Glucagon Replacement via Micro-Osmotic Pump Corrects Hypoglycemia and α-Cell Hyperplasia in Prohormone Convertase 2 Knockout Mice

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Prohormone convertase 2 (PC2) plays an essential role in the processing of proglucagon to mature active glucagon in pancreatic α-cells (J Biol Chem 276:27197–27202, 2001). Mice lacking PC2 demonstrate multiple defects, including chronic mild hypoglycemia and dramatic hyperplasia of the pancreatic α-cells. To define the contribution of mature glucagon deficiency to the hypoglycemia and α-cell hyperplasia, we have attempted to correct the defects by delivery of exogenous glucagon by micro-osmotic pumps. Intraperitoneal delivery of 0.5 μg glucagon/h in PC2−/− mice resulted in the normalization of blood glucose concentrations. Islet remodeling through the loss of hyperplastic α-cells was evident by day 11 after pump implantation; by 25 days postimplantation, PC2−/− islets were indistinguishable from wild-type islets. These rapid changes were brought about by induction of apoptosis in the α-cell population. Morphological normalization of islets was also accompanied by marked downregulation of endogenous proproglucagon gene expression, but with little or no change in the level of preproinsulin gene expression. Exogenous glucagon delivery also normalized hepatic expression of the gluconeogenic enzyme PEPCK. These results demonstrate that the lack of mature glucagon in PC2−/− mice is responsible for the aberrant blood glucose levels, islet morphology, and gene expression, and they confirm the role of glucagon as a tonic insulin antagonist in regulating glycemia. Diabetes 51:398–405, 2002

S ubtilisin-like prohormone convertases (PCs) proteolytically process many precursor proteins in the secretory pathway, including the maturation of active hormones from inactive prohormones in the regulated secretory pathway (1). PC1/3 and PC2 are thought to be the major convertases, acting on prohormones in multiple neuroendocrine tissues throughout the body. PC2−/− mice have both islet and central nervous system (CNS) processing defects (1,2), including a block in the conversion of proglucagon to mature glucagon in the pancreatic α-cells. This observation is consistent with earlier findings that PC2 is the predominant convertase in the α-cells and that PC2 is capable of independently processing proglucagon to mature glucagon (3,4).

The pancreatic islet is a dynamic structure that acts to control energy homeostasis. Changes in demand for islet hormones lead to alterations in the size and activity of islet cell populations. This is most generally noted for the β-cell population, where increased demand for insulin because of obesity, peripheral insulin resistance, or gestation leads to an enlarged β-cell population and increased activity of the β-cells (rev. in 5,6). When the increased demand is relieved, the β-cell population returns to normal levels. This ability to increase and decrease both activity and cell populations allows for a highly dynamic structure that is finely attuned to normal metabolic demands, but which may be disturbed in diabetes and other disorders.

PC2−/− mice exhibit marked hyperplasia and hypertrophy of the pancreatic α-cells, as well as chronic hypoglycemia and elevated levels of circulating glucagon precursors, but they have little or no active glucagon (2,7). These observations are consistent with the action of a feedback mechanism that regulates α-cell activity and the production of mature glucagon. Because PC2 is involved in the production of other hormones and neuropeptides (8,9), we have investigated the causal relationship between these observed defects and the lack of active glucagon. We show here that delivery of glucagon to PC2−/− mice is able to suppress the hypoglycemia, α-cell hyperplasia, and altered gene expression. These results demonstrate that the lack of mature glucagon in the PC2−/− mice is responsible for the above phenotypes and highlight novel mechanisms that regulate α-cell activity.

RESEARCH DESIGN AND METHODS

Glucagon pumps. Glucagon (Sigma, St. Louis, MO) was dissolved in a cetrimide solution (Sigma) at a molar ratio of 6 mol of cetrimide to 1 mol glucagon to maintain long-term solubility (J. Markussen, Novo Nordisk, Bagsvaerd, Denmark, personal communication). Glucagon solutions were made to concentrations necessary to deliver between 0.06 and 5.0 μg glucagon/h from 7- or 14-day micro-osmotic pumps (Alza, Palo Alto, CA). For example, 2 mg/ml glucagon in a 3.44 mmol/l cetrimide solution loaded into a 14-day pump with a flow rate of 0.25 μl/h delivered 0.5 μg glucagon/h. For every PC2−/− animal that received a glucagon/cetrimide–loaded pump, a PC2−/− littermate was implanted with a pump containing the same concentration of cetrimide alone. Pumps were implanted within the peritoneum of age-matched (4- to 6-month-old) animals (129Sv/c57BL/6J PC2−/− transgenic intercross) in accordance with institutional animal care and use committee–approved protocols. Briefly, animals were anesthetized by inhalation of metofane (Abbott Laboratories, Abbott Park, IL), an incision was made through the skin and muscle layers in the lower abdomen, a micro-osmotic...
RESULTS

Delivery of glucagon to PC2−/− mice via micro-osmotic pumps normalizes blood glucose levels.

PC2−/− mice produce no mature active glucagon and exhibit hypoglycemia compared with wild-type littermates (2,7). To determine a causal relationship between the lack of active glucagon and the chronic hypoglycemia, we provided long-term glucagon replacement to PC2−/− animals. Micro-osmotic pumps were loaded with glucagon to deliver 0.06–5.0 μg glucagon/h and implanted intraperitoneally. Because purified glucagon is not soluble in aqueous solution at the concentrations necessary for long-term delivery, it was dissolved with cetrimide solution to maintain solubility (see RESEARCH DESIGN AND METHODS). Blood glucose was assessed before pump implantation and on days 4 and 5 postimplantation (Fig. 1). Cetrimide alone had no effect on blood glucose levels. Glucagon delivered at rates of 0.06–0.25 μg/h also showed no effect. However, at delivery rates of ≥0.5 μg glucagon/h, blood glucose levels were elevated to levels similar to those of wild-type animals. Delivery of 0.5 μg glucagon/h in wild-type animals had no effect on blood glucose levels.

To determine the time course of blood glucose normalization, PC2−/− mice were implanted with micro-osmotic pumps that delivered 0.5 μg glucagon/h, and blood glucose levels were analyzed for 25 days. After implantation, blood glucose rose within 2 days to levels intermediate between PC2 knockout and wild-type mice (Fig. 2). By 12 days postimplantation, blood glucose levels were indistinguishable from those of wild-type animals and remained at this level for the remainder of the experimental period.

Delivery of mature glucagon to PC2−/− mice corrects islet morphology.

PC2−/− mice exhibit alterations in islet cell populations with dramatic hyperplasia of the α-cells (2). We assessed the effect of glucagon delivery to these mice on islet structure by morphometric analysis (see RESEARCH DESIGN AND METHODS). As observed previously, PC2−/− mice demonstrated a marked α-cell hyperplasia when compared with wild-type animals (ratio of α- to β-cell mass: 2.41 vs. 0.06) (Fig. 3). Delivery of 0.5 μg glucagon/h for 11 days caused a significant decrease in the α-cell mass. Continued delivery of glucagon for 25 days...
brought about a further dramatic decrease in the α-cell population, leading to normalization of islet structure (ratio of α- to β-cell mass: 0.05 vs. 0.06).

Qualitative analysis by immunofluorescent staining of the islets illustrated this effect on islet structure (Fig. 4). PC2<sup>−/−</sup> mice demonstrated a large α-cell mantle (red) surrounding the central core of β-cells (blue) (Fig. 4A), when compared with wild-type islets (Fig. 4B). After 11 days of glucagon delivery, there was a decrease in the size of the α-cell population (Fig. 4C); however, the α-cell mantle remained enlarged compared with the wild-type state. Delivery of glucagon for 25 days brought about a further decrease in the hyperplastic mantle of α-cells that surrounded the islets in PC2<sup>−/−</sup> mice (Fig. 4D).

FIG. 4. Time course of blood glucose changes in response to glucagon delivery in PC2<sup>−/−</sup> mice. The blood glucose levels of 4- to 6-month-old PC2<sup>−/−</sup> animals were tested before and after intraperitoneal implantation of osmotic pumps delivering 0.5 μg glucagon/h (●) or pumps delivering vehicle alone (3.44 mmol/l cetrimide) (●). Blood glucose levels of PC2<sup>−/−</sup> mice (●) and wild-type mice (▲) are shown as controls. Data are presented as the mean ± SD at each time point.

To assess intracellular effects of glucagon replacement on α-cell morphology, immunoelectron microscopy was carried out on samples from treated and untreated animals using an antibody that recognized both proglucagon and glucagon (Fig. 5). Wild-type islets contained well-granulated α-cells with immunoreactive glucagon (Fig. 5A). α-Cells from PC2<sup>−/−</sup> animals were hypertrophic, containing immunoreactive glucagon in secretory granules and exaggerated and stacked endoplasmic reticulum (ER) compartments (arrows) (Fig. 5B). After 25 days of glucagon replacement, the remaining α-cells demonstrated abundant secretory granules immunoreactive for glucagon (Fig. 5C), but no evidence of hypertrophy.

Normalization of islet structure is brought about by induction of apoptosis. To investigate the mechanism by which islet structure is normalized, we carried out TUNEL staining to detect apoptosis (Fig. 4). Delivery of glucagon to PC2<sup>−/−</sup> animals brought about a large induction of apoptotic activity that was evident on days 11 and 25 (Fig. 4C and D, green staining nuclei indicated by arrows). Apoptotic cells were rarely seen in untreated PC2<sup>−/−</sup> or wild-type animals (Fig. 4A and B). Apoptosis in the islets was nearly always limited to glucagon-positive cells, suggesting that α-cells are specifically targeted for removal when animals were treated with glucagon. Occasionally, glucagon-treated animals would show apoptotic acinar cells.

Delivery of mature glucagon to PC2<sup>−/−</sup> mice normalizes the expression of genes in both the pancreatic islet and liver. The inability of PC2<sup>−/−</sup> mice to produce active glucagon leads to excessive signaling of the α-cells to produce glucagon and a concomitant elevation in the expression of the preproglucagon gene. The elevated gene expression is brought about partly by increasing the number of α-cells present in the islets (Fig. 4A). Northern blot analysis of pancreatic total RNA demonstrated that delivery of glucagon brought about a large decrease in the expression of the preproglucagon transcript (Fig. 6A). This effect was specific for α-cells. Analysis of proinsulin transcripts showed little or no change in expression level with glucagon treatment (Fig. 6A). Analysis of levels of circulating insulin suggested that replacement of glucagon in PC2<sup>−/−</sup> animals did not lead to statistically significant changes in circulating immunoreactive insulin levels (Fig. 7) (see DISCUSSION).

Because the primary target organ of secreted glucagon is the liver, we were interested in determining the effect of the lack of glucagon and subsequent glucagon replacement on gene expression in the liver. Glucagon causes the liver to increase gluconeogenesis, which in turn leads to increased glucose secretion and increased blood glucose levels. The enzyme PEPCK is a rate-controlling enzyme in gluconeogenesis that is regulated primarily at the level of transcript abundance (13). We carried out Northern blot analysis on PEPCK on total liver RNA from wild-type and PC2<sup>−/−</sup> mice with or without glucagon replacement (Fig. 6B). Null mice demonstrated decreased expression of PEPCK compared with wild-type mice. The delivery of glucagon via micro-osmotic pumps led to an increase in PEPCK gene expression to levels similar to wild-type
DISCUSSION

To define the contribution of the lack of active glucagon to multiple defects in PC2−/− mice, we reintroduced glucagon via micro-osmotic pumps. These pumps allow sustained long-term delivery of soluble peptides and pharmacological agents. Glucagon demonstrates low solubility in aqueous solution, with a tendency to aggregate and precipitate over time (14,15). Cetrimide was included with glucagon to maintain hormone solubility at the concentrations necessary for continuous long-term delivery (see RESEARCH DESIGN AND METHODS). Cetrimide is an amphipathic compound that binds glucagon at a 6:1 molar ratio, protecting the hydrophobic regions of the hormone and thereby increasing solubility (J. Markussen, personal communication), and it is approved for use in surgical procedures because of its antibacterial properties. The use of cetrimide in the osmotic pumps in the described experi-

animals, indicating a stimulation of gluconeogenic activity as part of euglycemia.

FIG. 4. Changes in pancreatic islet morphology in response to glucagon treatment of PC2−/− mice. Immunofluorescence and fluorescent TUNEL staining was carried out on fixed and sectioned pancreata from PC2−/− animals receiving 0.5 μg/h glucagon for 11 (C) or 25 (D) days. Wild-type (B) and PC2−/− (A) animals were similarly stained. Glucagon is stained red, insulin is stained blue, and TUNEL is stained green. Apoptotic nuclei are indicated by arrows.
ments created no sign of inflammation or other side effects, and when used alone, it demonstrated no effects on blood glucose levels or islet structure.

Delivery of glucagon at a rate of ≈0.5 μg glucagon/h led to blood glucose normalization in PC2−/− animals, but it did not lead to changes in blood glucose levels in wild-type animals. These findings suggest that the hypoglycemic state in PC2−/− animals is attributable solely to the lack of glucagon in these animals. The delivery of glucagon (up to 5.0 μg/h) did not, however, induce hyperglycemia, suggesting that the PC2−/− animals, like the wild-type controls, maintain a normal glycemic set point that is attainable once active glucagon is available. We have observed that acute intraperitoneal injection of glucagon produces hyperglycemia in PC2−/− animals (data not shown), and therefore normal hyperglycemic responses can be elicited by the hormone.

To confirm the delivery of glucagon via the implanted osmotic pumps, serum glucagon concentrations were determined for overnight-fasted wild-type control animals that had received the minimal effective dose of 0.5 μg glucagon/h for the previous 6 days (data not shown). Under these conditions, an average concentration of ~2,000 pg glucagon/ml was measured vs. 113 pg/ml in control fasted animals. This represents a 20-fold increase in circulating glucagon levels compared with those normally seen, and it suggests that in PC2−/− animals, some impediment to glucagon action has occurred. This may be at the level of the availability or responsiveness of the glucagon receptor (16–18). Alternatively, because PC2−/−
Serum contains large amounts of intact proglucagon and partially degraded forms (2), it is possible that inactive forms of glucagon-related material circulate and may compete with administered glucagon for receptor binding (19).

Remodeling of islet structure through proliferation of cell populations have been observed previously, most notably through expansion of the β-cell population (5), as well as via induced apoptosis (20). Alteration in the size of the α-cell population by dietary and immune modulation has also been noted (21,22), as well as modulation by islets transplanted outside the pancreas (23). Our observations indicate that the lack of active glucagon leads to signaling of the α-cells to produce more glucagon, resulting in marked α-cell proliferation, whereas delivery of glucagon exogenously led to a steady decline of the hyperplastic α-cell population in PC2−/− animals.

α-Cells respond to multiple physiological cues, including hormonal, autonomic, and metabolic ones. Our present observations indicate that the α-cells are normally negatively regulated by feedback signals arising externally in response to its secretory product, glucagon. This is consistent with earlier work that demonstrated induction of α-cell hyperplasia by immune neutralization of circulating glucagon in mice and rabbits (22). Both insulin and somatostatin are thought to exert negative effects on α-cells (24). Although we were unable to detect an increase in circulating insulin-like material, it is possible that the increase in blood glucose brought about by glucagon replacement led to an intra-islet increase in insulin that affected α-cell activity. However, demonstration of actual differences in insulin production and secretion were obscured by the presence of markedly elevated levels of proinsulin and intermediate forms in the PC2−/− mice (25). Somatostatin processing is also blocked in PC2−/− animals (2); however, the predominate form in PC2−/− animals (somatostatin-28) is capable of signaling through the somatostatin receptor and therefore may be available to regulate α-cell activity (26). Work by Habener and colleagues (27) demonstrated that few pancreatic α-cells possess glucagon receptors, suggesting that the dramatic effect that we see in the α-cell population is unlikely to be caused by the direct action of glucagon on the α-cells. Neural input to the α-cell is predominately stimulatory, brought about by both sympathetic and parasympathetic innervation (28), and CNS detection of glucose levels may influence neural regulation of islet activity (29,30). Our observed increase in blood glucose in glucagon-treated animals indicates that CNS input to α-cell activity could lead to the observed effects. Additionally, locally secreted neurotransmitters from the β-cell (e.g., GABA) inhibit α-cell activity (31). Lastly, circulating fuels directly affect α-cell activity (32,33). These include α-cell stimulation by the amino acids glutamine, alanine, and arginine, as well as suppression by glucose (34). It is interesting to note, therefore, that glucose regulates α-cell activity both directly and indirectly (for CNS input, see above), and that glucagon replacement in PC2−/− mice leads to increased circulating glucose levels. Therefore, glucose is likely a mediator of the effects observed in PC2−/− animals and the reversal seen with the reintroduction of active glucagon. However, other signals may also contribute to the observed changes. Further studies will be necessary to identify all the factors that regulate α-cell activity.

TUNEL-stained islet sections demonstrated that the loss of hyperplastic α-cells is brought about by induction of apoptosis. This observation indicates that islet plasticity takes place by both α-cell population expansion (in the absence of active glucagon) and by α-cell removal (in the presence of exogenous glucagon). The observed α-cell apoptosis is in contrast to earlier findings using transplanted glucagonomas to suppress islet α-cell activity (20). In this work, no α-cell apoptosis was evident, suggesting that the implanted glucagonoma may be secreting factors other than glucagon that partially protect the endogenous α-cell population. This protective effect might be secondary to other metabolic perturbations. Specifically, the unidentified factor(s) secreted by the glucagonoma led to severe anorexia and concomitant hypoglycemia. The hypoglycemia might offer a protective effect for α-cells. Neither anorexia nor hypoglycemia was induced by the introduction of purified glucagon in our experiments. Animals that received glucagon treatment did not lose weight over the time course of the experiment (data not shown), and consistent with glucagon’s prohyperglycemic effects, blood glucose levels were elevated to normal levels. We conclude, therefore, that long-term delivery of purified glucagon can induce α-cell apoptosis when the demand for endogenous glucagon is obviated. However, like the previous observations on implanted glucagonomas, we also observed apoptosis in the acinar tissue of animals receiving glucagon. These findings suggest that communication between the endocrine and exocrine pancreas may take place via glucagon signaling.

Electron microscopy demonstrated that PC2−/− islets possess α-cells with an enlarged ER, in addition to secretory granules with immunoreactive proglucagon (Fig. 5). This observation is consistent with the demonstrated increased secretory activity of the α-cells in these animals (2,7). The addition of exogenous glucagon for 25 days led to normalization of cellular structure. Condensed chroma-
tin and fragmented nuclei in electron microscopy profiles are indicative of apoptosis (35); however, the majority of α-cells from glucagon-treated animals demonstrated near-wild-type cytoplasmic and nuclear structures. This is consistent with the TUNEL assay, which showed selective α-cell apoptosis. In this regard, although the signal for cell removal cannot be cell-autonomous (see above), there is clearly heterogeneity in the response of individual α-cells to exogenous signals, in keeping with previous findings (36).

Consistent with suppression of islet α-cells, glucagon gene expression in PC2−/− animals treated with glucagon was dramatically downregulated to levels similar to wild-type animals. This effect was specific for proproglucagon, whereas glucagon replacement had no effect on the levels of preproinsulin transcripts. The increase in blood glucose levels seen with glucagon treatment (Figs. 1 and 2) initially led us to expect a concomitant increase in insulin transcripts. A possible explanation for failure to observe this may be that our experiments were conducted using the minimal dosage of glucagon necessary to normalize blood glucose (0.5 μg/h) (Fig. 1); therefore, insulin transcription may not be highly stimulated. Moreover, recent findings by Hussain et al. (37) indicate that glucagon itself demonstrates suppressive effects on insulin gene transcription through the inducible cAMP early repressor. These competing forces may lead to a minimal net effect on transcription of the preproinsulin genes.

The lack of active glucagon in PC2−/− animals also shows gene expression in the liver by decreasing the expression of PEPCK, a key gluconeogenic enzyme. This observation is consistent with the observed hypoglycemia of these animals. Delivery of active glucagon led to normalization of PEPCK gene expression. These data indicate that normalization of blood glucose is, at least in part, due to increased gluconeogenesis and subsequent glucose release from the liver.

In summary, in PC2−/− mice, the observed defects of hypoglycemia, α-cell hyperplasia, and altered gene expression in the pancreas (glucagon) and liver (PEPCK) are caused by the lack of active glucagon and can be partially or completely reversed by restoring active glucagon via micro-osmotic pumps.

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