Benefits and limitations of reducing glucagon action for the treatment of type 2 diabetes

Safina Ali and Daniel J. Drucker

From the Department of Medicine, Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, 60 Murray street Mailbox 39, Toronto M5T 3L9

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Address correspondence to:

Dr. Daniel J. Drucker
Mt. Sinai Hospital
60 Murray Street Mailbox 39
Toronto Ontario M5T 3L9
d.drucker@utoronto.ca

Abstract:

Glucagon is secreted from the alpha cells of the pancreatic islets and regulates glucose homeostasis through modulation of hepatic glucose production. As elevated glucagon levels contribute to the pathophysiology of hyperglycemia in subjects with type 2 diabetes, reduction of glucagon receptor (Gcgr) activity represents a potential target for the treatment of T2DM. Herein we review current concepts of glucagon action in hepatic and extra-hepatic tissues and evaluate the therapeutic potential, mechanisms of action, and safety of reducing Gcgr signaling for the treatment of T2D.

Introduction:

Insulin and glucagon are pancreatic hormones which play pivotal roles in regulating glucose homeostasis and metabolism. Subjects with type 2 diabetes mellitus (T2D) have fasting hyperglucagonemia with impaired post-prandial glucagon suppression in the presence of defective insulin secretion and/or action. Inhibition of post-prandial glucagon secretion in diabetic subjects has been shown to substantially reduce blood glucose suggesting that glucagon contributes significantly to the hyperglycemia seen in subjects with T2D (16, 62, 63). These observations have prompted interest in reduction of glucagon secretion or blockade of glucagon receptor (Gcgr) activity for the treatment of T2D. In this review we summarize recent advances in understanding the actions of glucagon in hepatic and extra-hepatic tissues and provide an overview of the therapeutic potential of Gcgr inhibition for the treatment of T2D.

Glucagon synthesis and secretion:

Glucagon is a 29 amino acid peptide hormone encoded within a proglucagon precursor which also contains amino acid sequences for glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), oxyntomodulin and glicentin (**Figure 1**). Tissue specific post-translational processing of proglucagon in α-cells is mediated by prohormone convertase 2 (PC2), which cleaves proglucagon to liberate glucagon and leaves unprocessed the carboxyterminal major proglucagon fragment. In contrast, prohormone convertase 1/3 (PC1/3) cleaves proglucagon in intestinal L cells and brain to liberate GLP-1, GLP-2, oxyntomodulin and glicentin (3). Glucagon secretion by α-cells is highly regulated by multiple factors the most important of which are glucose and insulin (18) and reviewed in (61). Low glucose levels activate specific channels in the brain, specifically, the KATP channel (51), and on pancreatic α-cells to generate action potentials of sodium and calcium currents leading to glucagon secretion. However, whether the modulating effect of glucose on glucagon secretion is predominantly direct or indirect remains uncertain. Studies

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conducted with mouse and human α-cells show that glucose can directly inhibit glucagon secretion. In contrast studies with rat α-cells show that glucose regulates glucagon secretion in a paracrine manner (61). β-cell-derived products such as insulin, GABA, and zinc also inhibit glucagon secretion (5). The mechanisms responsible for insulin-mediated inhibition of α-cell glucagon secretion may involve insulin-mediated activation of GABA receptor translocation to the cell surface in an Akt-dependent manner (74). Similarly, secretion of zinc from β-cells appears to be important for suppression of glucagon secretion, and reduced zinc secretion promotes enhanced glucagon secretion in response to hypoglycemia (76). Nevertheless, experiments using rodent and human islets demonstrated that glucose-mediated suppression of glucagon secretion may occur independent of GABA or zinc, and requires functional KATP channels (48). Somatostatin inhibits glucagon secretion by inhibition of adenylate cyclase and cAMP production and genetic deletion of the somatostatin receptor subtype-2 is associated with mild hyperglucagonemia and defective glucose- and somatostatin-mediated suppression of glucagon secretion in isolated islets in vitro (65). Similarly the incretin hormone GLP-1 inhibits glucagon secretion in a glucose-dependent manner through mechanisms requiring the somatostatin receptor subtype-2 (15).

Glucagon action and the Gcgr

The major biological action of glucagon is to counteract the actions of insulin and maintain normoglycemia during the fasting state by inducing hepatic glucose production. Glucagon exerts its action on target tissues through activation of the Gcgr, a G protein coupled receptor (GPCR) member of the class II GPCR superfamily (33). Gcgr activation leads to signal transduction by G proteins (Gαs and Gq), whereby Gαs activates adenylate cyclase which causes cAMP production resulting in an increase in levels of protein kinase A. Gq activation leads to phospholipase C-mediated increases in intracellular calcium levels. Gcgr signaling in the liver results in increased hepatic glucose production by induction of glycogenolysis and gluconeogenesis along with

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inhibition of glycogenesis (34). The actions of glucagon to promote increased hepatic glucose production are extremely rapid and reflect changes in the activity of enzymes regulating gluconeogenesis and glycogenolysis. Glucagon-stimulated increases in cyclic AMP lead to activation of glycogen phosphorylase and inhibition of glycogen synthase. The actions of glucagon to control gluconeogenesis are mediated through coordinate regulation of the CREB (cyclic AMP-regulated binding protein), regulated transcription coactivator 2, histone acetyltransferase p300 and the nutrient-sensing deacetylase sirtuin 1 (SIRT1), resulting in increased expression of genes regulating gluconeogenesis (45). The Gcgr is also expressed in extra-hepatic tissues which include heart, intestinal smooth muscle, kidney, brain and adipose tissue (27) and much less is known about the action of glucagon in these tissues (**Figure 2**).

Glucagon and the cardiovascular system (CVS)

Pharmacological doses of glucagon activate adenylate cyclase in a beta-adrenoreceptor-independent manner leading to cyclic AMP (cAMP) production in the myocardium, and a positive inotropic and chronotropic effect. Accordingly glucagon is occasionally used for the treatment of poisoning caused by cardio-depressant drugs such as beta-blockers or calcium channel blockers (73). The inotropic effects mediated by glucagon in the CVS may be preferentially localized to the ventricular myocardium due in part to differential Gcgr expression in the ventricle compared to the atrium (23). Infusion of glucagon in perfused rat hearts at levels designed to achieve physiological concentrations of glucagon leads to induction of glycolysis and glucose oxidation, similar to insulin actions in the heart, that are mediated via PI3K-dependent, adenylate cyclase and cAMP-independent pathways (28). Hence, unlike the effects of glucagon in the liver that generally oppose insulin action, glucagon and insulin action in the heart may overlap in regard to stimulation of fuel metabolism.

Glucagon, the kidney and the gastrointestinal tract:

Glucagon stimulates adenylate cyclase and cAMP production in nephrons and in cell free preparations of human renal medullas (54, 75). Although the role of glucagon in the control of renal glucose output remains uncertain glucagon regulates the rate of kidney filtration, urea excretion and water re-absorption by the kidney (26), via direct and indirect mechanisms (4). Paradoxically, long term infusion of glucagon in mice leads to kidney injury through the development of hypertension, hypertrophy and increased proliferation of mesangial cells (40). Although the Gcgr is also expressed in the gut where it regulates motility (58, 69), very little is known about the physiological role of glucagon in the gut.

Glucagon and the endocrine pancreas:

Gcgr immunoreactivity and mRNA expression have been detected predominantly in β-cells from rodent pancreas, however subsets of α- and δ-cells also express the Gcgr (37). Additionally, glucagon has been shown to regulate cAMP production in beta cells. However glucagon-mediated cAMP production in β-cells is less potent than that induced by the incretin hormones GLP-1 and GIP (53). Nevertheless, glucagon induces insulin secretion in human subjects. Moreover, insulin secretion is increased from perfused pancreas and isolated beta cells in the presence of glucagon (37, 53). The stimulatory actions of glucagon on the islet β-cell may be mediated through dual activation of both the Gcgr and the GLP-1R (52). However, the molecular mechanism(s) and physiological importance of glucagon-stimulated insulin secretion require further elucidation. Even less is known about the role of the Gcgr in α-cells, however several studies have demonstrated Gcgr expression in at least a subset of rodent α-cells (37, 47). Glucagon stimulates cAMP production in a dose-dependent manner from rat and mouse α-cells (37, 47) and increases α-cell exocytosis in a PKA-dependent manner suggesting that it may regulate its own secretion (47). However, the importance of glucagon action on α-cells is uncertain.

Glucagon action in the brain and adipose tissue:

The proglucagon gene is expressed in the brainstem and to a lesser extent, in the hypothalamus (17), and afferent projections distribute proglucagon-derived peptides to diverse brain regions (35). Glucagon binds to brain membranes and mouse astrocytes and stimulates adenylate cyclase and cAMP production respectively (12, 31). Intracerebral administration of glucagon at doses likely to produce pharmacological levels of glucagon in the brain produces dose-dependent hyperglycemia in rodents through mechanisms requiring cholinergic and alpha-adrenergic neural pathways (1, 50). Glucagon infusion in the CNS also inhibits food intake (32), and the anorectic actions of glucagon require functional vagal afferents (72). Moreover, neutralization of endogenous glucagon via intraportal infusion of glucagon antibodies increased meal size in normal rats, effects that were abolished in rats with selective hepatic vagotomy (20). The satiety-promoting effects of glucagon may also involve suppression of ghrelin secretion, actions that require an intact hypothalamic-pituitary axis and ghrelin has been shown to regulate feeding behavior; suggesting that the satiety effect of glucagon could be mediated through ghrelin (2).

Although Gcgr expression is detected in adipose tissue, the role of Gcgr in the induction of lypolysis in adipose tissue is contradictory. Although glucagon increases lipolysis in rat and human adipocytes (29, 59), subcutaneous infusion of glucagon in abdominal adipose tissue of healthy male subjects had no effect on lipolysis (24). Hence the precise role of glucagon in the control of lipolysis remains uncertain.

Glucagon and the pathophysiology of Type 2 diabetes:

Type 2 Diabetes is characterized by impaired insulin secretion and/or action, and many subjects also exhibit inappropriate levels of circulating glucagon in the fasting and postprandial state. An increase in the glucagon:insulin ratio is likely an important determinant of the hyperglycemia seen in T2D patients (6, 7, 19). Consistent with the importance of glucagon for fasting hyperglycemia,

infusion of low doses of glucagon leads to the development of hyperglycemia (44), whereas suppression of glucagon secretion in the fasting state by somatostatin infusion significantly reduces hepatic glucose production (6) Lack of suppression of post-prandial glucagon secretion in subjects with T2D also plays an important role in the pathogenesis of postprandial hyperglycemia (22, 30, 63). The molecular mechanisms responsible for dysregulation of α-cell glucagon secretion in diabetic subjects remain unclear, but may include impaired glucose sensing by α-cells and/or resistance of α-cells to the inhibitory actions of insulin or other β-cell secretory products, such as zinc or GABA.

Reduction of Gcgr signaling for the treatment of diabetes:

Considerable preclinical evidence supports the targeting of glucagon action as an effective approach to reduction of hyperglycemia. Immunoneutralization of glucagon with a monoclonal antibody produced significant improvements in plasma glucose in rats with streptozotocin-induced diabetes (10). Similarly, glucagon antibodies markedly reduced hepatic glucose production and reduced the extent of hyperglycemia in normal and diabetic rabbits (9). Additionally, immunoneutralization of plasma glucagon, decreased hepatic glucose output and reduced glucose and HbA1c in ob/ob mice, providing further evidence for the central role of glucagon in the pathogenesis of diabetic hyperglycemia (67).

Both peptide and non-peptide glucagon receptor antagonists have been generated for use as experimental tools to block glucagon action (34). Consistent with data from glucagon immunoneutralization studies, Gcgr antagonists lower blood glucose in response to exogenous glucagon administration in non-diabetic rodents, and block the actions of endogenously elevated levels of glucagon leading to reduction of hyperglycemia in diabetic rodents (14, 36, 70). Several different classes of small-molecule based orally available Gcgr antagonists have been identified including tri-substituted ureas, benzimidazole, alkylidene hydrazide and beta alanine derivative. These molecules were active following oral administration in dogs, rhesus monkeys

and non-diabetic and diabetic rodents (38, 39, 42, 49). Furthermore, BAY27-995, a small molecule Gcgr antagonist, successfully blocked exogenous glucagon-stimulated glucose production in human subjects (60).

Complementary strategies for reduction of hepatic Gcgr signaling have utilized antisense oligonucleotide (ASO) to target hepatic Gcgr expression. Twice weekly intraperitoneal administration of Gcgr ASOs to db/db mice significantly reduced plasma levels of glucose, triglycerides, and free fatty acids without associated hypoglycemia (43). Similarly, Gcgr ASOs reduced hyperglycemia in ob/ob and db/db mice and Zucker diabetic fatty rats, together with a reduction in plasma and hepatic triglyceride content. Intriguingly, plasma levels of glucagon and GLP-1 were markedly elevated in rodents treated with Gcgr ASOs, in association with the development of α-cell hyperplasia and hypertrophy, findings that were reversible following discontinuation of ASO therapy (66). Taken together these studies demonstrate that transient inhibition of Gcgr expression and/or glucagon action can inhibit hepatic glucose production leading to improved glucose homeostasis in rodents.

Elimination of Gcgr signaling: insights from Gcgr-/- mice

Studies of mice with targeted disruption of the *Gcgr* gene (*Gcgr* -/-) have demonstrated that Gcgr-/- mice are viable and exhibit mild fasting hypoglycemia (21, 56). Unexpectedly *Gcgr* -/- mice markedly increased circulating levels of GLP-1, and multiple phenotypes consistent with enhanced GLP-1 action, including improved glucose homeostasis, reduced gastric emptying, decreased adiposity, increased lean body mass and resistance to streptozotocin (STZ)-induced diabetes and diet induced obesity (**Table 1)** (13, 21, 56, 68, 71). The improvement in glucose homeostasis likely reflects a reduction in fasting glycemia due to reduced hepatic glucose production, and improved β-cell function as a result of increased circulating levels of GLP-1 in Gcgr-/- mice (21). Hence, the extent to which the improved metabolic phenotype of *Gcgr* -/- mice reflects the direct loss of Gcgr signaling in liver vs. the contribution of enhanced GLP-1 action, remains to be determined, through

additional studies employing GLP-1 receptor antagonists and/or genetic loss of GLP-1 action in the context of reduced Gcgr signaling.

Inhibition of Gcgr signaling: potential limitations

Although inhibition of Gcgr signaling for the treatment of T2D shows promising results in rodent models with diabetes, partial or complete ablation of the Gcgr is associated with several unexpected phenotypes that merit careful consideration in light of therapeutic attempts to attenuate Gcgr action for the treatment of type 2 diabetes. Notably, loss of Gcgr signaling is associated with the development of islet hyperplasia and increased endocrine cell proliferation, detectable in rodents following partial transient reduction or genetic extinction of hepatic Gcgr signaling (21, 66). The long term safety of activating signaling pathways that promote increased islet cell proliferation has not been defined, however it is worth noting that rodent β-cells tend to exhibit a greater capacity for replication relative to human β-cells (57). Moreover, Gcgr-/- mice exhibit significant increases in pancreatic weight (21), likely reflecting changes in cell number within the exocrine pancreas, although a precise analysis of the cellular composition of the *Gcgr* -/- pancreas has not been forthcoming.

More recent studies have focused on the function and viability of *Gcgr* -/- hepatocytes. Exogenous administration of glucagon induces a hypolipidemic effect in multiple species (8, 25) and glucagon administration promotes mobilization of hepatic fat in lactating dairy cows (55). Consistent with these findings fasted *Gcgr*-/- mice exhibit significant defects in lipid synthesis, secretion, and oxidation (46), and fail to upregulate a gene expression program promoting lipid oxidation. Moreover glucagon exerts multiple hypolipidemic actions directly on hepatocytes, in part through a PPARα-dependent pathway. Furthermore high fat feeding of *Gcgr* -/- mice was associated with accelerated development of steatosis in some (46), but not all studies (13). These findings imply that a threshold level of Gcgr signaling is required for hepatocytes to regulate synthesis, secretion and oxidation of lipids, and marked attenuation of Gcgr signaling would be predicted to be associated with an increased risk of dyslipidemia and fatty liver.

Complementary studies have examined the role of the Gcgr as a determinant of hepatocyte viability. The class 2 family of G protein coupled receptors contains several members, notably the GIPR, GLP-1R, and GLP-2R that have been linked to control of cell survival. For example, increased GLP-1R signaling reduces β-cell death both in cell culture studies in vitro and in multiple preclinical studies in vivo (11, 41), whereas genetic disruption of the *Glp1r* is associated with enhanced susceptibility to apoptotic injury (41). Sinclair and colleagues demonstrated that glucagon administration exerted cytoprotective actions for hepatocytes cultured in vitro, or following glucagon administration to mice in vivo. Conversely, *Gcgr-/-* mice exhibit significantly enhanced susceptibility to experimental liver injury, either following exogenous administration of the pro-apoptotic Fas ligand, or after high fat feeding (64). Moreover, re-introduction of the Gcgr in *Gcgr -/-* mice by adenoviral gene transfer significantly attenuated the development of liver injury in vivo. These findings demonstrate that the Gcgr is an important regulator of hepatocellular survival, however the minimum level of Gcgr expression required for optimization of hepatocyte survival has not been determined.

Another aspect of Gcgr biology that requires additional attention is the ability of hepatocytes with reduced Gcgr signaling to mount an appropriate counterregulatory response to hypoglycemia. Unexpectedly, genetic elimination of the *Gcgr* augments the counter-regulatory response to hypoglycemia in *Gcgr -/-* mice (21). The finding that *Gcgr -/-* mice exhibit increased epinephrine-stimulated cAMP production in liver membranes may contribute to the maintenance of an appropriate counter-regulatory response in the absence of glucagon action. Nevertheless, more detailed studies are required that examine the relationship between reduced Gcgr signaling and the counter-regulatory response to hypoglycemia in human subjects.

<u>Summary:</u>

The central importance of glucagon action for regulation of hepatic glucose production, taken together with considerable pre-clinical and clinical evidence documenting the contribution of dysregulated glucagon secretion to the

pathophysiology of diabetic hyperglycemia, makes the Gcgr a logical target for the treatment of T2D. Moreover, compelling data from preclinical studies illustrates the therapeutic potential of Gcgr antagonists or molecules targeting the expression of the Gcgr. Nevertheless, the *Gcgr* -/- mouse exhibits several unexpected phenotypes, namely α-cell hyperplasia, and increased mass of the pancreas, that complicate ascertainment of the risk:benefit ratio for marked inhibition of Gcgr signaling. Moreover, the demonstration that complete reduction of Gcgr signaling increases the susceptibility to hepatosteatosis and hepatocellular injury raises further questions about the margin for safety in reduction of glucagon action. Furthermore, the central role of glucagon as the primary hormone responsible for the counter-regulatory response to hypoglycemia requires a critical evaluation of the extent to which reduction of glucagon action can be safely achieved in diabetic subjects treated with agents that may inadvertently lead to the development of hypoglycemia.. Future studies in normal and diabetic human subjects should identify the extent to which reduction of Gcgr signaling produces a compelling therapeutic benefit without incurring a risk of adverse events.

Figure 1. Structures of proglucagon (A) B) Tissue specific post-translational processing of proglucagon in the pancreas gives glicentin-related polypeptide (GRPP), glucagon, Intervening peptide-1 (IP-1) and Major proglucagon fragment (MPGF), whereas glicentin, oxyntomodulin (OXM), intervening peptide (IP-2), GLP-1 and GLP-2 are liberated from the brain and the intestine. S= signal peptide

Figure 2. Glucagon action in hepatic and extra-hepatic tissues. The actions of glucagon are well studied in the liver (solid grey arrow). The actions of glucagon in extra hepatic tissues including, kidney, GI tract, pancreas, adipose tissue, cardiovascular system and the central nervous system are less extensively defined (dashed grey arrow).

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Table 1.

Comparison of phenotypes in Gcgr-/- (increased GLP-1 action) versus Glp1r-/- mice (lack of GLP-1 action)

	Gcgr-/- (↑ Glp-1 action)	*Glp1r-/- (↓ Glp-1 action)*
Fasting glycemia	Hypoglycemia	Hyperglycemia
Oral and intraperitoneal glucose tolerance	Improved	Impaired
Susceptibility to STZ-induced diabetes	Decreased	Increased
Gastric emptying	Reduced	Normal

Figure 1

Heart

↑ Cardiac inotropic effect

↑ Glucose oxidation

↑ Glycolysis

Brain

↑ Glucose production

↑ Satiety

Kidney

↑ Rate of glomerular filtration and water re-absorption

Adipose Tissue

↑ Lipolysis?

Pancreas

Glucagon

Stimulation of insulin secretion by β-cells

Autoregulation of α-cells?

Intestine

↓ Gut motility

Liver

↑ **Hepatic glucose production**

↑ Glycogenolysis

↑ Gluconeogenesis

↓ Glycogenesis

↑ Hepatocellular survival

↑ Lipid oxidation

Figure 2