESCRTs for degradation have been shown to be involved in other GPCR trafficking, however, the knowledge on Gcgr is still very limited and represented by question marks.

internalization and a potential endosomal contribution to signaling. In fact, endo-lysosomal transfer of $G_{\alpha s}$ subunit but not β-arrestins have been observed upon glucagon stimulation, together with increased adenylate cyclase activity, suggesting sustained Gcgr signaling at the level of endosomes (8, 255, 390). This can be achieved through the different binding properties of β-arrestins in class B versus class A GPCRs. While β-arrestins bind to the common binding pocket in the transmembrane core of the receptor in class A GPCRs, they bind class B GPCRs in the C-terminal tail, leaving the binding site for G proteins free for interactions in endosomes. In fact, a second wave of G protein-induced and β-arrestin-mediated signalling from endosomal membranes has been reported (87, 210, 378). Why β-arrestins have not been shown to

traffic to endosomes upon glucagon treatment is puzzling in this regard, however, limitation of antibody sensitivity and resolution of the subcellular fractionation could have influenced this study (255).

The fate of the GPCRs is decided at the level of the endosomes, which determine their re-usage or disposal. Internalized GPCRs can be either recycled back to the plasma membrane via the recycling endosomes for re-sensitization and continued signaling or can be degraded through the lysosomal system for a transient response (Figure 2) (150). These fates are determined by multiple mechanisms in the endosomal system. Targeting GPCRs to recycling usually requires sequence-directed mechanisms involving the cis-sorting sequence in their C-terminal tails (151, 375). These are

recognized by multiple sorting complexes in the endosomal network, including the retromer and WASH (Wiskott-Aldrich syndrome protein and SCAR homolog) complexes (62, 252), highlighting the complexity of the recycling system. Interestingly, the recycling kinetics can be altered depending on the extracellular environment, suggesting sensitivity in the sorting machinery to external nutritional cues (151, 405).

The fate of the Gcgr is dependent upon the duration of glucagon stimulation. Acute glucagon injections have no effect on Gcgr protein levels, leading to Gcgr internalization and reoccurrence after 2 h (8), suggesting activated recycling (Figure 2, right side). As for other GPCRs, this is dependent on the C-terminus of Gcgr, as its truncation causes reduced internalization, phosphorylation of the receptor, and a complete block on recycling (33, 212), resulting in presumably enhanced Gcgr localization to endosomes. Although the concept of sustained endosomal signaling of Gcgr has been proposed (8, 255, 390), the resulting consequences on signaling under these conditions have not been investigated. This would be of interest also in comparison to the fact that GPCRs can couple to G proteins even without inducing G protein signaling (150), which has also been shown for the Gcgr, as its antagonist (des-His1-[Glu9]glucagon) also induces detectable internalization of Gcgr (255).

GPCR internalization can be regulated by additional posttranslational modifications, such as palmitoylation and ubiquitination (150). Ubiquitination is a strong signal for receptor downregulation through the endo-lysosomal system (160). Lysosomal targeting is especially important for chronically activated receptors to downregulate their activity. In addition, it is also thought to play a role in drug tachyphylaxis or tolerance (411), which is particularly relevant for class B receptors that are used as drug targets. Indeed, the use of the pharmacological inhibitor, the receptor activity modifying protein (RAMP2), has been shown to co-localize with Gcgr and induces a reduction in cell surface-bound Gcgr (46). Whether enhanced degradation is achieved under these conditions need to be elucidated.

Receptor downregulation involves the trafficking through Rab7 positive late endosomes, multivesicular body formation, and concomitantly fusion with lysosomes (62, 128, 399). Membrane receptors designed for degradation are ubiquitinated at lysine residues that are recognized by the endosomal sorting complex required for transport (ESCRT) machinery, which binds ubiquitinated cargo and sequester those into intraluminal vesicles in multivesicular bodies/late endosomes, leading to receptor downregulation (160, 362). Activation of this process has been shown to be beneficial for degrading toll-like receptor 4 (TLR4) thus reducing its signaling in nonalcoholic fatty liver disease (NAFLD) to NASH progression (432). Although investigated for other GPCRs, such as chemokine receptor CXCR4 and β2AR (194, 392), this cellular mechanism has not been shown for Gcgr. In fact, some GPCRs are not ubiquitinated (80), however, arrestins are known to recruit E3 ligases to GPCRs (130, 342, 343), thereby inducing ubiquitination and potentially

thereby marking the bound GPCR for downregulation. As for the Gcgr, prolonged and chronicle treatment with glucagon results in a net decrease of glucagon binding efficiency and colocalization with lysosomes (Figure 2, left side) (7, 212), suggesting receptor downregulation under these conditions. Whether chronic glucagon treatment enhances arrestin-2 ubiquitination and thus Gcgr trafficking to lysosomes has not been investigated but would be an interesting concept that could be exploited to downregulate Gcgr in conditions of type-2 diabetes, where the overactivation of glucagon signaling is contributing to enhanced glucose output.

In fact, many of the studies on GPCRs have been performed on other members of the family, hampering our knowledge of Gcgr trafficking and its connection to signaling. Most of the studies on Gcgr were performed 20-30 years ago, where the detection techniques were less developed and subcellular fractionation or overexpression studies in cell lines, which do not endogenously express the Gcgr, were used. Given the fact that a complex trafficking machinery is involved in GPCR sorting, tissues with endogenous levels of Gcgr might engage other regulatory trafficking proteins than cell lines with an overexpression of nonendogenous Gcgr. In addition, studies with iodinated glucagon might have given misleading results, as iodoglucagons have been reported to alter adenylate cyclase activity *in vitro* and exhibit hyperglycemia *in vivo* (73, 235). Thus, further studies are needed to shed light into the regulation of Gcgr trafficking and signaling under physiological conditions and to connect this to its function as a fasting-induced receptor.

Glucagon Effects on Food Intake

Albeit its classical function to increase blood glucose under conditions of hypoglycemia, glucagon also lowers food intake and body weight in rodents (23, 68, 326) and humans (118, 289, 327, 333, 359) (Figure 3). Glucagon's anorexigenic effect is driven by the liver-vagus-hypothalamus axis and is achieved via a decrease in meal size without affecting meal frequency (119, 227), taste aversion, or postprandial behavior (120). Consistent with its role as a meal terminating factor, circulating levels of glucagon rise during food intake (70, 221, 385) and preprandial inhibition of glucagon signaling (222, 227), or antibody-based blocking of glucagon action (222), increase meal size, while stimulation of glucagon signaling during a meal terminates food intake (118). Glucagon's role in satiety is substantiated by increases in glucagon secretion following ingestion of high carbohydrate, high protein, and high-fat meals (70, 221), while antagonism of endogenous glucagon via hepatic-infused glucagon antibodies increases spontaneous meal size (119). Early studies observed that glucagon reduces meal size in humans (333), rodents (119), and sheep (215) independent of meal frequency (119, 227). Moreover, glucagon infused directly into the hepatic portal vein of rats reduces food intake and this effect is lost in
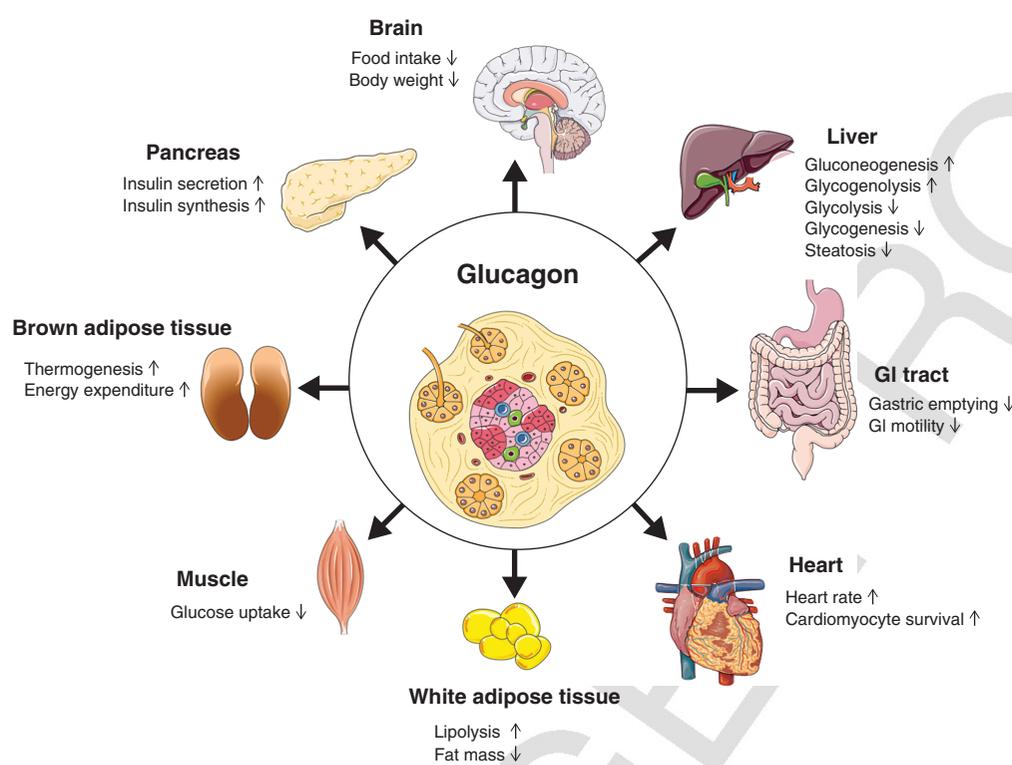
Figure 3 Schematic on the direct and indirect metabolic effects of glucagon.

hepatic-vagotomized rats (119), suggesting liver glucagon signaling mediates satiety via vagal afferents to the brain.

While the liver-hypothalamus axis regulates this process, direct neuronal glucagon signaling may also play a role. Acute intracerebroventricular (ICV) administration of glucagon decreases food intake acutely (1-4 h) in male mice in a dose-dependent manner and this effect was lost >6 h postadministration (307). Inhibition of PKA, the main downstream mediator of glucagon signaling, blunted glucagon's hypophagic effects, by decreasing Ca^{2+}-calmodulin-dependent protein kinase β (CaMKKβ) levels and AMPK activity (307). Further, ICV glucagon decreased expression of Agouti-related protein (AgRP) without changes in POMC, NPY, and CART; suggesting glucagon may decrease food intake via modulation of AgRP levels (307).

Genetically modified mice deficient for whole body Gcgr ($Gcgr^{-/-}$) also support glucagon's regulation of food intake. $Gcgr^{-/-}$ mice are resistant to diet-induced obesity (DIO), most likely due to a decrease in food intake compared to control animals (56). Interestingly, mice deficient for hepatic Gcgr ($Gcgr^{liver}$) are not resistant to DIO. Chronic Gcgr agonism via the long-acting Gcgr agonist, IUB288, in DIO mice reduces food intake (201, 274) in addition to increasing energy expenditure (201). However, the same glucagon-receptor agonism stimulates a similar suppression of food intake in $Gcgr^{liver}$ mice as compared to littermate controls (201), further supporting hepatocyte-independent

regulation and potentially implicating central Gcgr signaling in regulating HFD-food intake. Interestingly, chronic Gcgr agonism in lean male mice stimulates hyperphagia and a defense of their body weight (145), most likely to offset the increase in energy expenditure. Together these data suggest differential effects of glucagon on food intake depending on energy balance status.

While there is convincing data to support that central and liver-specific glucagon signaling both act to regulate food intake, the respective contributions of each pathway remain unclear. It is likely that both pathways work in concert with each other or selective pathways may dominate in a specific nutrient milieu. Regardless, further studies are needed to tease apart the contributions of central glucagon versus liver-mediated reductions in food intake.

CNS Regulation of Glucagon Action

While liver-regulated glucose homeostasis is well established (344), the hypothalamus likewise comprises a glucose-sensing network that is sensitive to hormonal signaling and known to modify peripheral glucose homeostasis (320, 347). Shimazu et al. (346) were the first to uncover that electrical stimulation, specifically in the VMH, resulted in an increase in blood glucose levels, accompanied by a decrease in liver glycogen. It is now appreciated that within the VMH there are both glucose excitatory (GE) neurons, which control peripheral glucose utilization, and glucose inhibitory (GI) neurons,

which control hepatic glucose production (347). Insulin and glucagon are well characterized in targeting peripheral tissues to mediate glucose homeostasis. However, there is a growing body of evidence that insulin is an important neuroregulatory peptide, involved in energy balance and glucose homeostasis (76). Despite greater appreciation for glucagon in energy balance beyond glucose metabolism, little attention has been given to its central actions.

Historically considered a diabetogenic hormone, glucagon signaling increases blood glucose levels via PKA-dependent signaling in the liver. However, this glycemic effect is transient, despite continuous intravenous glucagon infusion and lack of insulin (29, 92). This suggests an insulin-independent compensatory mechanism may be triggered to restore glucose homeostasis. A possible explanation for this effect may involve a negative feedback loop involving glucagon. Glucagon crosses the blood-brain barrier (13) and glucagon immunoreactivity has been identified in the hypothalamus (332), suggesting a potential physiological role for central glucagon signaling. Consistently, administration of glucagon to the mediobasal hypothalamus (MBH) decreases hepatic glucose production in clamped rats and improves glucose tolerance in nonclamped rats mediated via PKA-dependent signaling and hepatic vagal efferents (256). Similarly, central glucagon infusion also decreases hepatic glucose production in control, but not $Gcgr^{-/-}$ mice (256). While these data support that central glucagon signaling is sufficient to regulate hepatic glucose production, the physiological role of endogenous central glucagon signaling remains unclear.

Intriguingly, a high protein meal (65.4% protein) improves glucose tolerance, despite increasing glucagon signaling in the dorsal vagal complex (DVC) of the brainstem (223). DVC administration of either a Gcgr antagonist or a glucagon mAb blunts high protein diet-induced improvements in glucose tolerance, highlighting a role for endogenous central glucagon signaling in the regulation of glucose homeostasis. Interestingly, inhibition of Gcgr signaling on a normal protein diet (21.5% protein) did not alter glucose production, suggesting that DVC glucagon signaling may be important in specific nutrient states (e.g. high protein consumption).

Hormone resistance is common in rodents (67, 97) and humans with obesity (90, 425). Consistently, acute (3d) and chronic (3w) HFD-feeding resulted in Gcgr resistance in the MBH (256), indicating that hypothalamic Gcgr resistance may play a role in diet-induced hyperglycemia. Data support that the brain is sensitive to glucagon; however, most studies to date involve central glucagon administration, which may not reflect endogenous glucagon action. Further studies utilizing neuronal Gcgr knockout or central Gcgr-antagonist models will be essential for dissecting the endogenous role of central glucagon signaling. The focus of these studies will likely involve both the hypothalamus and the brainstem and the respective contributions of direct neuronal glucagon signaling versus indirect liver-brain communication. In addition, further studies are warranted to uncover whether central glucagon signaling mediates other facets of energy balance beyond peripheral glucose homeostasis.

Glucagon Effects on Energy Expenditure

Glucagon was first shown to increase energy expenditure in rats in 1960 (65) and has since then been confirmed in several human studies (205) (Figure 3). The energy expenditure effect in patients is rapid, with oxygen consumption elevated within minutes after intravenous glucagon infusion (366). In the fed state, glucagon's ability to stimulate energy expenditure is less effective compared to a robust increase by 100-200 kcal per day when administered in the fasted state (205). The magnitude of glucagon's energy expenditure effect in humans is similar to that of the β3-adrenergic receptor agonist mirabegron, which primarily targets the brown adipose tissue (BAT) (+203 kcal/day) (63), and to the energy expenditure increase detected during acute cold exposure (+193 kcal/day) (325).

Early studies investigating how glucagon leads to rapid increases in energy expenditure pointed to the BAT as the main responsible organ; this was based on studies showing that glucagon increases oxygen consumption in isolated BAT cells and BAT tissue explants from rats (181, 216), albeit at supraphysiological doses. In different animal models, glucagon elevates the temperature over interscapular BAT and augments blood flow into BAT (54, 157, 421). Moreover, in cold-adapted mice, which have more BAT glucagon's effect on energy expenditure is potentiated (77).

Substantial literature indicating that glucagon affects energy expenditure via BAT-dependent (181, 216) and -independent mechanisms. In animals with little (adult dog) or no functional BAT (pig) glucagon is still able to acutely increase energy expenditure (172, 403). Moreover, while BAT thermogenesis relies predominantly on the uncoupling protein 1 (UCP1), glucagon injection in mice lacking UCP1 increases energy expenditure to similar extent as in wild-type controls (17). In addition, mice with selective deletion of the Gcgr in BAT also increase their energy expenditure normally following glucagon injection (17). Collectively, this suggests that *in vivo* neither BAT *per se* nor Gcgr signaling in BAT are required for the acute energy expenditure effect of glucagon in mice. Fittingly, in humans, glucagon was recently shown to increase energy expenditure without increasing BAT activity in subjects specifically screened for functional BAT (325). An alternative, BAT-independent explanation for how glucagon mediates increased energy expenditure could comprise the engagement of multiple metabolic (predominantly catabolic) pathways. For example, liver oxygen consumption has been shown to increase by up to 20% during glucagon infusion in rats (57).

In addition to its acute effects, glucagon can also elevate energy expenditure chronically. Notably, glucagon fails to promote body weight loss in mice lacking liver glucagon receptor ($Gcgr^{liver}$) (201), suggesting that liver

Gcgr-signaling is necessary for the energetic response to glucagon. In the liver, glucagon stimulates the synthesis and release of fibroblast growth factor 21 (Fgf21) (64, 145), a circulating peptide hormone that regulates energy homeostasis (99, 129) via centrally mediated mechanisms (81, 284). Chronic glucagon treatment fails to augment energy expenditure and to prevent HFD-induced obesity in *Fgf21* null mice, suggesting that glucagons effect on energy expenditure requires Ffg21 signaling (145). Similarly, in obese liver-specific *Fgf21*-deficient mice, glucagon-mediated body weight loss is blunted (201), suggesting that specifically hepatic Fgf21 secretion contributes to the chronic effects of glucagon on energy expenditure. In addition to Fgf21, prolonged glucagon treatment increases circulating levels of bile acids in DIO mice (201). Bile acids are ligands for the farnesoid X receptor (FXR) (201) and both, bile acids and FXR, regulate energy expenditure (370). In liver-specific FXR knockout mice, the body weight lowering effects of glucagon is blunted, despite normal Fgf21 secretion (201); indicating that in addition to Fgf21, a hepatic bile acid—FXR axis contributes to the chronic effects of glucagon on energy expenditure.

It remains possible, that other factors that have been shown to be regulated by glucagon, like epinephrine, cortisol, and thyroid hormone (205) may play a role in glucagon's prolonged thermogenic effect. Also, glucagon can cross the blood-brain barrier (13) and the Gcgr is expressed in hypothalamus and brainstem regions, two sites known to modulate energy metabolism (168, 332). However, chronic ICV studies assessing the role of glucagon on energy expenditure are still missing.

Glucagon Action in the Heart

Traces of the Gcgr are expressed in the heart (1). Whole-body knockout of the Gcgr results in a lower intrinsic heart rate (268), whereas glucagon administration increases heart rate (chronotropic effect), contraction force (inotropic effect), and stroke volume in animals and humans (241, 287) (Figure 3). Glucagon fails to increase heart rate in Gcgr null mice (268). Mechanistically, glucagon triggers adenylyl cyclase activation through G_s protein-coupled signaling. Glucagon-mediated adenylyl cyclase activation occurs independently of the β-adrenergic system and its activation leads to an increase in cAMP levels, which engage the cyclic nucleotide-gated channels to elevate calcium concentrations in cardiac conduction tissue like the sinoatrial (SA) node (291). These effects are transient, lasting only several minutes rather than hours (287), because adenylyl cyclase quickly becomes uncoupled from the Gcgr (424), cAMP is rapidly broken down by phosphodiesterase (183), and receptor internalization reduces the number of available Gcgrs (164).

In the context of cardiac health following injury, like myocardial infarction, the role of augmenting versus diminishing cardiac glucagon signaling has been investigated

in several preclinical studies. In mice, glucagon treatment impairs survival after myocardial infarction, whereas cardiac-specific deletion of the Gcgr markedly improves survival rates compared to wild-type mice (1). Similarly, treatment with monoclonal Gcgr antagonistic antibody ameliorates onset and progression of heart failure (114, 187, 341). Whether Gcgr antagonism improves heart health in humans has not been tested.

Glucagon Regulation of Hepatic Metabolism

The fundamental aspect of liver glucagon action is its function on increasing hepatic glucose output (308). Initially recognized as a hormonal factor that counter-regulates the hypoglycemic effects of insulin, glucagon was later identified to increase hepatic glucose production through stimulation of glycogenolysis and gluconeogenesis, while at the same time inhibiting glycogenesis and glycolysis (308). Glucagon's role on hepatic glucose production is most prominent via intra-portal injection and is absent in hepatectomized rats (25). Consistent with this observation, glucagon has been identified to be secreted into the portal vein from the pancreas and reaches the liver at a much higher concentration than the in the systemic circulation (365), indicating an acute and preferential effect on the liver.

Regulation of glycogenolysis

Upon short-term starvation, glucagon induces rapid mobilization of hepatic glycogen stores leading to an immediate increase in hepatic glucose output (176). This is achieved via glucagon signaling through PKA (144) and activation of glycogen phosphorylase kinase (GPK), which leads to phosphorylation and activation of glycogen phosphorylase (GP) initiating glycogen breakdown (Figure 4) (144). Besides this, glucagon has also been shown to reduce acetylation of hepatic GP thereby enhancing its activity (430). In addition to stimulating glycogen breakdown, glucagon also inhibits the activity of glycogen synthase, causing an overall net increase in glycogenolysis (292), thereby channeling glucose into the plasma. Glucagon thus becomes an important counter regulatory hormone during conditions of hypoglycemia as a direct access to release hepatic glycogen (352). Thus, the glucose releasing effect of glucagon is directly proportional to glycogen levels, as seen in fasted animals or patients with liver cirrhosis (61).

Regulation of gluconeogenesis

After depletion of glycogen stores upon longer starvation, glucagon activates gluconeogenesis to increase hepatic glucose output and to maintain blood glucose levels (292). This is achieved by allosterically modulating the activity of several enzymes shifting the metabolic flux from
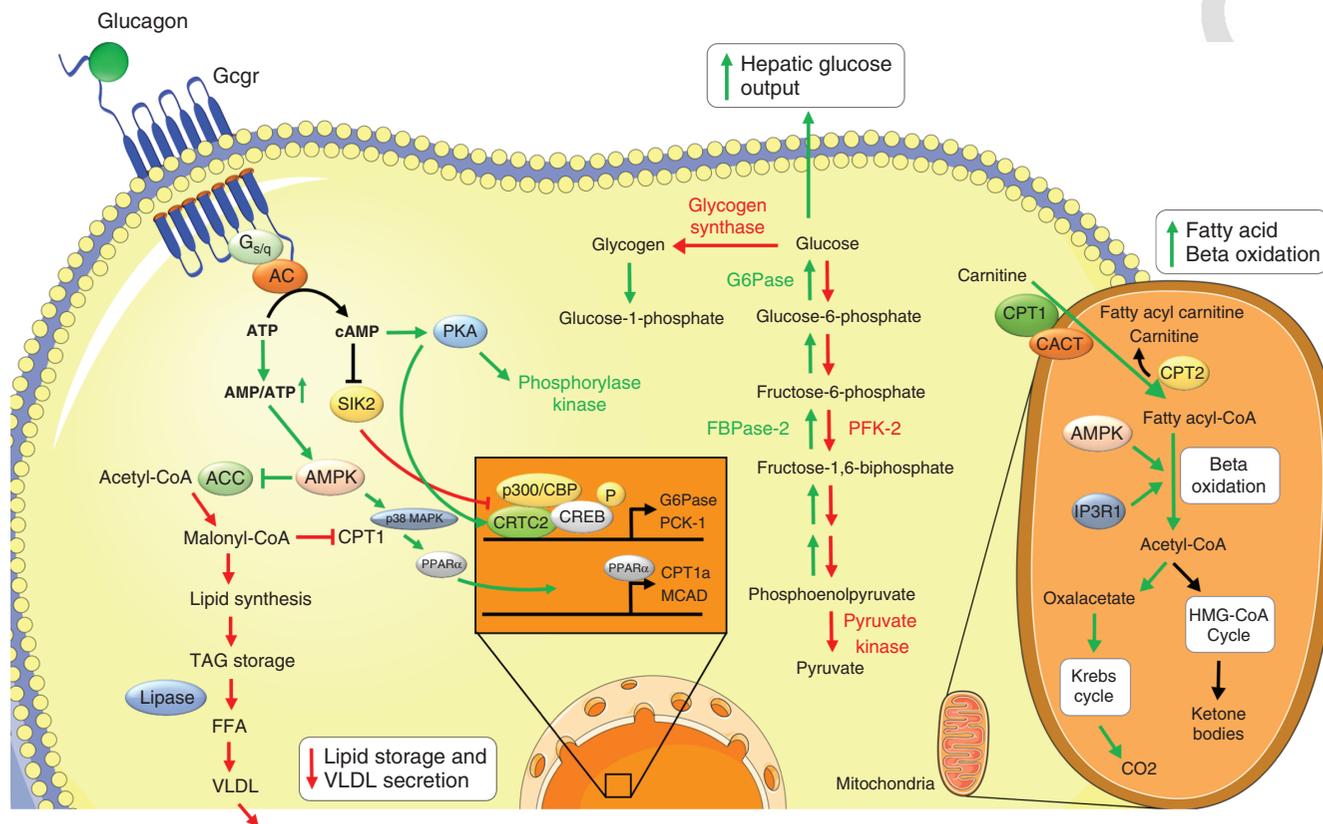
Figure 4 Glucagon effects on hepatic glucose and lipid metabolism. Activation of glucagon receptor by glucagon in hepatocyte stimulates adenylate cyclase-/cAMP-/PKA-dependent phosphorylation of Creb and dephosphorylation/nuclear translocation of Crtc2. p-Creb induces transcription of gluconeogenic genes *G6Pase* and *Pck1*. PKA activates phosphorylase synthase and inhibits glycogen synthase, thus stimulating glycogen breakdown. In addition, PKA activates FBPase2 and inhibits PFK-1 and pyruvate kinase, thereby enhancing gluconeogenesis and inhibiting glycolysis. By AC dependent inhibition of SIK2, glucagon stimulates activation of p300, which facilitates transcription of gluconeogenic genes. p-CREB induces transcription of *Ppar-α* that enhances transcription of β-oxidation genes *Cpt1* and *Mcad*. ATP to cAMP conversion leads to enhanced AMP/ATP ratio leading to AMPK activation and inhibition of ACC. This results in inhibiting the conversion of acetyl-CoA to malonyl-CoA by ACC and subsequent decreases the lipid synthesis pathway. As a consequence malonyl-CoA formation is reduced which induces an accumulation of Cpt1. Cpt1 enhances fatty acyl-CoA transport into mitochondria and induces β-oxidation. In addition, glucagon stimulates AMPK and mitochondrial IP$_3$R1 further activating β-oxidation. Acetyl-CoA subsequently enters Krebs cycle for ketone body formation during prolonged starvation. Abbreviations, AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; Creb, cAMP-responsive element-binding protein; G6Pase, glucose 6 phosphatase; Pck1, phosphoenol pyruvate carboxykinase 1; FBPase 2, fructose 2,6-bisphosphatase; PFK-1, phospho-fructokinase 2; Crtc2, Creb-regulated transcription coactivator 2; SIK2, salt-inducible kinase 2; Ppar-α, peroxisome proliferator-activated receptor alpha; Cpt1, carnitine palmitoyltransferase 1; Mcad, medium-chain acyl-CoA dehydrogenase; ATP, adenosine triphosphate; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; IP3R1, inositol triphosphate receptor 1; FFA, free fatty acid; TAG, tri-acyl glycerol; VLDL, very low-density-lipoprotein; LCAD, long-chain acyl-CoA dehydrogenase.

glycolysis to gluconeogenesis (176). Glucagon binding to its receptor induces the production of cAMP causing PKA activation. Then, PKA phosphorylates and inhibits the activity of phospho-fructokinase 2 (PFK-2), a bifunctional enzyme acting on fructose 2,6-bisphosphatase (FBPase 2) and 6-phosphofructo-2 kinase (409). Inhibition of PFK-2 activates FBPase 2 and inhibits 6-phospho fructo-2-kinase, causing a rapid reduction in the secondary metabolite fructose-2,6-bisphosphate [F(2,6)P$_2$], shifting the flux toward gluconeogenesis (409). PKA also phosphorylates pyruvate kinase causing a reduction in its activity. This enhances fructose-1,6-bisphosphate, which lowers pyruvate levels leading again to reduced glycolysis and redirection of substrate toward gluconeogenesis (Figure 4) (176, 409).

Activation of PKA strongly depends on maintaining high cAMP levels. Thus, controlling cAMP amounts is crucial for downstream signaling. Interestingly, a recent paper has shown another level of regulation of cAMP-PKA signaling, through controlling phosphodiesterase 4B (Pde4b) transcription (431). Pde4b is responsible for the degradation of cAMP thereby terminating signaling (182). Glucagon-stimulated nuclear factor-kappa B2 (NF-κb2) (p52) binding to PDE4B promotor inhibits its transcription, thus strengthening cAMP action (431).

Besides direct modulation of enzyme activity by phosphorylation, transcriptional regulation by glucagon also enhances gluconeogenesis. Glucagon signaling increases phosphorylation of Creb at serine residue 133 and dephosphorylation and nuclear translocation of its co-activator,

Creb-regulated transcription coactivator 2 (Crtc2) (see above) (237). Phosphorylated Creb binds to DNA and promotes expression of its target gluconeogenic genes *G6Pase*, *Pepck*, *Pgc1α,* and hepatocyte nuclear factor 4 (*Hnf4*), thereby enhancing glucose output (Figure 4).

In addition to the transcriptional regulation, glucagon has been shown to facilitate gluconeogenic gene transcription by regulating histone modifications that alter chromatin environment for gene induction. Other than Crtc2, Creb is also associated with coactivators—histone acyl transferase p300 and Creb-binding protein (Cbp) (237). Glucagon dephosphorylates p300 at Ser89, thereby increasing its activity (3). This is achieved by adenylyl cyclase-mediated inhibition of salt-inducible kinase 2 (Sik2) (237). p300 in turn, acetylates Crtc2 at Lys628, enhancing the transcription of *G6Pase* and *Pepck* (154, 237). Importantly, Crtc2 interaction with p300/Cbp is essential for their recruitment to Creb target genes and subsequent transcription (Figure 4) (312). Cbp and p300 are known to acetylate H3K27 lysine residue at enhancers, facilitating a chromatin environment more accessible to TF binding (48) and an enhanced transcript elongation rate of RNA polymerase 2 (354). Furthermore, p300/Cbp also directly acetylate lysine residues in TFs such as p53 (314). Substantiating this, p53 has been shown to promote gluconeogenic gene expression (133). Aside from p300, glucagon stimulation also recruits other histone acyltransferases. Glucagon induced nuclear translocation of Crtc2 has been shown to recruit lysine acetyltransferase 2B (Kat2b/Pcaf) to gluconeogenic genes (311). Kat2b then enhances histone H3 acetylation at Lys9 (H3K9Ac) promoting gene transcription and further potentiating Crtc2 occupancy at Creb-binding sites. Along with Kat2b, WD repeat-containing protein 5 (Wdr5), a core subunit of histone methyltransferase (HMT) is also recruited and exhibits concerted action with Kat2b on enhancing H3K9Ac (311).

Besides Creb, the Forkhead box protein O1 (Foxo1) is a major transcriptional regulator of gluconeogenic gene expression. Foxo1's nuclear binding activity is modulated by acetylation/deacetylation cycles, where acetylation reduces and de-acetylation enhances binding of Foxo1 to gluconeogenic gene promoters (303). Inhibition of Foxo1 activity by E26 oncogene homolog 1 (Ets-1) (230) is attenuated by glucagon-mediated downregulation of Ets-1, reducing its acetylation (230). In addition, glucagon rapidly dephosphorylates class IIa histone deacetylases (HDACs), facilitating their translocation to the nucleus and concomitant deacetylation of Foxo1 (257). Sirtuins, another class of deacetylases involved in metabolic control, are also regulated by glucagon. Here, sirtuin 6 (Sirt6) deacetylates and thus activates the general control nonrepressed protein 5 (Gcn5/Kat2a), causing Pgc1α acetylation and reduction of its gluconeogenic gene transcriptional activity (79). By reducing the expression of Sirt6, glucagon indirectly enhances the activation of Pgc1α (78). Altogether, glucagon activates gluconeogenic gene transcription by modulating the deacetylation of transcriptional

co-regulators. For a more detailed description on glucagon-mediated histone acetylation and its implication in glucagon biology visit a recent review by Zhang et al. (429).

In addition to acetylation, gluconeogenic gene expression is enhanced through histone methylation by protein arginine methyltransferase 5 (Prmt5) (376). Glucagon stimulates Crtc2 interaction with Prmt5 thereby increasing the methylation (H3R2me2) of gluconeogenic genes, while the downregulation of Prmt5 reduces gluconeogenic gene expression and circulating glucose levels (376). Altogether, these studies show the importance of histone modifications, chromatin environment, and TF binding/interaction for Creb activity and adds to the complexity in the regulation of gene transcription by glucagon (132).

Regulation of amino acid metabolism

Gluconeogenesis is a substrate driven process, wherein substrates from other tissues such as adipose-derived glycerol or muscle-derived AAs contribute to gluconeogenesis in the liver, also known as the Cori cycle (161). There is no indication of Gcgr expression in the skeletal muscle or adipose tissue in humans (238), indicating that glucagon may not directly mobilize precursors for gluconeogenesis from these tissues (240). Thus other mechanisms, such as catecholamines and cortisol, participate in precursor mobilization from muscle and fat.

However, glucagon stimulates AA influx into hepatocytes providing AA substrates for gluconeogenesis (2). This is achieved by glucagon-stimulated expression of the AA transporters for alanine (A system) and glutamine, histidine, and asparagine (N system) in the liver, resulting in increased AA uptake (197, 233). After influx, these AAs are further processed to be used as precursors for gluconeogenesis. Essential for this is their deamination, after which the amine groups enter the urea cycle for excretion (334). For this, glucagon induces the rapid deamination of glutamine, resulting in an immediate increase in ureagenesis and AA metabolism (16, 217). How the deamination is acutely regulated is unclear. The rapid increase in ureagenesis by glucagon is induced by allosteric activation of Sirt3 and Sirt5, which in turn increases the activity of ornithine transcarbamylase (Otc) and carbamoyl phosphate synthetase 1 (Cps1), critical enzymes in ureagenesis (146, 273). In addition, glucagon also induces the transcript levels of enzymes involved in the urea cycle through the cAMP-PKA-Creb mediated pathway (349). Particularly, enzyme *N*-acetyl glutamate synthetase (Nags) transcription is enhanced by glucagon, driving AA flux toward ureagenesis (156). Altogether, glucagon primes the hepatocytes for the uptake of AAs from the circulation, which are used as precursors of gluconeogenesis in periods of long-term starvation (61, 240).

The importance of hepatic glucagon signaling in AA metabolism is further supported by studies, where Gcgr antagonism causes hyper-aminoacidemia, due to reduced uptake of circulating AAs and decreased ureagenesis (112,

267). Hyper-aminoacidemia has been suggested to be a factor for increase in α-cell mass (350). In fact, interfering with liver glucagon signaling through liver-specific deletion of Gcgr results in pancreatic α-cell hyperplasia (112), suggesting a liver to α-cell axis. Importantly, the increase in circulating AAs upon Gcgr ablation then further stimulates glucagon secretion from α-cells (410), creating a vicious cycle of overproduction of glucagon. This partly explains the hyper-glucagonemia observed after ablation of liver Gcgr signaling. In particular, arginine, alanine, and proline have been shown to stimulate the secretion of glucagon from α-cells (110), while glutamine induces α-cell mass (72). In addition, the pancreatic amino-acid transporter, Slc38a5 was found to play a vital role in α-cell hyperplasia induced by liver Gcgr inhibition in mice and its absence prevented hyperplasia development (72). The occurrence of hyperglucagonemia and hyperaminoacidemia is observed in type-2 diabetes patients (313) and patients with NAFLD (406, 408), underlining the association between these conditions and metabolic diseases. These data emphasize the need to further clearly characterize the role of glucagon on hepatic AA metabolism and to delineate the underlying mechanisms regulating the liver-α-cell axis.

Regulation of mitochondrial metabolism and hepatic calcium signaling

Gluconeogenic flux is deeply linked to respiration rate and ATP production (302). Indeed, glucagon stimulates mito-chondrial oxygen consumption correlating well with the physiological requirement for energy production during gluconeogenesis (31, 422). Interestingly, glucagon via signal-ing through G_q and PLC-mediated IP_3 formation increases intracellular and mitochondrial calcium levels as one of the ways to enhance mitochondrial respiration (55). This is achieved by release of intracellular calcium stores from the ER (94), through cAMP-mediated regulation of inositol triphosphate receptor (IP_3R) by several independent mech-anisms (reviewed in (368)). This includes cAMP-activated PKA phosphorylation of IP_3R2, the major subtype expressed in hepatocytes, at serine residue 937 resulting in enhanced burst of IP_3R channel gating (21). More importantly, cAMP directly delivered to IP_3R2 signaling junctions on the ER potentiates its response to IP_3 independent of PKA or Epac, as observed through nuclear patch-clamp recordings (373), suggesting a direct role for cAMP in sensitizing the IP3R2. Subsequent increase in cytosolic calcium stimulates gluco-neogenesis either by directly modulating enzyme activity of pyruvate carboxylase and Pepck or by modulating the expres-sion of gluconeogenic genes (3, 278). The later is mediated by cytosolic calcium sensors such as calmodulin-dependent kinases and calcineurin, which together increase the nuclear transcription of *Foxo1*, *Creb,* and *Crtc2*, thereby enhancing gluconeogenesis (3, 278). Additionally, cytosolic calcium also regulates glycogenolysis through stimulation of the phosphorylase kinase and activation of GP (3, 278). Recently,

Perry et al. (290) have shown that glucagon stimulates hepatic gluconeogenesis through activation of mitochondria localized IP_3R1-mediated stimulation of mitochondrial fat oxidation and lipolysis, indicating the physiological importance of this process in glucagon biology.

Calcium release from the ER occurs either directly into the cytosol, as described above, or can be taken up into the mitochondria thought mitochondria/ER contact sites (42). Mitochondrial calcium influx stimulates mitochondrial oxidative metabolism and electron transport. This is mediated by increasing the activity of calcium-sensitive dehydroge-nases of the TCA cycle: pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase (258). Additionally, direct activation of the mitochondrial ATPase through calcium stimulates ATP synthesis (258). Besides this, glucagon stimulated mitochondrial calcium influx accumu-lates adenine nucleotides via the mitochondrial ATP-Mg/P_i carrier (SCaMC-3/slc25a23), which serve as precursors for gluconeogenesis (358).

Carbon source for glucose production during gluconeoge-nesis is provided by pyruvate and acetate as well as alanine, glutamine, and glycerol. Recently, the carbon share from glutamine has been shown to be enriched upon glucagon stimulation in hepatocytes (72). It is proposed that mitochon-drial calcium influx following glucagon treatment stimulates the activity of α-ketoglutarate dehydrogenase paving the way to increased anaplerotic flux from glutamine. Consistent with this, deletion of glutaminase (*Gls2*), the enzyme involved in conversion of glutamine to glutamate, results in reduced glucagon stimulated glutamine turnover and decreased fast-ing blood glucose levels in mice (72). Importantly, a mutation at human *GLS2* locus causes enhanced glutaminase activity stimulating glutamine influx and is connected with higher fasting blood glucose in humans (259). Altogether, these data reveal the main function of glucagon on calcium influx and mitochondrial respiration is to tune the system for maximal gluconeogenic capacity.

Glucagon action on lipid metabolism in the adipose tissue and liver

Consistent with glucagon's main function during fasting, where lipid mobilization is needed to provide energy through β-oxidation and production of ketone bodies (321), glucagon has been connected to lipid metabolism since the 1960s (44, 286). Subsequently, glucagon has been shown to reduce plasma cholesterol (43, 141), triglycerides (43, 141, 240), and esterified fatty acid levels (45). The involvement of the adipose tissue in these effects has been investigated in mice, where small amounts of Gcgr are also detectable (35). Here, glucagon stimulates lipolysis through cAMP-PKA-hormone sensitive lipase (HSL)-mediated pathway thereby, however, increasing circulating FFA levels (155, 348, 361). Despite this, there has been no solid evidence of Gcgr expression in human adipocytes (417) and glucagon induced lipolysis was only obtained at supra-physiological concentration in

human adipocytes (possibly through glucagon stimulated catecholamine secretion) rather than at physiological levels (124). Consistent with the fact that Gcgr expression is highest in the liver, these data indicate hepatic Gcgr signaling to be the primary regulator of lipid metabolism by glucagon.

Glucagon affects liver lipid metabolism through inhibition of lipogenesis and stimulation of lipolysis (111). In hepatocytes, glucagon activates AMPK and p38 MAPK which leads to nuclear translocation and transcriptional activation of peroxisome proliferator-activated receptor alpha (*Ppar-α*) that in turn increases the transcript level of fatty acid oxidation gene-carnitine palmitoyltransferase-1a (Cpt-1a) (240, 355). Cpt-1a enables catabolism of long-chain fatty acids by converting them to acyl-carnitines (240, 355). These acyl-carnitines are then transported into mitochondria thereby activating β-oxidation wherein fatty acids are degraded to acetate. Acetate and CoA combine to form acetyl-CoA, which then condenses with oxaloacetate to form citrate ultimately entering citric cycle. This process enhances fatty acid catabolism and inhibits glycolysis (Figure 4).

Apart from transcriptional activation, glucagon also regulates lipid metabolism by acetylation and deacetylation, similar to its control of gluconeogenesis. Here, the activity of forkhead transcription factor A2 (Foxa2) is increased upon its acetylation (394) *via* adenylyl cyclase mediated inhibition of Sik2 and subsequent enhancement of p300 activity (237). Foxa2 then induces the transcription of β-oxidation genes such as Cpt-1 and medium-chain acyl-CoA dehydrogenase (Mcad) (394). Recruitment of Kat2b/Pcaf by glucagon acetylates cAMP-responsive element-binding protein H (Crebh) at Lys294 (199), which induces its nuclear localization and interaction with PPARα, leading to increased transcription of fibroblast growth factor 21 (*Fgf21*). Fgf21 then increases energy expenditure and inhibits lipogenesis (200) as described above. In addition, glucagon induces the expression of Sirt3 (207), that in-turn deacetylates and enhances activity of long-chain acyl-CoA dehydrogenase (Lcad) (166). Lcad, a key mitochondrial fatty acid oxidation enzyme, reduces triglyceride accumulation and stimulates fatty acid oxidation.

Glucagon-induced cAMP formation shifts the intracellular AMP/ATP ratio to an energy-depleted state sufficient to activate AMPK (19). This leads to phosphorylation and inactivation of acetyl-CoA carboxylase, causing a reduction in malonyl-CoA formation. As accumulation of Malonyl-CoA inhibits Cpt-1 induced β-oxidation, reducing its production will redirect FFAs from re-esterification as triglyceride to β-oxidation (75). FFAs are either stored as triglycerides or are processed by lipases to be released as very-low-density-lipoprotein (VLDL) into circulation. As FFAs are used for β-oxidation, VLDL secretion is also downregulated in this process (Figure 4) (22, 24).

Consistent with the allosteric activations, acute and long-term administration of glucagon in mice *in vivo* showed reduced plasma FFA (111), TG (111, 140), and phospholipid (140) concentrations along with decreased hepatic triglyceride content (111, 158), which is dependent upon Gcgr, as *Gcgr⁻/⁻* mice and glucagon antagonists do not show this effect (121, 240). Also, *Gcgr* knockdown in *db/db* mice increases plasma low-density-lipoprotein (LDL) cholesterol, liver triglycerides, and liver cholesterol, which is accompanied by increases in lipogenic genes including fatty acid synthase, acetyl-CoA carboxylase, stearoyl-CoA desaturase 1, and elongation of very long-chain fatty acids protein (147), further supporting evidence for a role of glucagon in lipid metabolism. In fact, humans with hyperglucagonemia exhibit a decrease in lipoprotein particle turnover and induced β-oxidation (301, 419), confirming its clinical relevance. These observations have hampered the pharmacotherapeutic use of Gcgr antagonists as treatment options for the hyperglycemia in type-2 diabetic patients (see below) (142, 189, 328). Thus, there is a pressing need for identification of clear mechanisms and pathways mediating the glucose and lipid metabolic effects downstream of Gcgr upon ligand activation.

Regulation of ketone body metabolism

During prolonged starvation, the liver produces ketone bodies that provide energy fuel for the brain. Glucagon functions to stimulate ketogenesis, a process occurring in the mitochondria of perivenous hepatocytes, which transforms fatty acids (FAs) into acetoacetate (AcAc) and 3-hydroxy butyrate (3HB) (152). FAs shuttled into the mitochondria via Cpt-1 undergo β-oxidation to form acetyl-CoA, to enter the citric cycle or for utilization in ketone body formation. Since the activity of the citric cycle is reduced under long-term starvation, as all intermediates are used for gluconeogenesis, acetyl-CoA becomes available for ketone body formation (220). Glucagon stimulates the activity of hepatic mitochondrial HMG-CoA synthase, a key rate-limiting enzyme for AcAc formation, and thereby enhances ketone body production (Figure 4) (304). This is achieved by lowering the concentration of succinyl-CoA, which inactivates HMG-CoA synthase, thus increasing ketogenesis (304). Interestingly, elevated blood glucagon levels have been shown to contribute to increased circulating ketone bodies and metabolic acidosis in diabetic ketoacidosis and alcoholic ketoacidosis, suggesting its human relevance (220). In fact, in uncontrolled insulin-deficient diabetic patients hyperglucagonemia was found to be essential for ketosis rather than hyperglycemia (191, 253). However, recent conflicting data implies a limited role for glucagon in ketogenesis, since interruption of glucagon signaling has no effect on fasting stimulated ketosis (41), emphasizing the need to revisit the direct role of glucagon in ketone body metabolism.

Regulation of bile acid metabolism

Synthesized from cholesterol in hepatocytes, bile acids have emerged as pivotal modulators of lipid, glucose, and energy

metabolism in the liver (340). Cyp7a1 is the first and rate-limiting enzyme in bile acid biosynthetic pathway. Glucagon represses the gene expression of *CYP7A1* in human and rat hepatocytes, through PKA-dependent phosphorylation and inactivation of HNF4α (351). Importantly, chronic Gcgr agonism increases circulating bile acid levels in DIO mice and induces body weight reduction (140, 201). While bile acids are ligands for FXR and induce energy expenditure, the weight lowering effect of chronic Gcgr agonism was reduced in liver-specific-FXR knockout mice (201). This opens the possibility for bile acid-FXR axis in hepatocytes mediating the glucagon-stimulated effects on energy expenditure.

Glucagon and Fgf21

Fibroblast growth factor 21 (Fgf21) was first described in 2000 as a novel FGF with high homology to the endocrine Fgf19 (277). Fgf21 is secreted via coat protein complex II vesicles (400) in response to diverse nutritional stressors including fasting (11, 109, 171), a ketogenic diet (10, 11) a low protein diet (165, 218, 219), and carbohydrate refeeding (170, 330). First reported as a novel metabolic regulator in 2005 (195), Fgf21 has been shown to have pluripotent effects, including regulating energy expenditure (59, 331), thermogenesis (98, 398), fatty acid oxidation (300), glucose metabolism (40, 247, 250, 331), and body weight (59) in rodents. As such, Fgf21 has emerged as an appealing therapeutic for the metabolic syndrome (9, 171, 177).

Consistent with fasting-induced Fgf21 secretion, acute glucagon administration increases plasma FGF21 levels in rodents (64, 145) and humans (145, 149). This is a direct effect of liver glucagon signaling, as glucagon treatment in mouse primary hepatocytes increases both *Fgf21* gene expression and Fgf21 in the cultured media (145). Consistently, this effect is lost in hepatocytes isolated from mice deficient for Gcgrs (Gcgr; $Gcgr^{-/-}$ and $Gcgr^{-liver}$) (145, 201). This regulation is consistent and more robust in mice treated either acutely (145) or chronically with the potent Gcgr agonist IUB288 (145, 201). Fasting-induced Fgf21 is regulated by Ppar-α (109, 171) and glucagon signaling regulates Ppar-α transcriptional activity (240). While it is logical to assume glucagon regulates Fgf21 in a Ppar-α dependent manner, this has yet to be definitively elucidated. Glucagon also regulates Fgf21 secretion in rat primary hepatocytes via posttranslational modifications mediated in a PKA and Epac-dependent manner, with no differences in gene expression (64). This model-specific difference in glucagon-mediated *Fgf21* gene regulation may be a result of differences in the model organism, time of treatment, or culture conditions. While Fgf21 is regulated by multiple factors, $Gcgr^{-/-}$ mice are refractive to fasting-induced liver Fgf21 expression, suggesting glucagon is the primary stimulator of Fgf21 in a fasted state (240).

Mice deficient for Fgf21 ($Fgf21^{-/-}$) are likewise refractive to Gcgr-mediated increases in EE and prevention of DIO with no genotypic differences in food intake, suggesting glucagon regulates energy balance via Fgf21. Further, overexpression of liver Fgf21 and administration of recombinant Fgf21 increases EE in a brain-dependent manner (331). While it has yet to be elucidated, it is plausible that glucagon regulates energy balance via central Fgf21 action. Chronic Gcgr agonism additionally decreases plasma cholesterol, liver triglycerides, and increases day-time locomotor activity (145). Fgf21 likewise regulates plasma cholesterol and locomotor activity, highlighting Fgf21 as an important mediator of specific glucagon actions. While Fgf21 is sufficient to mediate Gcgr-prevention of body weight gain on high-fat diet, hepatic Fgf21 is only partially responsible for the weight loss effects of Gcgr-agonism in DIO mice (201). These observations may be due to differential glucagon-mediated mechanisms regulating obesity prevention versus treatment.

Pharmacological Actions of Glucagon in Type-1 and Type-2 Diabetes

Insulin deficiency is traditionally viewed as the major culprit in diabetes. In the early 1970s, however, Roger Unger proposed that elevated postprandial glucagon levels are an equally critical factor underpinning diabetes (272, 384). Indeed, postprandial glucagon levels are higher in all forms of diabetes, including type-1 and type-2 diabetes (383). It is postulated that in patients with diabetes, a relative excess of glucagon compared to the decrease in insulin drives excessive hepatic glucose production, contributing to fasting hyperglycemia (313) and greater postprandial glucose excursion (339). In support, in patients with type-2 diabetes, hepatic gluconeogenesis is increased compared to age- and BMI-matched nondiabetic control subjects. Therefore, attenuating glucagon action has been investigated as a treatment of diabetes. The first seminal study to explore this concept used somatostatin to inhibit endogenous glucagon production in patients with type-1 diabetes and observed a decrease in blood glucose levels (125). Similarly, somatostatin administration ameliorated hyperglycemia in dogs rendered diabetic by either alloxan or by removal of the pancreas (126, 324). Subsequently, genetic mouse models have been used to explore the metabolic consequences of lack of glucagon signaling. Glucagon-receptor knock-out mice ($Gcgr^{-/-}$), in which the ratio of insulin to glucagon signaling is shifted entirely to the side of insulin, have lower blood glucose levels, are more glucose tolerant, and are resistant to HFD-induced insulin resistance (56, 228). Remarkably, $Gcgr^{-/-}$ mice are even resistant to STZ-induces hyperglycemia and β-cell destruction (56), without exhibiting signs of hypoglycemia (121).

In light of these observations, strategies to pharmacologically suppress Gcgr signaling have received a lot of attention in recent years for the potential treatment of diabetes. In the preclinical models, Gcgr antagonists improve glucose tolerance in mouse models of diabetes (104, 226, 267). Similarly, Gcgr antibodies decrease glucose levels and improve glucose tolerance in diabetic rodents and monkeys (138, 202, 280, 423) and anti-sense oligonucleotide-mediated reduction of

hepatic Gcgr expression similarly ameliorated hyperglycemia in diabetic mice (232).

In healthy humans, single administration of a Gcgr antagonist reduced glucagon-induced glucose production. In longer-term trials, Gcgr antagonists lower fasting and postprandial blood glucose concentrations, as well as HbA1c levels in patients with type-2 diabetes (63, 189, 388). Similarly, antisense oligonucleotides also improve HbA1c in people with diabetes (389), while monoclonal antibodies against the Gcgr diminish glucagon-induced glucose excursions (209).

These encouraging clinical data, however, have been associated with significant side effects that have thwarted the clinical use of these agents. Increased hepatic transaminases have been seen with Gcgr antagonists (20, 142, 189, 193, 388) and humanized monoclonal antibodies (209), suggesting adverse effects on the liver. Gcgr antagonists increase LDL cholesterol (20, 139) and liver fat (142). Another concern pertains to pre-clinical studies showing that Gcgr antibodies cause α-cell hyperplasia (138, 280), which also has been observed in global $Gcgr^{-/-}$ mice (121) as well as in liver-specific $Gcgr^{-/-}$ mice (239). Whether this translates into serious clinical side effects will have to be determined in longer-term studies, but the concern that this hyperplasia may become malignant has to be carefully assessed.

However, in light of the support of glucagon in the regulation of lipid metabolism, Gcgr agonism may be useful for treatment of hepatic steatosis (336), to which there are no FDA-approved therapeutics (428). Chronic Gcgr agonism decreases liver triglycerides and plasma cholesterol in DIO mice (145, 201, 274). This effect is dependent on liver Gcgr signaling, as the benefits of Gcgr agonism on dyslipidemia are lost in mice deficient for hepatic Gcgr (Gcgr^liver) (145, 201, 274). These pharmacological effects are consistent with results from clinical trials utilizing GLP1R and Gcgr dual agonist. Treatment with the dual agonist reduces liver triglycerides and plasma cholesterol (6). While glucagon monotherapy has not been tested directly in clinical trials, the dual agonists are superior to GLP1 agonism alone in reducing hepatic steatosis in rodent models (66, 299). These additional metabolic actions of glucagon warrant further study as promising avenues for the treatment of obesity and hepatic steatosis.

Outlook and Future Questions

Identified nearly a century ago in a process to optimize insulin purification, glucagon has ever since been stigmatized for its hepatic effects to increase blood glucose. Long overshadowed by the monumental importance of insulin, recent years have witnessed a renaissance of glucagon pharmacology with acknowledged applications that go far beyond its initial use as a life-saving rescue medication for severe hypoglycemia. A plethora of studies nowadays testify glucagon pharmacological value to improve body weight and lipid metabolism and dual-agonists targeting the receptors for glucagon and GLP-1 are in clinical development for the treatment of type-2 diabetes.

Acknowledgments

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