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Glucagon Deficiency Reduces Hepatic Glucose Production and Improves Glucose Tolerance In Adult Mice

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The major role of glucagon is to promote hepatic gluconeogenesis and glycogenolysis to raise blood glucose levels during hypoglycemic conditions. Several animal models have been established to examine the in vivo function of glucagon in the liver through attenuation of glucagon via glucagon receptor knockout animals and pharmacological interventions. To investigate the consequences of glucagon loss to hepatic glucose production and glucose homeostasis, we derived mice with a pancreas specific ablation of the α-cell transcription factor, Arx, resulting in a complete loss of the glucagon-producing pancreatic α-cell. Using this model, we found that glucagon is not required for the general health of mice but is essential for total hepatic glucose production. Our data clarifies the importance of glucagon during the regulation of fasting and postprandial glucose homeostasis. (Molecular Endocrinology 24: 1605–1614, 2010)

Glucagon, the product of pancreatic α-cells, is a major counteracting hormone to insulin in regulating glucose homeostasis (1–3). Glucagon promotes hepatic gluconeogenesis and glycogenolysis while inhibiting glycogen synthesis and glycolysis in response to hypoglycemia (4, 5). In addition, glucagon has been shown to play a major role in the development of hyperglycemia in both type 1 and type 2 diabetes mellitus (1–3, 6, 7). As elegantly demonstrated in both man and dog, glucagon plays an important role in the regulation of basal hepatic glucose production (HGP), as well as maintaining glucose levels in the postprandial state (8–12).

Because the development of severe hyperglycemia requires the presence of glucagon, suppressing glucagon action in the liver has been an attractive approach to reverse the metabolic consequences of insulin deficiency. This was first evident when somatostatin, a glucagon suppressant, was shown to restore glucose levels to the normal range in insulin-deficient humans and dogs (6, 10, 13, 14). This observation was further confirmed recently, when the potent glucagon suppressant, leptin, was shown to not only correct the hyperglycemia in the moribund insulin-deficient rodents but also restore the animals to full health (7).

The importance of glucagon signaling in regulating glucose homeostasis has also been demonstrated using genetically modified mouse models and by pharmacological interventions that suppress glucagon activity (15–24). Mice lacking the glucagon receptor (GR) (Gcgr⁻/⁻) display modest fasting hypoglycemia and improved glucose tolerance, as well as reduced adiposity and circulating triglycerides (16, 18). When challenged with a high-fat diet, Gcgr⁻/⁻ mice are resistant to obesity and exhibit reduced body weight and improved glucose tolerance (15). In addition, knockdown of GR expression in mice using antisense oligonucleotides (ASOs) lead to improved

Abbreviations: Arx, Aristaless-related homeobox; ASO, antisense oligonucleotide; CREB, cAMP response element-binding protein; CRTC2, CREB-regulated transcription coactivator 2; FFA, free fatty acid; GIR, glucose infusion rate; GR, glucagon receptor; GTT, glucose tolerance test; H&E, hematoxylin and eosin; HGP, hepatic glucose production; P, postnatal day; PAS, periodic acid-Schiff; P-CREB, phospho-CREB; PEPCK, phosphoenolpyruvate carboxykinase; Rd, glucose disposal rate; STZ, streptozotocin.
glucose metabolism in ob/ob mice and decreased HGP (19, 20). Furthermore, high-affinity glucagon neutralizing antibodies, which effectively reduce circulating glucagon, can also lower glucose levels in several animal models (22–24). Recently, it has been reported that although devoid of most, if not all, of the glucagon-derived peptides, glucagon null (Gcggfp/gfp) mice are born without gross abnormalities but display lower glucose levels at 2 wk of age (25).

Collectively, there is ample evidence to support the direct link between hyperglucagonemia and hyperglycemia in diabetes. In this study, we derived mice with a conditional deletion of the X-linked aristaless-related homeobox (Arx) gene to study the effects of glucagon during HGP and glucose homeostasis. Arx has been shown to be the key gene directing endocrine progenitor cells toward the α-cell lineage in the developing pancreas (26–28). Arx null animals do not develop α-cells and display severe hypoglycemia shortly after birth (28). Surprisingly, we find that mice with a pancreas-specific deletion of Arx have a normal life span despite lack of glucagon-producing α-cells, which is in agreement with what was recently reported in the Gcggfp/gfp animals (25). This discovery shows that additional defects must be responsible for the perinatal lethality in Arx null mice (28). However, adult Arx-deficient mice show relative hypoglycemia compared with control animals after fasting and display improved glucose tolerance. Our results confirm and expand what was previously known regarding the role of glucagon during basal and postprandial glucose homeostasis.

**Results**

**Derivation of mice deficient for Arx in the pancreas**

To directly investigate the consequence of a complete loss of glucagon on HGP, glucose homeostasis, and overall health, we derived pancreas-specific Arx mutant mice. Mice with a floxed allele of Arx (Fig. 1A), Arxfl/fl (29), were bred to mice carrying a transgene with Cre-recombinase under control of the Pdx1 promoter (30). Pdx1-Cre;Arxfl/fl and Pdx1-Cre;Arxfl/fl (Arx deficient) mice were born at the expected Mendelian distribution and showed no significant differences in size or appearance at any age examined for either gender when compared with littermate controls (data not shown).

To evaluate the specificity and efficiency of Cre-mediated deletion of Arx, we first used PCR analysis of genomic DNA to determine whether exon 2 of the Arx gene was excised in the pancreas of postnatal d 2 (P)2 Arx-deficient mice. Primers were designed to amplify a 402-bp product only detectable when the Arx gene was deleted. The PCR analysis using genomic DNA obtained from pancreas and liver indicated that gene ablation occurred only in pancreatic cells of Arx-deficient mice and not in liver cells (Fig. 1B). Concordantly, mRNA levels of Arx were reduced by approximately 95% in P7 Arx-deficient pancreas, whereas no significant differences were detected in the stomach and intestine (Fig. 1C) (data not shown). These data demonstrate that efficient and specific loss of Arx expression in the Arx-deficient pancreas is achieved by P7.

**Arx-deficient mice display a loss of α-cells and a significant increase in β, δ and PP-cells**

To evaluate whether glucagon-producing α-cell differentiation is perturbed in Arx-deficient mice, we performed immunohistochemical analysis for glucagon ex-
pression in sections of Arx-deficient and control animals at 3–6 months of age. Similarly to what was reported in Arx null animals (28), we observed a dramatic reduction in the number of glucagon-producing cells in the Arx-deficient pancreas, whereas the number of glucagon-expressing cells in the stomach and intestine were not affected (Fig. 2, A and B, and Supplemental Fig. 1A, published on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org) (data not shown). Furthermore, the number of somatostatin-expressing δ-cells and insulin-expressing β-cells was increased within the islets of Arx-deficient animals (Fig. 2, C–F). Morphometric analysis confirmed a 99% decrease, as well as 1.5- and 2-fold increases, in the number of glucagon-, insulin-, and somatostatin-producing cells in the pancreas, respectively (Fig. 2, B, D, and F). Interestingly, we also observed an increase in the number of pancreatic peptide-producing PP cells in our Arx-deficient mice (Supplemental Fig. 1, B and C), which is in disagreement with what was previously reported in the Arx null animals, where a normal number of PP cells was reported (28).

**Arx-deficient mice are healthy with lower fasting blood glucose levels**

It has been reported that Arx null mice die perinatally (28, 31), and this lethality was attributed to abnormal endocrine function likely resulting in hypoglycemia (28). Strikingly, despite the complete loss of α-cells, our Arx-deficient mice were born at the expected Mendelian ratio and are outwardly indistinguishable from control littermates at all ages examined (up to 14 months). Body weights did not differ between the two groups (data not shown). Because glucagon is thought to be critical in maintaining fasting glucose homeostasis, we measured blood glucose levels in both young (6 wk) and older (3–6 month old) Arx-deficient mice after 0-, 16-, and 24-h fasts. Although we did not detect a significant difference in glucose levels between Arx-deficient and control animals after a 0- or 16-h fast in both age groups (Fig. 3, A and B), the difference in glucose levels did reach significance after the 24-h fast (Fig. 3, A and B). These results suggest that glucagon is required for maintaining normal glucose levels during a prolonged fast regardless of the animals’ age.

**Arx-deficient mice display improved glucose tolerance test (GTT) with no detectable plasma glucagon**

To determine whether glucagon is also critical in maintaining postprandial glucose homeostasis, we performed a GTT on 6-wk-old and 3- to 6-month-old animals. Although we did not observe a difference between Arx-deficient and control mice at 6 wk of age (Fig. 3C), we found a significant improvement of glucose tolerance in 3- to 6-month-old Arx-deficient mice (Fig. 3D). To determine whether the improved glucose tolerance was associated with the loss of glucagon in the Arx-deficient mice, we measured plasma glucagon levels by RIA in 3- to 6-month-old mice after a 16-h fast and found no detectable glucagon in the peripheral blood of Arx-deficient mice.
animals (Fig. 3E). Interestingly, although we did observe an increase in β-cell mass in the Arx-deficient pancreas (Fig. 2, C and D), we did not detect a significant increase of plasma insulin levels in the Arx-deficient mice after a 16-h fast (Fig. 3E). To determine whether more insulin is secreted in the Arx-deficient mice upon glucose stimulation, we measured the plasma insulin levels during the first 30 min of a GTT in 3- to 6-month-old mice and found no significant increase in insulin secretion from the Arx-deficient animals (Fig. 3F). In fact, we noticed a trend of reduced insulin secretion in the Arx-deficient mice. These data suggest that the lack of glucagon contributes to the improved glucose tolerance in adult Arx-deficient mice.

**Arx-deficient mice have reduced HGP**

Glucagon helps to maintain blood glucose levels by promoting glycogenolysis and gluconeogenesis, resulting in increased HGP. To assess whether glucagon production in the Arx-deficient animals was affected, we performed a hyperinsulinemic/euglycemic clamp study on 2-month-old mice while the blood glucose levels were maintained between 60–70 mg/dl (control, 64.1 ± 2.6 mg/dl; and mutant, 64.9 ± 2.1 mg/dl). Although there was no difference in the glucose disposal rate (Rd), the glucose infusion rate (GIR) trended higher in Arx-deficient animals, suggesting that more glucose was needed to keep the blood glucose levels steady. This would only occur if there was lower HGP, which was indeed evident in our Arx-deficient mice (Fig. 4A). These data demonstrate that glucagon deficiency results in reduced HGP in Arx-deficient mice.

**Arx-deficient mice have increased quantities of glycogen in the liver**

Next, to determine whether there are histological differences in the liver of the Arx-deficient mice, we compared hematoxylin and eosin (H&E) stained sections from 3- to 6-month-old control and Arx-deficient mice. Although there were no gross morphological differences in the liver between control and Arx-deficient animals (Fig. 4B), glycogen granules were clearly larger and more plentiful in Arx-deficient mice, suggesting that lack of glucagon results in accumulation of glycogen in the liver (Fig. 4B). Periodic acid-Schiff (PAS) staining, which labels glycogen granules in the liver, further confirmed the presence of increased glycogen in the Arx-deficient liver (Fig. 4D). Furthermore, hepatic glycogen content in the Arx-deficient mice was also significantly increased (Fig. 4C). These data suggest that in the absence of glucagon production, enhanced glycogen synthesis or suppressed hepatic glycogenolysis occur in the liver of Arx-deficient mice, resulting in excess glycogen granules.
mRNA levels of major gluconeogenic genes are down-regulated in fasted Arx-deficient mice

To investigate whether hepatic gluconeogenesis is impaired in Arx-deficient mice, we next examined the expression of genes involved during this process by quantitative PCR analysis. We found that mRNA levels of phosphoenolpyruvate carboxykinase (PEPCK) (70% reduction) tyrosine amino transferase (76% reduction), and glucose 6 phosphotase (60% reduction) were significantly down-regulated in the Arx-deficient liver after a 16-h fast (Fig. 4E). Levels of peroxisome proliferators-activated receptor-γ coactivator 1α mRNA were also reduced, although not statistically significant (Fig. 4E). Furthermore, the level of PEPCK protein was reduced by 60% in Arx-deficient mice (Fig. 4, F and G). Thus, as expected, expression of genes involved in hepatic gluconeogenesis was impaired in the absence of glucagon. Interestingly, levels of phospho-cAMP response element-binding protein (P-CREB) were unchanged in the Arx-deficient liver, in agreement with recent findings that glucagon action is mediated by the CREB coactivator CREB-regulated transcription coactivator 2 (CRTC2) and is independent of CREB phosphorylation (28, 32–34). These results suggest that during a 16-h fast, glucagon is required to fully activate gluconeogenic genes in the liver to promote glucose production. To determine whether other fight-or-flight hormones, i.e. catecholamines, were increased in the Arx-defi-

FIG. 4. Arx-deficient mice exhibit increased fat and glycogen in the liver. A, GIR HGP, and Rd were measured during the clamp from 2-month-old male control and Arx-deficient mice. Values are mean ± SEM from five mice per group. *, P < 0.05 between genotypes. B, Liver histology of 3- to 6-month-old male control and Arx-deficient paraffin-embedded liver sections stained with H&E or (D) Periodic acid-Schiff (PAS) (magnification, ×40). An enlarged portion of the image is provided in the box. C, Glycogen levels were measured in liver taken from mice at 3- to 6-months of age after a 16-h fast. Values are mean ± SEM from three mice per group. *, P < 0.05 between genotypes. E, Real-time PCR analysis measured relative mRNA levels of genes involved in gluconeogenesis from 3- to 6-month-old male after a 16-h fast. Tat, Tyrosine amino transferase. Values are mean ± SEM from four mice per group. *, P < 0.05 between genotypes. F, Western blot analysis using liver samples of 3- to 6-month-old male control and Arx-deficient animals to detect PEPCK (63 kDa), P-CREB (43 kDa), and tubulin (50 kDa). G, Mean intensity of PEPCK and P-CREB normalized to tubulin as quantified from Western blot analysis.
cient animals to maintain glucose homeostasis, we next measured both epinephrine and norepinephrine and detected an increase, although not statistically significant, in Arx-deficient mice (Fig. 5C).

**Arx-deficient mice have higher fat content in the liver**

In addition to the increased number of glycogen granules in the Arx-deficient liver (Fig. 4, B and D), we noticed large vacuoles in the hepatocytes of Arx-deficient mice (Fig. 5A). We confirmed the presence of excess lipid droplets in the liver sections of the Arx-deficient mice by Oil red O staining (Fig. 5A). At the same time, plasma triglyceride and ketone levels were both increased in Arx-deficient mice, although not statistically significant (Fig. 5B).

**STZ-treated Arx-deficient mice exhibit improved blood glucose**

Excess levels of circulating glucagon lead to overproduction of hepatic glucose, which in turn contributes to the severe hyperglycemia observed in diabetic patients. To investigate whether endogenous hyperglycemia can be eliminated in our Arx-deficient mice, devoid of glucagon-producing α-cells, we next treated both control and Arx-deficient mice with the β-cell toxin streptozotocin (STZ) (35). After treating mice with STZ, we measured glucose levels every other day for 24 d in both control and Arx-deficient mice. Monitoring of the blood glucose levels in control mice revealed that the β-cells were efficiently ablated by the STZ treatment, because these mice began to display elevated glucose levels as early as 1 wk after treatment onset. The control mice, within 2 wk of STZ treatment, were severely diabetic with blood glucose levels more than 350 mg/dl (Fig. 5D). Unlike controls, blood glucose levels of STZ-treated Arx-deficient mice remained between 100 and 180 mg/dl at all time points examined (Fig. 5D). These data suggest that the diabetes resulting from β-cell deficiency is glucagon-dependent in mice, just as had been seen in dog and man (6, 10, 13, 14).

**Discussion**

For the past 35 yr, the action of glucagon has been investigated using potent glucagon suppressors and by targeting the GR via genetic ablation, ASOs, and neutralizing antibodies (6, 10, 13, 14, 16, 21, 25). Recently, glucagon null (Gcg<sup>−/−</sup>) animals have been reported that display improved glycemic control and hyperplasia of islet α-cells, but these mice do not allow to separate the effects of GLP1 and GLP2, also produced from the glucagon gene, from those of glucagon itself (25). In this study, we have derived mice lacking pancreatic α-cells to address the requirement for glucagon in the normal life of an animal and regulation of glucose homeostasis. It has previously been shown that Arx null mice exhibit perinatal lethality, which was attributed to anomalous endocrine pancreatic function, because Arx null mice show severe hypoglycemia 2 d after birth (26, 28). To our surprise, despite the complete loss of glucagon-producing cells in the pancreas and no detectable circulating blood glucagon, Arx-deficient animals are born alive and have a normal life span, which is similar to what was reported in the
Gcggfp/gfp animals (25). The findings presented above suggest that the cause of death is independent of the loss of glucagon-producing cells in the Arx null animals. In addition, reports from others have suggested that Arx null mice likely die from either seizures and/or olfactory bulb defects, caused by Arx deficiency in the brain (29, 31). Furthermore, we have detected an increase of catecholamines levels in the Arx-deficient animals, indicating that other stress hormones are likely to compensate for the loss of glucagon in Arx-deficient mice allowing normal growth and development.

Although we detected a modest increase in β-cell mass in the Arx-deficient mice, we did not observe a significant increase in circulating blood insulin levels after a 16-h fast. This would suggest that the slightly reduced levels of basal glucose seen in the Arx-deficient animals are caused by the absence of glucagon and are not due to an increase in circulating insulin levels. Furthermore, we detected an improvement of postprandial glucose homeostasis in the Arx-deficient mice. However, this change was not evident until the animals reached 3–6 months of age. Although it is not clear why we did not observe a difference in the GTT in 6-wk-old Arx-deficient and control animals, the likely explanation is the involvement of other hormones in these young animals. Interestingly, although not statistically significant, we did notice a trend of reduced insulin secretion during the first 30 min of a GTT in the Arx-deficient animals. However, the exact cause of this reduction remained to be further investigated, although this is likely the consequence of lower glucose levels in our mutant mice. Lastly, we have also observed an increase in the number of somatostatin-producing and pancreatic polypeptide-producing cells and their distribution throughout the medulla of the islet. The increase of δ-cells in our Arx-deficient animals is reminiscent of what was reported in the Arx null animals due to the critical role of Arx in repressing δ-cell lineage in the developing pancreas (26, 28). Together with the increased levels of plasma catecholamines and the increase number of somatostatin-producing and pancreatic polypeptide-producing cells and their distribution throughout the medulla of the islet. The increase of δ-cells in our Arx-deficient animals is reminiscent of what was reported in the Arx null animals due to the critical role of Arx in repressing δ-cell lineage in the developing pancreas (26, 28). Together with the increased levels of plasma catecholamines and the increase number of somatostatin-producing and pancreatic polypeptide-producing cells and their distribution throughout the medulla of the islet. The increase of δ-cells in our Arx-deficient animals is reminiscent of what was reported in the Arx null animals due to the critical role of Arx in repressing δ-cell lineage in the developing pancreas (26, 28)

It has recently been shown that PEPCK, one of the key enzymes regulating gluconeogenesis, alone only has a weak influence during this process (37, 38). In our Arx-deficient animals, we found reduced expression of genes regulating hepatic gluconeogenesis, including a 60% reduction in the PEPCK protein levels. Burgess et al. (37) showed that the protein levels of PEPCK have to be reduced by 80% or more before any effects are observed. However, the lack of glucagon in our mice impacts the expression of several gluconeogenic genes other than PEPCK, which likely collectively affect glucose production.

It has been demonstrated by several studies that upon glucagon stimulation, CRTC2, a cofactor of CREB, gets dephosphorylated, then translocates into the nucleus, and forms transcriptional complexes with P-CREB to activate gluconeogenesis in the liver (32–34, 39). The sustained levels of P-CREB, in our Arx-deficient mice, further strengthens the importance of glucagon for CRTC2 recruitment in initiating hepatic gluconeogenesis, which is independent of CREB phosphorylation.

Although the Arx-deficient, Gcggfp/gfp, Gcgr−/−, and GR-ASO-treated mice all exhibit lower blood glucose and improved glucose tolerance, Gcggfp/gfp, Gcgr−/−, and GR-ASO mice have additional phenotypes not present in the Arx-deficient mice. For instance, Gcgr−/− and GR-ASO-treated mice display supraphysiological levels of glucagon, and α-cell hyperplasia was observed in the Gcggfp/gfp and Gcgr−/− mice (16, 19, 20, 25). Similar to our Arx-deficient mice, mRNA levels of several gluconeogenic and glycogenolytic enzymes were decreased in the GR-ASO-treated mice (19). Furthermore, mice deficient in prohormone convertase 2, which lack mature α-cells, also display lower blood glucose levels, α-cell hyperplasia, and improved glucose tolerance similar to the Gcgr−/− mice (21, 40). However, interpretation of this model is complicated by the fact that prohormone convertase 2 is also required for processing of other endocrine hormones (40, 41). Finally, it was recently reported that ectopic expression of paired box gene 4 in the mouse pancreas results in overproduction of insulin-secreting β-cells at the expense of α-cells, and these transgenic mice display improved glucose tolerance and increase in plasma insulin levels at 3 wk of age (42).

During a prolonged fast, the body activates both HGP and ketogenesis. Ketogenesis is fueled by an increase in free fatty acid (FFA) levels resulting from lipolysis in adipose tissue, which is no longer suppressed by insulin. However, because FFA levels were not elevated in the Arx-deficient mice, one of the likely explanations for the excess glycogen and fat in the liver of Arx-deficient mice is lack of glucagon, which results in enhanced hepatic
glucose entry and overaccumulation of carbon to be stored as glycogen and fat. Furthermore, the relative hypoglycemia in our Arx-deficient mice could potentially cause a small increase in the rate of lipolysis, thus compensating for the loss of glucagon stimulation, maintaining FFA levels at normal levels. At the same time, the rate of ketogenesis may have had a small increase caused by the slight hypoglycemia, thus increasing the conversion of FFA to ketone bodies. Elevated levels of epinephrine and norepinephrine would also stimulate this. In addition, limitation of the assay, mixed background of the animals, and the number of animals used may have prevented us from observing a sustained increase of FFA levels.

In this study, we have derived and analyzed one of the first mouse models with a complete ablation of α-cells in the adult pancreas to directly study the impact of glucagon during basal and postprandial glucose homeostasis. We have demonstrated that although glucagon is critical during a prolonged fast and postprandial glucose homeostasis, it is not essential for the health of an animal. Our results have extended the role of glucagon in regulating glucose homeostasis and provided further evidence that glucagon suppression or elimination can limit the consequences of insulin deficiency in diabetes.

Materials and Methods

Animals and breeding strategy

The derivation of the ArxΔloxP and Pdx1-Cre transgenic line has been reported previously (29, 30). All mice were kept on a mixed background. ArxΔloxP and ArxΔloxP;Pdx1-Cre mice were mated to generate ArxΔloxP;Pdx1-Cre or ArxΔloxP;Pdx1-Cre mutant mice. Littermate heterozygous mice were indistinguishable from control animals. The Children’s Hospital of Philadelphia’s Institutional Animal Care and Use Committee approved all animal experiments.

GTTs and analytical procedures

Overnight fasted animals were injected ip with 2 g of glucose (Sigma, St. Louis, MO) per kilogram of body weight. Blood glucose values were monitored at 0, 15, 30, 60, 90, and 120 min after injection using an automatic glucometer (One Touch Ultra; LifeScan, Milpitas, CA). For hormone/lipid measurement, after injection using an automatic glucometer (One Touch Ultra; LifeScan, Milpitas, CA). After the color reaction, sections were dehydrated and mounted with histomount (Invitrogen). Sections were counterstained for H&E before dehydration to help visualize the tissue morphology. Slides were then given to the Children’s Hospital of Philadelphia pathology core for slide scanning. PAS staining on deparaffinized liver sections (6 μm) was done by placing the slides sequentially in 0.5% periodic acid solution, Schiff reagent, hematoxylin, 0.5% acid alcohol, and saturated lithium carbonate. Oil red O staining was performed using frozen liver tissue.

Quantitative PCR analysis

Tissues were homogenized in TRIzol reagent. The RNA was recovered by chloroform extraction and then purified using RNeasy mini kit (QIAGEN, Germantown, MD). RNA was reverse-transcribed using 0.5 μg Oligo (dT) primer, Superscript II Reverse Transcriptase, and accompanying reagents (Invitrogen). Real-time PCR reactions were set up using the Brilliant SYBR Green PCR Master Mix (Stratagene, La Jolla, CA). All reactions were performed in triplicate with reference dye normalization and median cycling threshold values used for analysis. Primer sequences are available upon request.

α-, β-, And δ-cell mass

Pancreata of male 3- to 6-month-old control and mutant mice was removed, weighed, fixed in 4% paraformaldehyde overnight at 4 °C, and embedded in paraffin. Sections (7 μm) with maximum footprint were used for insulin, glucagon, and somatostatin immunostaining. Images were taken under ×4 magnification, and pancreatic tissue positive for insulin, glucagon, and somatostatin staining were measured using Aperio software. Cell mass was obtained by measuring the fraction of strong positive pixels to total tissue area and multiplying by the pancreatic weight. Three sections were used per pancreas with three control and mutant pancreata analyzed.

Hyperinsulinemic-euglycemic clamp

An indwelling catheter was inserted in the right internal jugular vein under sodium pentobarbital anesthesia and extended to the right atrium. After a 6-h fast, a 120-min hyperinsulinemic-euglycemic clamp was conducted with a continuous infusion of human insulin (Humulin; Novo Nordisk, Princeton, NJ) at a rate of 2.5 mU/kg/min to raise plasma insulin within a physiological range. Tail blood samples (20 μl) were collected at 10- to 20-min intervals for the immediate measurement of manufacture’s instructions. Specifically, EDTA (final concentration, 1 mM) and sodium metabisulfite (final concentration, 4 mM) were added to the whole blood to prevent degradation.

Histology

Tissues were fixed in 4% paraformaldehyde, then embedded in paraffin. Slides (7-μm sections) were cut, then deparaffinized. Primary antibodies were glucagon (1:3000; Milliopore, Billerica, MA), insulin (1:1000; Millipore), PP (1:50; Invitrogen, Carlsbad, CA), and somatostatin (1:3000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The sections were then incubated with either a fluorescent secondary antibody (1:600) or a biotinylated secondary antibody (1:200). The biotinylated antibody was followed by incubation with the ABC elite reagent and color reaction using the diaminobenzidine substrate kit according to the recommendation from the manufacturer (Vector Laboratories, Burlingame, CA). After the color reaction, sections were dehydrated and mounted with histomount (Invitrogen). Sections were counterstained for H&E before dehydration to help visualize the tissue morphology. Slides were then given to the Children’s Hospital of Philadelphia pathology core for slide scanning. PAS staining on deparaffinized liver sections (6 μm) was done by placing the slides sequentially in 0.5% periodic acid solution, Schiff reagent, hematoxylin, 0.5% acid alcohol, and saturated lithium carbonate. Oil red O staining was performed using frozen liver tissue.

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plasma glucose concentration, and 20% glucose was infused at variable rates to maintain plasma glucose at basal concentrations. Blood glucose was sustained at 60–70 mg/dl, and glucose levels were checked every 10 min during the procedure to ensure a steady state. Insulin-stimulated whole-body glucose flux was estimated using a prime-continuous infusion of HPLC-purified [3-3H]glucose (10 μCi bolus, 0.1 μCi/min; PerkinElmer Life and Analytical Sciences, Boston, MA) throughout the clamps. To estimate insulin-stimulated glucose transport activity in individual tissues, 2-deoxy-d-[1-14C]glucose (2-[14C]DG; PerkinElmer Life and Analytical Sciences) was administered as a bolus (10 μCi) at 45 min before the end of clamps. Blood samples (20 μl) were taken at 77, 80, 85, 90, 100, 110, and 120 min after the start of clamps for the determination of plasma [3H]glucose, [3-3H]glucose, [3H]2O2, and 2-[14C]DG concentrations. Additional blood samples (10 μl) were collected before the start and at the end of clamps for measurement of plasma insulin concentrations. All infusions were done using a Programmable Syringe Pump BS-8000 (Braintree Scientific, Inc., Braintree, MA). The rates of basal glucose turnover and whole-body glucose uptake are measured as the ratio of [3H] GIR (dpm) to the specific activity of plasma glucose. HGP during clamp is measured by subtracting the GIR from the whole-body glucose uptake (Rd).

**STZ treatment**

Diabetes was induced by ip injection of STZ (50 mg/kg body weight; Sigma-Aldrich, St. Louis, MO) for five consecutive days after a 4-h fast. Blood glucose measurements for d 1–5 were performed after a 4-h fast. Blood glucose levels from d 6 onwards were performed after a 1-h fast.

**Hepatic glycogen content**

One hundred milligrams of frozen liver samples were homogenized in 6% perchloric acid and then mixed with 100 μg amyloglucosidase (Sigma) for 2 h. Glucose was measured using the Amplex Red kit (Invitrogen).

**Western blot analysis**

Protein from small liver sections was extracted by repeatedly freezing/thawing the sample, then homogenizing the remaining tissue followed by a final sonication step; 20 μg/lane of the extract was separated by 10% SDS-PAGE and subsequently transferred to a nitrocellulose membrane. This assay was performed using mouse monoclonal tubulin antibody (Sigma), rabbit polyclonal P-CREB antibody (Cell Signaling, Danvers, MA), or rabbit polyclonal PEPCk antibody (Cayman Chemical Co., Ann Arbor, MI). Analysis of the resulting blot was performed using mean intensity of P-CREB or PEPCk normalized to tubulin.

**Statistical analysis**

All error bars represent SEM, calculated by dividing the SD of each group by the square root of n. A t test was performed to measure significance.

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**References**

5. Consoli A 1992 Role of liver in pathophysiology of NIDDM. Diabetes Care 15:430–441
10. Liljenquist JE, Bloomgarden ZT, Cherrington AD, Perry JM, Rabin D 1979 Possible mechanism by which somatostatin-induced glucagon suppression improves glucose tolerance during insulinopenia in man. Diabetologia 17:139–143


