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Glucagon and Amino Acids Are Linked in a Mutual Feedback Cycle: The Liver–α-Cell Axis

Diabetes 2017;66:235–240 | DOI: 10.2337/db16-0994

Glucagon is usually viewed as an important counterregulatory hormone in glucose metabolism, with actions opposing those of insulin. Evidence exists that shows glucagon is important for minute-to-minute regulation of postprandial hepatic glucose production, although conditions of glucagon excess or deficiency do not cause changes compatible with this view. In patients with glucagon-producing tumors (glucagonomas), the most conspicuous signs are skin lesions (necrolytic migratory erythema), while in subjects with inactivating mutations of the glucagon receptor, pancreatic swelling may be the first sign; neither condition is necessarily associated with disturbed glucose metabolism. In glucagonoma patients, amino acid turnover and ureagenesis are greatly accelerated, and low plasma amino acid levels are probably at least partly responsible for the necrolytic migratory erythema, which resolves after amino acid administration. In patients with receptor mutations (and in knockout mice), pancreatic swelling is due to α-cell hyperplasia with gross hypersecretion of glucagon, which according to recent groundbreaking research may result from elevated amino acid levels. Additionally, solid evidence indicates that ureagenesis, and thereby amino acid levels, is critically controlled by glucagon. Together, this constitutes a complete endocrine system; feedback regulation involving amino acids regulates α-cell function and secretion, while glucagon, in turn, regulates amino acid turnover.

Glucagon was discovered in 1923 as a hyperglycemic substance (1), and subsequent research documented its actions on glycogenolysis and gluconeogenesis. Indeed, glucagon's actions on the cAMP system and its interactions with the glucagon receptor provided substrates for the Nobel prizes of Earl Sutherland, Christian de Duve, and Martin Rodbell (2). With the development of the radioimmunoassay for glucagon (3), delineation of its physiological role became possible, and Akira Ohneda working with Roger Unger, the nestor of modern glucagon research, demonstrated the reciprocal regulation of glucagon secretion by glucose (4). In elegant studies in both experimental animals and humans, typically involving blockade of glucagon (and insulin) secretion by the hormone somatostatin (5,6), it was demonstrated that hepatic glucose production, and thereby the regulation of blood glucose in the fasting state, is maintained in a balance between the inhibitory actions of insulin and the stimulatory actions of glucagon, with each hormone being approximately equally important for the regulation (7).

In further studies, inappropriate secretion of glucagon was observed in various forms of diabetes (8), and pioneering studies by Gerich et al. (9) pointed to an essential role for glucagon in the development of the hyperglycemia and ketoacidosis of type 1 diabetes, leading Unger and Orci (10) to suggest that inappropriate glucagon secretion underlies all forms of diabetic hyperglycemia. This hypothesis spurred attempts to develop glucagon receptor antagonists, which on one hand would allow conclusive studies about the essentiality of glucagon for diabetic hyperglycemia and, on the other hand, might be useful therapeutic agents for diabetes therapy (11). Eventually, the pharmaceutical industry managed to develop small molecule, orally available glucagon receptor antagonists (12,13), which have demonstrated excellent glucose-lowering efficacy

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Received 15 August 2016 and accepted 31 October 2016.

in clinical studies in patients with type 2 diabetes, in fact providing convincing proof for the importance of (inappropriate?) secretion of glucagon for the hyperglycemia of type 2 diabetes (13).

In the delineation of the physiological role of a hormone, it may be useful to study conditions of hormone excess and hormone deficiency. Among endocrinologists with an interest in glucagon, such conditions were eagerly searched for, employing radioimmunoassays to reveal hyper- and hyposecretion. The latter turned out to be difficult because the glucagon concentrations in plasma are low and measurements, therefore, challenging in terms of assay sensitivity (14). In addition, there were specificity problems, caused by (as it turned out) gut-derived, alternative products of proglucagon, the glucagon precursor (15,16), so conditions with glucagon deficiency were not easily identified. Regarding hyperglucagonemia, patients with a glucagon-secreting tumor were eventually described (17), and with more cases being collected, it became possible to define a "glucagonoma syndrome" (18,19). Some glucagonoma patients did indeed show hyperglycemia, but not all, and overt diabetes is not the rule. It was assumed that this might reflect what is seen after prolonged infusion of glucagon, where the initial elevation of plasma glucose rapidly wanes (20,21). This evanescent glucose response to glucagon was much debated and may be due to the simultaneous stimulation of insulin secretion, whereby hepatic glucose production is reduced (21). The possible consequences of glucagon deficiency remained enigmatic, but because of the interest in the development of glucagon receptor antagonists for possible clinical use, it was important to identify the consequences of glucagon lack. Intensive research from several groups had identified glucagon secretion as the first-in-line defense against hypoglycemia in humans (22), and subcutaneous glucagon injections were used to treat insulin-induced hypoglycemia in patients with type 1 diabetes. Therefore, the expected outcome of glucagon antagonism was severe hypoglycemia. Surgical glucagon deficiency brought about by experimental pancreatectomy turned out not to solve the problem because of apparent production of glucagon from sites outside of the pancreas (as had been suspected since 1948 [23] and subsequently confirmed in humans [24,25]). The development of a high-affinity monoclonal neutralizing antibody against glucagon enabled Brand et al. (26) to bring about a state of apparently complete glucagon deficiency. This resulted in significant but very slight lowering of fed and postprandial glucose levels in rats, mice, and rabbits (26–29), whereas antibody treatment in combination with an extensive fast (up to 48 h) and a 10-h fast plus complete adrenergic blockade (sympathetic activation representing the second defense line against hypoglycemia [22]) did not cause hypoglycemia (27). The actions of glucagon appear to be mediated by a single glucagon receptor (30); therefore, it was also possible to create another condition with lack of glucagon signaling, namely glucagon receptor knockout mice (31). These animals have similar plasma glucose levels as those treated with antibodies and also do not develop hypoglycemia, although they show pronounced changes in levels of the enzymes thought be to be regulated by glucagon: pyruvate carboxylase, glycogen phosphorylases, phosphoenolpyruvate carboxykinase, fructose 1,6-bisphosphatase, and glucose 6-phosphatase. Obviously, life-long global deletion of the glucagon receptor may be associated with important adaptive changes, but the similarity with the immunoneutralization results and with results from further studies employing acute small interfering RNA–induced downregulation of the glucagon receptor (32) was reassuring. Two important lessons emerged from these studies: *1*) glucagon is not essential for the maintenance of fasting glucose levels (for this, cortisol/corticosterone is more important because it provides gluconeogenic substrates for gluconeogenesis [33]) and, as a consequence, *2*) glucagon antagonism does not cause hypoglycemia. The latter was of course of particular interest to those trying to develop glucagon receptor antagonists for diabetes therapy, and subsequent studies of the antagonists confirmed that they do not cause hypoglycemia (unless combined with inappropriate amounts of insulin) (11,13). Note that these conclusions are not incompatible with a role for glucagon in the regulation of normal and diabetic postprandial and fasting glucose levels, as indicated from studies with glucagon antagonism and deletions of the glucagon receptor (34).

Thus, neither glucagon deficiency nor glucagon excess had quite the expected consequences for plasma glucose regulation. In contrast, there were other, unexpected consequences of the hyperglucagonemia of the glucagon-producing tumors. The main features of the glucagonoma syndrome include a characteristic skin rash termed necrolytic migratory erythema, which is observed in most cases (82% of patients), often with painful glossitis and angular stomatitis (35). Other characteristics are normochromic normocytic anemia (61%), weight loss (90%), deep vein thrombosis (50%), and depression (50%). Mild hyperglycemia is seen in many (80%), but overt diabetes is only observed in about 30% and is related to pancreatic damage. The skin disease is characterized by superficial epidermal necrosis, fragile blister formation, crusting, and healing with hyperpigmentation (36). The histological appearance is quite unique and involves sudden death of the cells of the stratum basale. A biochemical feature of the syndrome is extreme hypoaminoacidemia, which may involve all or only selected plasma amino acids (36,37). Since the skin is a tissue with an extremely high cell turnover, it was soon suspected that the low plasma amino acid levels might be involved, and, indeed, several subsequent studies have shown that infusions of mixed amino acids may lead to a complete resolution of the condition (37–39). These observations would point to an important role for glucagon in amino acid metabolism, confirmed in elegant

clamp studies demonstrating a critical role for glucagon in the disposal of amino acids (40). As already mentioned, one of glucagon's established effects is to promote gluconeogenesis (41). Among its actions on the liver, increased activity of key enzymes responsible for gluconeogenesis (phosphorylase kinase, phosphoenolpyruvate carboxykinase, fructose 1,6-bisphosphatase, glucose 6-phosphatase) is evident. During prolonged fasting, where glucagon secretion may be elevated (42), gluconeogenesis from glucogenic amino acids derived from the tissues may be particularly prominent. The glucose-alanine cycle, where alanine during prolonged fasting is released to the blood stream from the muscles, converted to pyruvate in the liver, and used as a substrate for gluconeogenesis, is an example (43). Both alanine transferase, responsible for conversion of alanine to pyruvate, as well as the key enzymes responsible for gluconeogenesis of this cycle are regulated by glucagon. Note, however, that the extent of this cycle is dependent on the supply of alanine rather the actions of glucagon. Indeed, in mice with genetically induced deletion of glucagon gene products, mainly the enzymes related to hepatic amino acid metabolism were affected (as opposed to those regulating gluconeogenesis), and a major finding was hyperaminoacidemia (44). In addition, the isolated effect of glucagon on gluconeogenesis is short-lasting because it cannot provide substrates (45). Pancreatectomized humans exhibit pronounced hyperaminoacidemia, which may be rapidly relieved by glucagon administration (46). Patients with cirrhosis of the liver show hyperaminoacidemia and invariably have hyperglucagonemia (47,48) and impaired ureagenesis in response to glucagon (49).

Amino acid metabolism is complex and serves many purposes. Amino acids may be divided into three categories: glucogenic, ketogenic, or both glucogenic and ketogenic, depending on their ability to be transformed into pyruvate or related products of the Krebs cycle (the glucogenic amino acids) to contribute to the production of glucose (gluconeogenesis) or into acetyl-CoA (acetoacetyl-CoA), which cannot bring about a net increase in glucose production but supplies the Krebs cycle. Importantly, amino acids contain nitrogen and in the liver undergo transaminations and deaminations, eventually leading to ureagenesis. Glucagon strongly promotes ureagenesis (50), in this way removing from the body the ammonia that results from transamination, the accumulation of which potentially would be dangerous to the brain. The urea instead is eliminated with the urine. Indeed, it can be demonstrated, using glucagon immunoneutralization, that glucagon importantly regulates both the capacity for and the rate of urea formation in experimental animals (51). Particularly in diabetic animals with accelerated ureagenesis, glucagon immunoneutralization resulted in normalization of ureagenesis. In patients with glucagonoma, a similarly increased ureagenesis rate was found together with accelerated clearances of most amino acids; lean body mass was reduced in parallel (52). Conversely, in patients with impaired glucagon secretion (chronic pancreatitis), the capacity for urea formation is reduced as a consequence of glucagon deficiency (53). Thus, it seems clear that excessive (long-term) production of glucagon has a major and potentially dangerous influence on plasma amino acid turnover, whereas glucose metabolism is less affected. However, amino acid metabolism may also be acutely affected, with significant reductions observed after minutes to hours after glucagon administration (45).

Now let us return to glucagon deficiency. As mentioned, the effects of glucagon receptor deletion in the knockout mice on plasma glucose and glucose tolerance were rather inconspicuous. Are there other prominent features of these animals? Indeed, the most dramatic consequence of the deletion is dramatic α-cell hyperplasia and hyperglucagonemia, the latter reaching a similar degree to that found in patients with glucagon-producing tumors (31), i.e., 100- to 1,000-fold elevations. And this is not solely a mouse phenomenon. A number of humans with inactivating mutations in the glucagon receptor gene have been described (11). These patients also develop massive α-cell hyperplasia (sometimes even appearing as an abdominal swelling), leading to dramatic hypersecretion of glucagon and other proglucagon-derived products from their hyperplastic α-cells (54–58). The massive hyperplasia suggests than an α-cell growth factor is produced in response to loss of glucagon action. In elegant experiments, Longuet and colleagues (59,60) showed that selective knockout of the glucagon receptor in the liver resulted in an identical phenotype to that of the global knockout, suggesting that the factor (or factors) is derived from the liver. A similar phenomenon results from blocking glucagon action with glucagon antagonists (11) (also in humans, where tumor-like levels of circulating glucagon may be reached [13]), and although there is data to indicate that the hypersecretion (and probably hyperplasia) is reversible (13), this phenomenon remains a challenge for the clinical development of glucagon receptor antagonists for diabetes therapy. But what might the nature of the hepatic growth factor be? In endocrinology, factors that stimulate the secretion of hormones often also exert trophic actions on the endocrine glands. Hypoglycemia is not known as a trophic factor for the α-cells, but the cells also respond to other metabolic substrates, namely amino acids. It has been known since the early days of the glucagon radioimmunoassay that amino acids are powerful stimulants of glucagon secretion (61,62), and, indeed, intravenous infusion of arginine is widely used as a test for the secretory capacity, not only of the insulin-producing but also the glucagon-producing cells. An intravenous arginine infusion (5 g) generally results in up to 10-fold increases in peripheral plasma levels (63). Recently, research from several laboratories has investigated the possibility that amino acids might provide the growth stimulus to the α-cells in the glucagon receptor knockout ($Gcgr^{-/-}$)

mice. Thus, Solloway et al. (64) demonstrated that inhibition of the glucagon receptor leads to reduced catabolism of amino acids in the liver and increased plasma levels of amino acids, which then induce proliferation of pancreatic α-cells. Furthermore, inhibition of mTOR attenuated the proliferation of α-cells but without normalization of the plasma levels of amino acids (the liver was still without glucagon signal and thus unaffected). Similarly, antagonism of the glucagon receptor with glucagon antagonists or pancreatectomy in humans led to increasing amino acid levels (12,46).

As also suggested by Solloway et al. (64), these studies strongly support the idea that the growth factor responsible for the α-cell hyperplasia, and presumably also the hyperglucagonemia after *Gcgr* deletion, is one or more of the plasma amino acids. This supports the idea that a normal function of amino acids is to maintain and regulate the secretion of glucagon, partly by regulating the α-cell number. As already mentioned, the regulation by amino acids of glucagon secretion is very powerful, exceeding what is observed in response to falling blood glucose (with the exception of the branched amino acid, which may actually inhibit glucagon secretion) (62). In turn, the effect of glucagon released from the α-cells, when reaching the liver, would be to accelerate ureagenesis and increase amino acid clearance, thereby reducing plasma amino acid levels. A missing glucagon signal to the liver, in terms of regulation of glucose production, is apparently easily compensated for by decreasing insulin levels (because of falling glucose levels) and increasing cortisol levels to provide more amino acids to the liver. Such circuits might involve hypothalamic glucose-sensing with subsequent sympathetic activation of the adrenal cortex. Normally, glucagon secretion may be stimulated by this mechanism (65). However, without effective glucagon signaling (e.g., impaired liver function, diabetes), the liver is unable to accelerate ureagenesis and the rise in amino acid levels can no longer be controlled (53,66). Indeed, there is evidence that the fasting hyperglucagonemia of type 2 diabetes parallels development of signs of impaired liver function (nonalcoholic fatty liver disease) (67). It was recently proposed that angiopoietin-like 4, an inhibitor of lipoprotein lipase from the adipose tissue, might link glucagon antagonism and α-cell hyperplasia (68). However, the authors used an inefficient glucagon receptor antagonist, and the changes observed both with respect to glucagon secretion and α-cell hyperplasia were miniscule and clearly unrelated to the changes observed after effective antagonists and *Gcgr* deletion.

Taken together, it appears that a perfect feedback loop exists between the liver and the α-cells of the pancreas (Fig. 1), with amino acids as the link to the α-cells and with glucagon as the link to the liver. These data support a concept where a major role for glucagon in physiology and pathophysiology is to regulate amino acid metabolism and the idea that its role in glucose metabolism, although important, is discounted when disturbances in amino acid balance require restoration by glucagon.

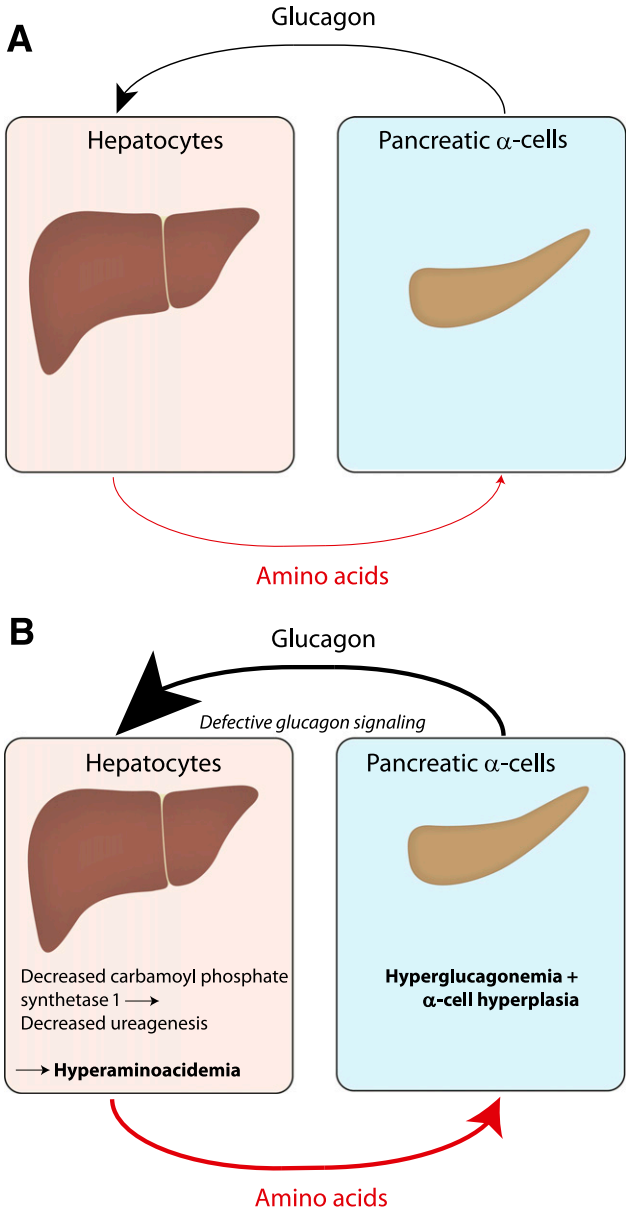

Figure 1—A physiological role of glucagon: regulation of amino acid metabolism. The proposed circuit includes hepatocytes in the liver and pancreatic α-cells. *A*: Under normal circumstances glucagon increases enzymes essential for the regulation of amino acid metabolism (ureagenesis) including in particular carbamoyl phosphate synthetase 1. Carbamoyl phosphate synthetase 1 is the first of two limiting steps in urea cycle, which takes place in the mitochondria and leads to the formation of carbamoyl phosphate from $NH3^{-}$, $HCO3^{-}$, and ATP. Amino acids then stimulate the secretion of glucagon. *B*: Upon disturbances of hepatic glucagon signaling, plasma levels of amino acids increase, causing hypersecretion of glucagon and eventually hyperplasia of pancreatic α-cells.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

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