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Insulin and Glucagon: Partners for Life

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In August 2016, several leaders in glucagon biology gathered for the European Association for the Study of Diabetes Hagedorn Workshop in Oxford, England. A key point of discussion focused around the need for basal insulin to allow for the therapeutic benefit of glucagon blockade in the treatment of diabetes. Among the most enlightening experimental results presented were findings from studies where glucagon receptor-deficient mice were administered streptozotocin to destroy pancreatic beta cells or had undergone diphtheria toxin-induced beta cell ablation. Here, key features of the discussion are summarized as we reached a consensus. Agents that antagonize glucagon may be of great benefit for the treatment of diabetes; however, sufficient levels of basal insulin are required for their therapeutic efficacy.

Hyperglucagonemia and dysregulated glucagon secretion have been implicated in contributing to hyperglycemia in patients suffering from type 1(1,2) and type 2(3-5) diabetes mellitus. These observations have supported continued efforts aimed at understanding the bihormonal relationship between insulin and glucagon and the investigation of glucagon-based therapeutic approaches. Herein, we review much of the seminal work in glucagon biology and highlight recent mechanistic studies that elegantly utilize the glucagon receptor-deficient mouse model to further assess the ability of blocked glucagon signaling to counteract insulin deficiency.

Glucagon was originally isolated as a hyperglycemic substance(6), and when the radioimmunoassays became available(7), it was revealed that glucagon secretion was inversely regulated by plasma glucose concentrations(8), supporting its role as a major glucose-regulating hormone. In studies of fasting patients with type 1 diabetes that were maintained at near euglycemia with insulin infusions, it could be demonstrated that plasma glucose and ketone levels increase rapidly after termination of the insulin infusion; importantly, this was paralleled by increases of plasma glucagon concentrations(9). Further, if somatostatin (which strongly inhibits glucagon secretion) was infused simultaneously, the rise in not only glucagon but also in plasma glucose and ketones could be strongly reduced, but not when glucagon was replaced(1,9). These and several other related observations led Unger and Orci to propose in 1975 that diabetic hyperglycemia in general was inseparably associated with inappropriate/unopposed glucagon secretion(10). This proposal caused considerable debate, particularly regarding the pathophysiology of type 1 diabetes, where the existing dogma of the clinical features being entirely due to lack of insulin was not easily
abandoned. The strongest support for the traditional belief was from demonstrations of missing or inappropriately low secretion of insulin in patients with diabetic ketoacidosis and the observation of immediate relief of the condition upon insulin administration.

In 1977, Barnes et al., using advanced (for the time) assay technology, reported full-scale development of diabetic ketoacidosis in totally pancreatectomized subjects without measurable glucagon secretion, leading the authors to conclude that glucagon is not essential for the development of ketoacidosis in diabetes(11). At that time, several reports had appeared showing production of glucagon from extrapancreatic sites in experimental animals (in particular cats and dogs)(12), but in Barnes’ patients, glucagon levels were immeasurable, suggesting that extrapancreatic secretion of glucagon might not occur in people. However, Orci and colleagues identified glucagon-positive cells in human stomach that showed ultrastructure features consistent with an alpha cell(13), suggesting humans may indeed have extrapancreatic glucagon which could contribute to hyperglycemia. Subsequent research with even more sophisticated techniques has now demonstrated that pancreatectomized patients may have remarkably large amounts of glucagon secreted from the gastrointestinal tract(14). Therefore, it remains possible that glucagon can contribute to the diabetic phenotype, even in patients with total pancreatectomy.

For many years, it was tacitly accepted that glucagon might play a role, but that the lack of insulin was believed to be the predominant hyperglycemic factor in diabetes, and this remained textbook dogma. However, the evidence for a role for glucagon was intriguing enough to spur interest in developing antagonists of glucagon action, and many (generally futile) attempts at this were made. Initially, these were mainly peptide-derived glucagon receptor antagonists that were invariably partial agonists, and the antagonistic effect was not sufficiently robust(15). However, evidence started to mount that glucagon might also play a role in the hyperglycemia of type 2 diabetes, including important pioneering studies from the laboratories of Alain Baron(4) and Robert Rizza(16). To provide proof of concept for the use of glucagon antagonism in diabetes, Brand et al(17) normalized blood glucose of mildly alloxan diabetic rabbits (a type 2 diabetes-like model) by administration of a high affinity monoclonal glucagon antibody, which could effectively neutralize circulating glucagon activity. The same antibody could also normalize glycemia in intermediate-dose streptozotocin (STZ)-diabetic rats (a type 2 model), but it was ineffective in more severely diabetic animals caused by high-dose STZ (a type 1 model)(18), supporting the general concept regarding the role of glucagon in contributing to hyperglycemia but also indicating there may be a minimal insulin requirement for glucagon antagonism to be effective.

These and other studies provided further impetus to develop glucagon receptor antagonists, and several pharmaceutical companies became engaged in the hunt. Support for those pursuits was bolstered by findings from studies characterizing a new experimental model: the glucagon receptor (Gcgr<sup>-/-</sup>) knockout mouse(19;20). These animals showed lower blood glucose levels and significantly improved glucose tolerance but displayed similar insulin levels compared with control mice, further supporting the glucagon pathway as a therapeutic target. Soon thereafter, glucagon receptor antisense oligonucleotides (ASOs) were demonstrated to robustly reduce hyperglycemia in several rodent models of type 2 diabetes(21;22). In agreement with effects of the glucagon neutralizing antibodies, Gcgr null mice or normal animals administered the glucagon receptor ASOs did not develop hypoglycemia, only a mild lowering of fasted and fed plasma glucose concentrations were observed. Based on the lack of severe hypoglycemia, the lesson learned was that glucagon antagonism appears relatively safe and therefore a potential therapy for type 2 diabetes. In subsequent years, several small molecule glucagon receptor antagonists were developed and shown to act as powerful (oral) antidiabetic agents in both preclinical and in clinical studies of type 2 diabetes(23). The appearance of unexpected side effects (increasing plasma levels of glucagon (see below), hepatic transaminases, and LDL(24)) halted development of some, but not all
of these molecules. Importantly, the clinical results with the antagonists demonstrated unequivocally that inappropriate secretion of glucagon is responsible for a major part of the hyperglycemia of type 2 diabetes(24).

On this background, it was little less than a sensation when in 2011 Lee et al.(25) from the laboratory of Roger Unger reported that in Gcgr<sup>−/−</sup> mice (developed by Maureen Charron(20)), repeat doses of STZ did not cause a diabetic phenotype. The same group subsequently showed(26) that reconstitution of hepatic glucagon receptors in these mice by adenovirus-mediated delivery resulted in a full-blown diabetic state, which resolved as the transgene expression waned. In further studies, a similar effect was shown in normal mice using a glucagon receptor antagonist antibody(27). These findings provided new fuel to the old hypothesis that glucagon is responsible for diabetic hyperglycemia, and the old debate flared up again: what is more important - insulin lack or glucagon excess?

This discussion was a major theme at an EASD sponsored symposium on glucagon recently held in Oxford (21<sup>st</sup> EASD-Hagedorn Oxford Workshop: glucagon, the alpha cell and intra-islet paracrine relationships; chairs: Jens Juul Holst and Patrik Rorsman, August 2016), with representatives from many of the groups who had been engaged in this debate and who had carried out experimental work to understand these new observations. Several studies were presented, and at the final discussion, a consensus was reached about the probable mechanisms behind the observations from Roger Unger’s and several other laboratories.

First, let us consider more of the important published results on this theme. Thorel et al. from the laboratory of Pedro Herrera were able to produce acute glucagon deficiency using diphtheria toxin in transgenic animals expressing the human diphtheria toxin receptor under control of the proglucagon promoter(28). These animals lost almost all of their alpha cells, but the phenotype was unremarkable; however, hyperglycemia after STZ administration was not prevented by the glucagon cell ablation. The authors concluded that the small amount of pancreatic glucagon left after the glucagon cell ablation must be sufficient to maintain the metabolic effects of glucagon, including the type 1-like diabetic phenotype resulting from STZ treatment. Similarly, Steenberg et al. also generated acute glucagon cell-depleted mice using diphtheria toxin(29), but the massive reduction in pancreatic glucagon cells and glucagon content did not result in improvement of the severe type 1 diabetic phenotype after large doses of STZ. Inspired by Thorel’s conclusion that even a small amount of glucagon would suffice to produce the metabolic effects of glucagon(28), these investigators also administered a glucagon neutralizing antibody (same monoclonal antibody as used in the original studies by Brand et al.(18)) as well as a glucagon receptor antagonist, previously shown to potently antagonize glucagon action in vivo(30) but both approaches were unable to resolve the hyperglycemia.

Additional important contributions to the discussion were data provided by Damond et al. in April 2016(31). These authors hypothesized that incomplete destruction of beta cells might underlie some of the differences observed in the various prior studies. Using traditional STZ treatment in Gcgr<sup>−/−</sup> mice, they reproduced the observations of complete absence of hyperglycemia (Figure 1, inverted purple triangles). However, realizing that after conventional STZ treatment a residual beta cell mass was present and thus insulin secretion remains, these investigators employed a diphtheria toxin ablation approach that had previously been shown to result in near total beta cell ablation(32). Importantly, now after diphtheria toxin-induced ablation, full-blown diabetes developed in the Gcgr<sup>−/−</sup> mice. Similarly, insulin blockade with the potent insulin receptor antagonist S961 administered after conventional STZ treatment resulted in development of a full type 1 diabetic phenotype in the Gcgr<sup>−/−</sup> mice (Figure 1, purple diamonds). Similar observations have been made by researchers in the Holland and Unger laboratories using the PANIC ATTAC mouse, a model of triggered beta cell apoptosis. Here, glucagon receptor antagonist antibodies failed to lower blood
glucose in severely diabetic PANIC ATTAC mice (Holland and Unger; unpublished observations). Very recently, Neumann et al.(33) assessed mice with double knockout for the insulin and glucagon receptor genes kept alive with exogenous insulin and islet transplants; these studies indicated that the metabolic manifestations associated with complete lack of insulin cannot be overcome by \textit{Gcgr} inactivation, and that lack of glucagon signaling was associated with modest reductions in blood glucose and ketones but not survival. Taken together, the cumulative evidence from all of these studies indicates that lack of glucagon signaling efficiently compensates for the consequences of insulin insufficiency, but only if residual insulin action persists after beta cell loss.

Unlike their \textit{Gcgr}\textsuperscript{+/−} counterparts, \textit{Gcgr}\textsuperscript{−/−} animals remain normoglycemic after two STZ injections (blue vs purple inverted triangles), but develop hyperglycemia after additional insulin blockade with the insulin receptor antagonist S961 (purple diamonds). Mice were injected with STZ at days 0 and 7 (200 and 150 mg/kg, respectively) to ablate beta cells and/or treated with S961 between days 15 and 21 (osmotic pump, 40 nmol) to inhibit insulin signaling. Random-fed glycemia is shown. From Damond et al.(31)

It has been proposed that secretion of alpha cell-generated glucagon-like peptide-1 (GLP-1) might contribute to some of the antidiabetic effects of attenuated glucagon signaling. In agreement with the original findings by Gelling et al.\textsuperscript{(20)}, Steenberg and colleagues\textsuperscript{(29)} noted that the \textit{Gcgr}\textsuperscript{−/−} mice develop massive alpha cell hyperplasia and hypersecretion of glucagon (the same is observed after glucagon neutralizing antibody, glucagon receptor antagonist antibody, or glucagon receptor ASO administration). Similar findings are made in humans with inactivating mutations in the glucagon receptor\textsuperscript{(34)}. Interestingly, the hyperplastic islets in mice also appear to produce GLP-1 (another product processed from proglucagon). This finding is in line with original observations by both the Bloom and Habener laboratories suggesting proglucagon processing in islets generates some alpha cell-derived GLP-1\textsuperscript{(35;36)}. The potential involvement of alpha cell-produced GLP-1 in antidiabetic effects was first supported by the glucagon receptor ASO experiments\textsuperscript{(22)} and later by studies with a glucagon receptor antagonist antibody\textsuperscript{(37)}. The role of GLP-1 in glucose lowering that results from blunting glucagon signaling was investigated by Jun et al. from the Eli Lilly and Company laboratories using a double knockout mouse model harboring deletion of both the \textit{Gcgr} and \textit{Glp1r}\textsuperscript{(38)}. Similar to Unger’s findings, these studies showed that STZ-induced diabetes did not develop in \textit{Gcgr}\textsuperscript{−/−} mice. However, in the double knockout mice, the same STZ treatment resulted in significant hyperglycemia, amounting to about half of that seen in control mice given STZ\textsuperscript{(38)}. Also, administration of a glucagon receptor antagonizing antibody reversed hyperglycemia in STZ treated mice (in agreement with results from Wang et al. using a different antibody\textsuperscript{(27)}), but importantly, the antibody was only partially effective in \textit{Glp1r}\textsuperscript{−/−} mice\textsuperscript{(38)}. Judging from these results, actions exerted via the GLP-1 receptor appear to play an important role in the glucose-lowering phenomenon. Therefore, increased alpha cell-produced GLP-1 might be responsible for some of the antidiabetic effects of glucagon blockade, independent of its insulinotropic actions. Traditionally, GLP-1 is not thought to influence hepatic glucose production, but this has been challenged in recent studies\textsuperscript{(39)}, in which GLP-1 appeared to directly inhibit hepatic glucose production in mice and humans\textsuperscript{(40;41)}.

Can the GLP-1 hypothesis also explain part of the efficacy demonstrated by glucagon receptor antagonist antibodies, which appear to be nearly as effective as \textit{Gcgr} knockout in preventing STZ-induced hyperglycemia? As mentioned above, there is little doubt that strong glucagon antagonism results in alpha cell hyperplasia and hyperglucagonemia in rodents\textsuperscript{(22;38)} and non-human primates\textsuperscript{(42)}. Prevention of glucagon action by glucagon receptor antibodies in humans would, therefore, be expected to have similar results. With the currently available data, it is unclear whether there are significant differences in the degree and rate of alpha cell proliferation induced by glucagon receptor antagonist antibodies in rodents versus primates, and the implications of alpha
cell hyperplasia following long-term treatment need to be carefully assessed. Further, the kinetics of GLP-1 secretion by alpha cells after blocking glucagon signaling also needs investigating.

The consensus conclusion from the total body of work, is that complete insulin lack, as in severe long-standing type 1 diabetes, locks the liver in a state where blunting of glucagon action is unable to down-regulate glucose production. However, this applies only to conditions of severe insulin deficiency. Experimentally, a threshold seems to exist in the severity of diabetes, above which only insulin or combinatorial treatment can effectively normalize blood glucose. Perhaps this threshold is coincident with ketosis, as beta cell depletion sufficient to induce ketosis cannot be completely restored by glucagon antagonism. The control of ketogenesis is equally importantly regulated, and some observations suggest ketogenesis may be more selectively dependent on glucagon action than glucose production(43). It is important to note that ketosis was reduced in further studies of the diphtheria toxin beta cell ablated $Gcgr^{-/-}$ animals (Damond and Herrera, Figure 2) in agreement with observations that $Gcgr$ gene deletion attenuated hyperketonemia in InsKO pups(33). Given the important role of glucagon signaling in regulating hepatic lipid oxidation(44), it will be important to determine if chronic treatment with glucagon antagonists promotes hepatosteatosis.

In mice with normal glucagon signaling, diphtheria toxin-mediated beta cell destruction leads to a sharp increase in circulating levels of ketone bodies. This increase is attenuated but not entirely abolished in beta cell-ablated $Gcgr^{-/-}$ mice. The corresponding random blood glucose levels averaged over one month are shown on the lower panel. Experimental procedures were performed as described(31). Briefly, adult (10-12 weeks old) RIP-DTR;$Gcgr^{-/-}$ males(32) and(20) were injected with diphtheria toxin to induce beta-cell ablation. One month later, beta-hydroxybutyrate levels were measured from plasma using an enzymatic assay (MAK041, Sigma). *, p < 0.05; **, p < 0.01; vs unablated $Gcgr^{-/-}$ mice. ##, p < 0.01; beta cell ablated: $Gcgr^{-/-}$ vs $Gcgr^{+/+}$. Mann-Whitney U test.

In the entire interval of relative insulin insufficiency, which covers late-stage type 2 diabetes and probably also type 1 diabetes with residual insulin production, (inappropriate) glucagon levels are responsible for hepatic glucose production to an extent, where a complete normalization of glucose levels may be achieved if glucagon actions are prevented. Therefore, glucagon antagonism remains an important target for antidiabetic therapy in conditions where basal insulin action remains, and should be further investigated(44).

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Figure 1 - *Gcgr*<sup>−/−</sup> mice become hyperglycemic after efficient insulin signaling blockade. Unlike their *Gcgr*<sup>+/−</sup> counterparts, *Gcgr*<sup>−/−</sup> animals remain normoglycemic after two STZ injections (blue vs purple inverted triangles), but develop hyperglycemia after additional insulin blockade with the insulin receptor antagonist S961 (purple diamonds). Mice were injected with STZ at days 0 and 7 (200 and 150 mg/kg, respectively) to ablate beta cells and/or treated with S961 between days 15 and 21 (osmotic pump, 40 nmol) to inhibit insulin signaling. Random-fed glycemia is shown. From Damond et al.(31)

Figure 2 - The lack of glucagon action mitigates hyperketonemia development after beta cell ablation. In mice with normal glucagon signaling, diphtheria toxin-mediated beta cell destruction leads to a sharp increase in circulating levels of ketone bodies. This increase is attenuated but not entirely abolished in beta cell-ablated *Gcgr*<sup>−/−</sup> mice. The corresponding random blood glucose levels averaged over one month are shown on the lower panel. Experimental procedures were performed as described(31). Briefly, adult (10-12 weeks old) *RIP-DTR;Gcgr*<sup>−/−</sup> males(32)and(20) were injected with diphtheria toxin to induce beta-cell ablation. One month later, beta-hydroxybutyrate levels were measured from plasma using an enzymatic assay (MAK041, Sigma). *, p < 0.05; **, p < 0.01; vs unablated *Gcgr*<sup>+/−</sup> mice. ##, p < 0.01; beta cell ablated: *Gcgr*<sup>−/−</sup> vs *Gcgr*<sup>+/−</sup>. Mann-Whitney *U* test. “