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Enhanced β-cell mass without increased proliferation following chronic mild glucose infusion

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Jetton TL, Everill B, Lausier J, Roskens V, Habibovic A, LaRock K, Gokin A, Peshavaria M, Leahy JL. Enhanced β-cell mass without increased proliferation following chronic mild glucose infusion. *Am J Physiol Endocrinol Metab* 294: E679–E687, 2008. First published January 29, 2008; doi:10.1152/ajpendo.00569.2007.— The physiological mechanisms underlying pancreatic β-cell mass (BCM) homeostasis are complex and not fully resolved. Here we examined the factors contributing to the increased BCM following a mild glucose infusion (GI) whereby normoglycemia was maintained through 96 h. We used morphometric and immunochemical methods to investigate enhanced β-cell growth and survival in Sprague-Dawley rats. BCM was elevated >2.5-fold over saline-infused control rats by 48 h and increased modestly thereafter. Unexpectedly, increases in β-cell proliferation were not observed at any time point through 4 days. Instead, enhanced numbers of insulin$^+$ cell clusters and small islets (400–12,000 μm^2; ~23- to 124-μm diameter), mostly scattered among the acini, were observed in the GI rats by 48 h despite no difference in the numbers of medium to large islets. We previously showed that increased β-cell growth in rodent models of insulin resistance and pancreatic regeneration involves increased activated Akt/PKB, a key β-cell signaling intermediate, in both islets and endocrine cell clusters. GI in normal rats also leads to increased Akt activation in islet β-cells, as well as in insulin$^+$ and insulin$^-$ cells in the common duct epithelium and endocrine clusters. This correlated with strong Pdx1 expression in these same cells. These results suggest that mechanisms other than proliferation underlie the rapid β-cell growth response following a mild GI in the normal rat and involve Akt-regulated enhanced β-cell survival potential and neogenesis from epithelial precursors.

β-cell growth; β-cell adaptation; insulin signaling

β-CELLS exhibit a substantial capacity for growth in response to a range of physiological and pathophysiological factors (4, 12, 44), although the homeostatic mechanisms that regulate normal β-cell growth and development in the adult are poorly understood. The precise contributions of proliferation of existing β-cells vs. new cell development (neogenesis) from pancreatic epithelial precursors, apoptosis, and clearance, and of hypertrophy of β-cells, are also unknown and are likely dependent on the model under study. However, proliferation is widely considered to play a predominant role (1, 8, 9, 22, 37).

Although the molecular bases of β-cell growth and regeneration remain mostly unresolved, recent studies suggest that the insulin-signaling pathway may play key roles in compensatory β-cell expansion due to increased insulin requirements (10, 15, 28). Principal intermediates in this cascade include putative regulators of the gene encoding pancreatic-duodenal homeobox-1 (Pdx1) and include insulin receptor substrate-2 (Irs-2), protein kinase B (PKB/Akt), and the forkhead transcription factor Foxo1 and have been implicated as having roles in the growth, survival, and differentiation of β-cells (20, 21, 23, 35). Most recently, β-cell glucose signaling through glucokinase has also been shown to impact β-cell mass (BCM) (47).

We previously (19) investigated β-cell regeneration following partial pancreatectomy in normal insulin-sensitive Sprague-Dawley rats. We observed that the growth factor-activated Akt/PKB, implicated in regulating β-cell proliferation, cell size, differentiation (neogenesis), and survival (reviewed in Refs. 5, 10, 14), was activated in a subset of Irs-2$^+$/Pdx1$^+$ epithelial cells in the common pancreatic duct epithelium. This suggested a possible regulatory mechanism whereby enhanced signaling through Irs-2 and Akt mediates duct-derived β-cell differentiation during islet regeneration. We have made similar observations (17) of activated Akt/Pdx1$^+$ cells among small ducts and acini in postweanling Zucker fatty rats during a period of increased islet neogenesis. Whether these proteins may play a general role in neogenesis in other models of BCM compensation is not known.

Genetically normal rodent models of β-cell growth and regeneration have typically relied on pancreatic manipulations (i.e., toxins, trauma) that evoke biological responses that may not reflect physiological β-cell compensation. The most widely studied factor affecting β-cell growth and function is glucose, which is known to have marked effects on both Irs-2 expression and Akt activation (31, 40, 46), in turn promoting β-cell survival (2, 46), at least partially due to its negative regulation of Foxo1 activity (33). Here we have used a more physiologically relevant model of β-cell growth, a mild glucose infusion (GI) to examine Akt signaling in normal rats. These animals maintain virtual normoglycemia by, initially, increased insulin secretion that is later followed by enhanced β-cell growth likely mediated by Akt.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (200 g) were obtained from Harlan and housed in the University of Vermont (UVM) Animal Facility at least 5 days before being used. The guidelines set forth by the UVM Institutional Animal Care and Use Committee (IACUC) were strictly adhered to for these studies, and all procedures were reviewed and approved by the IACUC.

Glucose infusion procedure. The basic protocol for chronic intravenous GIs has been well documented (26, 27). After induction of general anesthesia, the external jugular vein was cannulated with Silastic tubing after exposure and then tunneled under the skin, coursing dorsally and exiting between the scapulae as detailed previously (26).

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Once body weights had risen to presurgery values (usually 2–3 days) the infusion commenced with saline (0.45% NaCl) or 20% glucose (wt/vol) diluted in sterile saline. Rats were infused for 1–4 days with a syringe pump at an infusion rate of 2 ml/h (dosage = 1.8 $g \cdot kg^{-1} \cdot h^{-1}$). Control groups consisted of saline control, stress control (harnessed and catheterized but no infusion), and unmanipulated rats. After the infusion period, rats were euthanized and the pancreata were processed for microscopic or biochemical evaluation. A minimum of five rats per group were used in each morphometric assay, whereas four rats per group were used in the phospho-Akt localization experiments (see below).

Plasma glucose and insulin measurements. Daily fed glucose levels were determined in whole blood with a glucose meter (Freestyle, TheraSense) at 9:00 AM, and the plasma was subsequently analyzed for insulin with a rat insulin enzyme immunoassay kit (Alpco Diagnostics).

Tissue processing. Pancreata were rapidly excised, cleared of fat and lymph nodes, blotted, and weighed before immersion fixing overnight in 4.0% paraformaldehyde in 0.1 mmol/l phosphate buffer at 4°C. After being washed in several changes of PBS, tissues were dehydrated and embedded in paraffin.

For the detection of phospho-Akt, pancreata were rapidly excised (<20 s), cut into smaller pieces, and then paraformaldehyde fixed for 1.5 h. They were then washed, equilibrated in 30% sucrose/phosphate buffer, embedded in OCT medium, sectioned at 5 μm in a cryostat, and immediately stained as detailed below.

BCM measurements. The proportion of islet β-cell surface area vs. surface area of the whole pancreas was determined planimetrically. Five-micrometer paraffin sections throughout the entire pancreas were mounted as ribbons on microscope slides to facilitate section counting. Three sections (≥300 μm apart) from the broadest pancreatic sections were analyzed for BCM measurement (n = 5–10 for each group and time point) after insulin immunohistochemistry and hematoxylin counterstaining as detailed previously (20). We found that this sampling strategy permits detection of a 20% difference in β-cell surface area between two animal groups.

Analysis of β-cell clusters/small islets. To obtain information on potential changes in endocrine cell cluster/islet size dynamics, the relative surface area and number of β-cell clusters and very small islets (>400 μm^2, or >23-μm average diameter) were tallied for each animal, using the same sections used for BCM measurements. β-Cell cluster size was categorized into one of six classes: 400–599, 600–3,999, 4,000–7,999, 8,000–11,999, 12,000–15,999 μm^2 (up to ~143-μm diameter). β-Cell clusters (400–599 μm^2, or ~23- to 28-μm diameter) and small islets (600–3,999 μm^2, or ~29- to 71-μm diameter) may be newly formed because of their increased prevalence after GI, and thus potentially neogenic. For measuring β-cell clusters <400 μm^2 we microscopically imaged 10 × 0.3-mm^2 fields for each animal and counted clusters of 1–4 cells. Image files were analyzed with NIH Image J as indicated above and entered into Microsoft Excel for statistical analyses. Significance was determined by Student's t-test, where $P < 0.05$ was considered significant.

β-Cell, duct, and acinar proliferation. At least two slides per adult pancreas were immunohistochemically stained for the proliferation marker Ki-67 and insulin as previously detailed (17). The number of Ki-67^+ nuclei per 1,000–1,500 islet β-cells or per 300 common duct cells was counted for each animal. Acinar cell proliferation was determined in the GI and saline groups by determining the relative number of Ki-67^+ acinar cells in six 400× fields per rat at the 24-, 36-, and 48-h time points.

β-Cell size measurements. Islet β-cell size was determined by double immunofluorescence labeling and confocal imaging of insulin and GLUT2 (Alpha Diagnostics) to mark β-cell surface boundaries. A nuclear counterstain was also used in an effort to maximize the number of cells optically sectioned through the middle of the cell. Pseudocolored images were merged with Adobe Photoshop, and β-cell boundaries were outlined, the surface area measured (μm^2), and β-cell volumes estimated (NIH Image J).

Multiple-labeling immunofluorescence. Pancreas sections were incubated overnight at 4°C in antibody mixtures of sheep anti-amylase (Biogenesis), guinea pig anti-insulin or C-peptide (Linco), rabbit or guinea pig anti-glucagon (Linco), or sheep anti-somatostatin (Cortex). Staining for the glucose sensor proteins glucokinase and GLUT2 was accomplished with appropriate antibodies (sheep anti-glucokinase; gift of Mark Magnuson, Vanderbilt University and Alpha Diagnostics, respectively). For staining of Pdx1, a rabbit antiserum was used (from Dr. Chris Wright, Vanderbilt University). PTF1 (P48) antibody was from Dr. Ray MacDonald (University of Texas Southwestern). Pax6 antiserum was purchased from Chemicon. A cyclin D2 antibody was purchased from Santa Cruz Biotechnology. A polyclonal antiserum to Irs-2 was obtained from Upstate. Labeling of phospho-Akt required rapidly fixed and processed frozen sections that were stained with antiserum specific for activated Akt, using a mouse monoclonal anti-phospho-S^{473}Akt (Cell Signaling) or a sheep anti-phospho-S^{473}Akt (Upstate).

Immunoblot analyses. Purified islets were lysed in ice-cold buffer consisting of 50 mmol/l HEPES (pH 7.5), 1% (vol/vol) Nonidet P-40, 2 mmol/l sodium orthovanadate, 100 mmol/l sodium fluoride, 10 mmol/l sodium pyrophosphate, 4 mmol/l EDTA, 1 mmol/l PMSF, and protease inhibitors. The lysate was sonicated and spun, and the protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce). Fifty milligrams of protein per lane was separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and then probed overnight at 4°C with the relevant antibodies followed by enhanced chemiluminescence detection. Antibodies used to probe for total protein and activated (phosphorylated) forms of Akt and BAD were obtained from Cell Signaling. Polyclonal antibody to Bcl-2 and Akt1/2 was obtained from Santa Cruz Biotechnology. Total Akt1/2 and actin served as loading controls for phosphorylated Akt and Bcl-2, respectively. Dilutions for the antibodies used for immunoblotting were as follows: phosphorylated Akt 1:200, total Akt1/2 1:100, Bcl-2 1:500, actin 1:1,000.

Analysis of β-cell apoptosis. For determination of the apoptotic β-cells, a modified terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining protocol was used (18). Pancreas or cultured islet sections were immunofluorescently stained for insulin (CY2) and counterstained with 0.5 μg/ml Hoechst. Adjacent slides were stained with rabbit-anti-cleaved caspase 3 (R&D Systems). TUNEL labeling of short-term cultured islets was accomplished by isolating islets from GI rats by standard methods and incubating in RPMI 1640 + 10% FBS at 37°C for 1 h and 24 h before fixing and processing for frozen sections.

Confocal imaging. Sections were imaged with a Zeiss 510 Meta confocal microscope (UVM Microscopy Imaging Facility). Images were acquired with the Zeiss software and formatted on a Macintosh G5 running Adobe Photoshop.

RESULTS

We first examined the parameters influencing compensatory growth of β-cells in 20% (wt/vol) GI rats compared with saline-infused, stress control, and unmanipulated control rats. While a 35% and 50% glucose instillation caused a pronounced hyperglycemia within 24 h (Ref. 3; data not shown), a 20% GI resulted only in a subtle increase in blood glucose levels at 24–48 h (Fig. 1*A*). Plasma insulin concentrations were significantly increased in the GI group at 24 h after the start of the infusion but unexpectedly returned to normal levels by 48 h (Fig. 1*B*). By contrast, rats infused with 35% (n = 11) and 50% (n = 5) glucose exhibited 9-fold and >12-fold increases in insulin concentrations at both 24-h and 48-h time points, respectively ($P < 0.0001$ for all; not shown).

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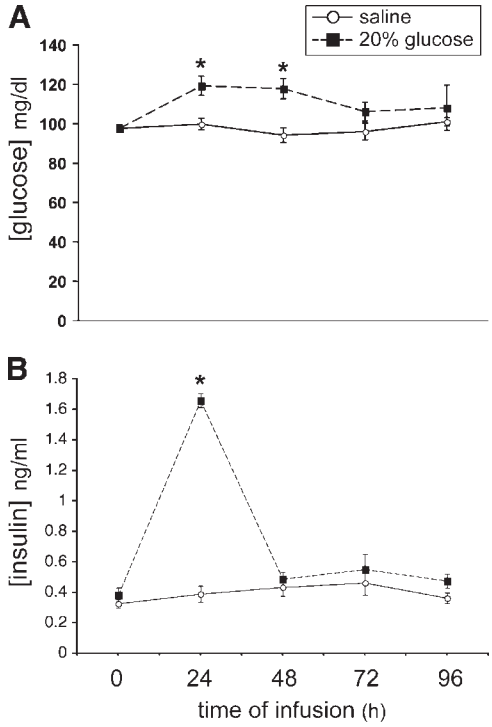

Fig. 1. Blood glucose (*A*) and plasma insulin (*B*) profiles of glucose-infused rats. *A*: virtual normoglycemia prevailed throughout the infusion period in 20% glucose infusion (GI) rats, although a small elevation in blood glucose concentration was observed at 24 and 48 h. *B*: plasma insulin values were ~4-fold increased at 24 h in the GI group but were normalized by 48 h onward; $n = 12$ (saline control) or 16 (20% GI). *$P < 0.001$.

BCM is rapidly increased in GI rats. To examine the effects of glucose on β-cell growth, BCM was measured in 20% GI rats and control rats (Fig. 2*A*). Whereas saline-infused control rats exhibited BCM values of ~4 mg, GI rats displayed an >2.5-fold increase in BCM at 48 h of infusion and increased by >3-fold at 96 h of infusion (Fig. 2*A*). Stress control rats and unmanipulated rats displayed morphometric measurements that were nearly identical to those of the saline-infused rats (not shown). No significant differences in pancreatic weight were observed within the 24- to 48-h interval in the GI group. Since the estimation of BCM is a product of both pancreas weight and relative β-cell surface area, we compared β-cell surface area at 24-, 36-, and 48-h duration (Fig. 2*B*). We detected a significant increase in β-cell surface area only at the 48-h time point (>2.5-fold increase, $P < 0.001$). Therefore, substantial β-cell growth occurs in the interval between 36 and 48 h of infusion in the GI rats. This correlates well with an ~40% increase in pancreatic insulin content under these conditions (26).

β-Cell proliferation is not increased in GI rats. Since β-cell proliferation has been recently described as the principal or sole means of β-cell expansion in the adult (9), we measured proliferation in GI rats with the marker Ki-67. Unexpectedly, a negative trend in β-cell proliferation frequency was observed in both groups after 24 h but remained suppressed in the GI group at 48 and 96 h (Fig. 3*A*). β-Cell nuclear cyclin D2 immunoreactivity, previously shown to increase correlating with heightened proliferation following GI in mice (1), was not different between the groups at 48 h of GI in rats (Fig. 3*C*). Because several models of β-cell growth and regeneration involve transient pancreatic duct proliferation (13, 43, 45) that may give rise to new islet cells, we also quantified proliferation in the large common pancreatic duct of each rat. No significant differences were detected in the GI group compared with the control rats except at 48 h, when duct proliferation was reduced >50% (Fig. 3*B*). Acinar cell proliferation was also measured in GI rats; no differences were observed among the groups at any time point.

β-Cell cluster and small islet prevalence are increased in GI rats. We next quantified the relative number and size of islets. We first analyzed "normal"-size islets (≥144-μm diameter), the principal size population amenable to standard isolation procedures. We found no significant differences in the number of these normal-size islets (~8 islets/50-mm^2 pancreas fields) or size (~185-μm mean diameter) among the groups at 24, 36, or 48 h of infusion. However, analyses of β-cell clusters and small islets of from 23- to 123-μm diameter (400–11,999 μm^2) revealed significant increases in the GI group compared with the control rats at 48 h (Fig. 4*B*). Also, a trend for increased prevalence was also observed in the next larger size class, 124–143 mm in diameter. Similar to the latent growth trends observed in the β-cell surface area measurements (Fig. 2*B*), no changes in the prevalence of β-cell clusters/small islets were observed between the groups at 24 (not shown) or 36 (Fig. 4*A*) h of infusion. However, at 48 h, an ~1.8-fold increase in the occurrence of β-cell clusters/small islets in the

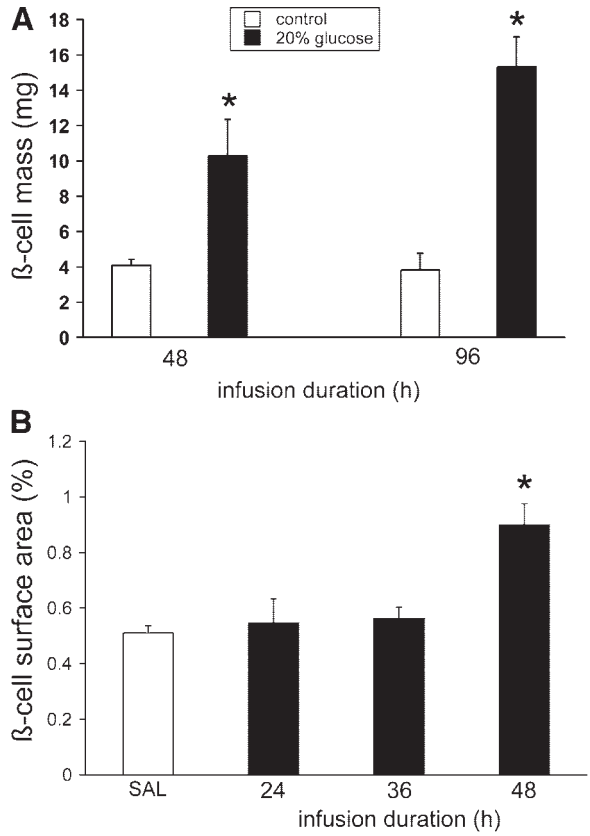

Fig. 2. β-Cell mass increases in glucose-infused rats. *A*: β-cell mass measurements reveal 2- to 3-fold increases in the GI group (*$P < 0.01$). *B*: β-cell surface area comparison in the 20% GI group between the 24-, 36-, and 48-h time points. Although no differences in pancreatic weights were observed between the GI groups, an increase in β-cell area was detected only after 36 h in the GI group (*$P < 0.001$); $n = 5$ for each group. SAL, saline.

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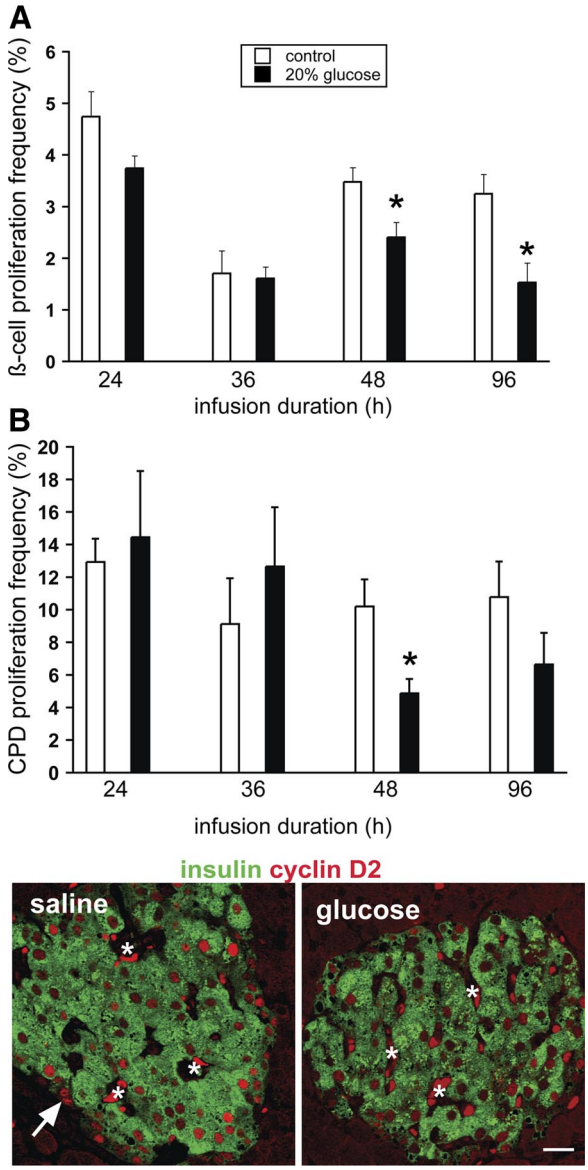

Fig. 3. β-Cell and ductal proliferation in 20% glucose-infused rats. *A*: no increases in β-cell proliferation were observed. Proliferation frequency actually decreased at 48 and 96 h (*$P < 0.05$). *B*: in a similar pattern, common pancreatic duct (CPD) proliferation frequency was reduced at 48 h (*$P < 0.05$); $n = 5$ for each group. *C*: representative islet fields showing nuclear cyclin D2 immunostaining (red) and insulin (green). Note that the prevalence and staining pattern of cyclin D2 is comparable in β-cells of both islets, suggesting similar replicative potential. Non-β-cells also exhibit cyclin D2 immunoreactivity (arrow). Bright red bodies mark erythrocytes in capillaries (*); $n = 3$ for each group. Scale bar, 10 μm.

smaller size classes (23- to 28-μm and 29- to 71-μm diameter) was observed as well as ~1.6-fold increases in the next largest size classes (72- to 100-μm and 101- to 123-μm diameter). Hence, the greater than twofold increase in BCM in the GI rats might be the result of an abrupt surge of both neogenesis and small islet growth in the interval between 36 and 48 h of infusion, with no changes in the average size or number of normal-size islets (≥144 μm).

The generation of new islets could occur through the proliferation of single, scattered endocrine cells, or differentiation from progenitors (classical neogenesis), and their aggregation. To begin to address these possibilities, we microscopically analyzed single β-cells and small clusters up to four cells in cross section. On the basis of serial section and confocal analyses, we confirmed that these clusters were distinct bodies. In the 48-h GI group, the smallest of the β-cell clusters, single cells and doublets, were increased by >50% (Fig. 5*A*, $P < 0.05$). Similar to a previous study (32), most of these appeared to be embedded within the acinar compartment. Ki-67$^+$ β-cells of these clusters were only rarely encountered, and singlet/doublet insulin$^+$ cells were never found to be Ki-67$^+$ (Fig. 5, *B*, *D–G*). However, individual β-cells or closely opposed doublets routinely appeared to have daughter nuclei and/or, perhaps, were binucleate (Fig. 5, *B* and *D*), a characteristic of acinar cells (36). Resembling islets, endocrine cell clusters were composed of centrally oriented β-cells with peripheral non-β-cells (Fig. 5, *C* and *D*). We could not detect any qualitative differences in clusters between the control and GI groups, with the exception that cells bordering these clusters exhibited a trend of a higher proliferation frequency (>2.0-fold, not significant) in the 48-h GI group compared with the

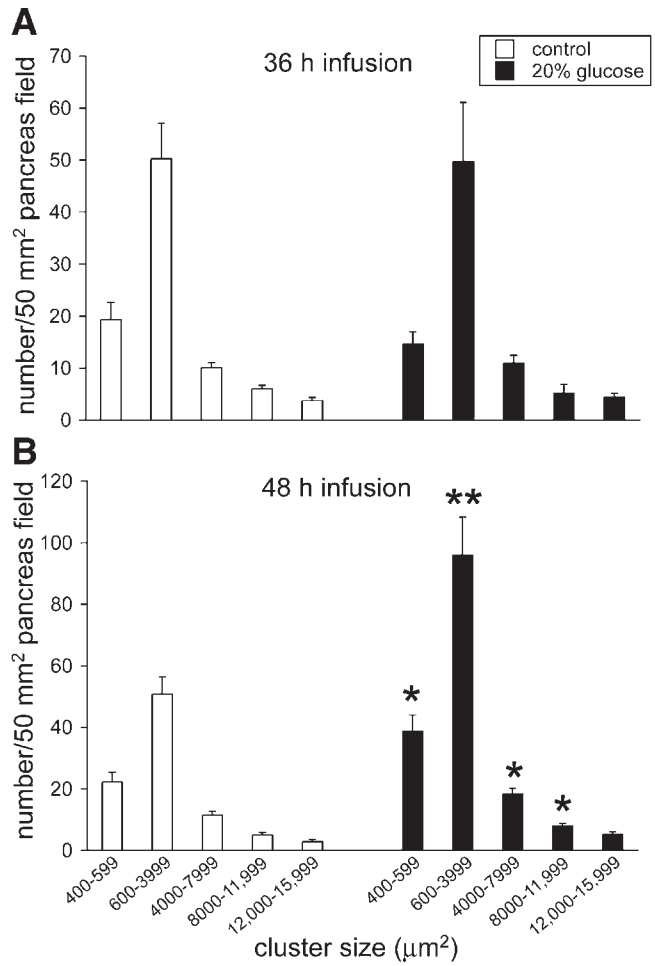

Fig. 4. β-Cell cluster/small islet prevalence in 20% glucose-infused rats. *A*: no differences in the relative number of β-cell cluster/small islets between the groups were seen before 36 h of infusion. *B*: at 48 h, significant 1.6- to 1.9-fold increases in the prevalence of clusters and small to medium size islets were observed (*$P < 0.05$, **$P < 0.01$). A trend of increased prevalence (1.9-fold, $P = 0.055$) was also observed in the next larger size class, 12,000–15,999 μm^2 (~124- to 143-μm diameter); $n = 5$ for each group.

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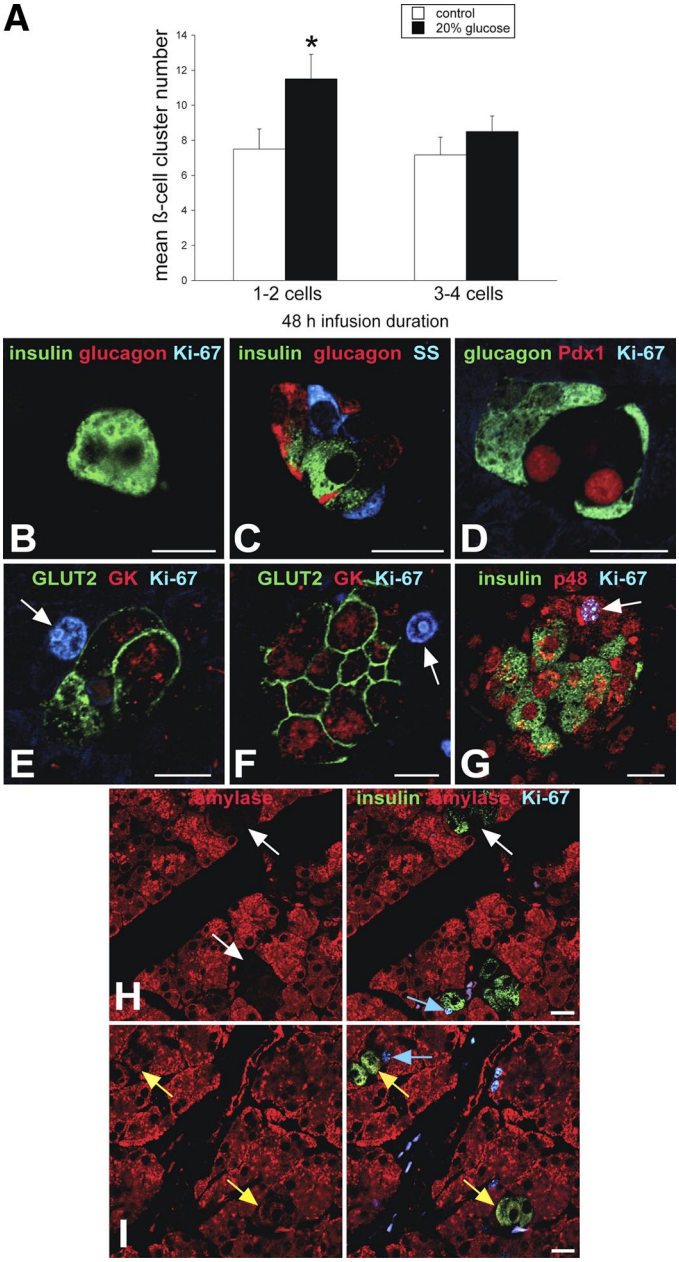

Fig. 5. β-Cell cluster characterization. *A*: the smallest class of β-cell cluster sizes was composed of 1–2 cells in cross section and increased in prevalence by 48 h in 20% GI rats (*$P < 0.05$); $n = 5$ for each group. *B–I*: β-cell clusters/small islets of 48-h GI rats. Except for their relative numbers, these clusters were indistinguishable from those of control rats. All clusters were closely associated with capillaries. *B*: single β-cells and doublets were consistently Ki-67$^-$ (nonproliferating). *C*: a typical small endocrine cell cluster composed of central β-cells and peripheral non-β-cells. SS, Somatostatin. *D*: another cluster exhibiting central pancreatic-duodenal homeobox-1 (Pdx1)$^+$ cells, probably β-cells, surrounded by α-cells. *E*: a β-cell cluster expressing the glucose sensor proteins glucokinase (GK) and GLUT2 at levels comparable to islets (not shown). Note a nearby Ki-67$^+$ cell (arrow). *F*: a very small islet with central GK$^+$/GLUT2$^+$ β-cells and a closely associated Ki-67$^+$ nonendocrine cell (arrow). These insulin$^-$ replicating cells are most often in the acinar compartment. *G*: a small islet with central β-cells (green) and a peripheral p48$^+$/Ki-67$^+$ replicating cell (arrow), probably in the endocrine compartment. *H*: representative field of typical amylase$^-$ β-cell clusters (white arrows) showing amylase alone (*left*) and amylase with insulin (green) and Ki-67 (blue) (*right*). Note the peripheral Ki-67$^+$ cell (blue arrow). *I*: another field showing less frequent β-cell clusters with low to moderate levels of amylase immunoreactivity (yellow arrows). Note a peripherally located Ki-67$^+$ acinar cell (blue arrow). Scale bars, 10 μm.

control group (Fig. 5, *E–I*). Most of these proximal proliferating cells were acinar cells (Fig. 5*I*), as confirmed by amylase costaining, whereas others were insulin$^-$ but P48$^+$ in the cluster/small islet periphery (Fig. 5*G*).

Unlike islets with well-defined methods for their isolation, these small endocrine clusters have not been well characterized. They may be functionally competent because of the expression of β-cell glucokinase and GLUT2 in a pattern similar to islet β-cells (Figs. 5, *E* and *F*).

Other β-cell growth mechanisms play only minor roles in short-term GI rats. Individual β-cell size as well as β-cell death and clearance rates also influence BCM. We therefore measured β-cell size in islets from 48- and 96-h-infused rats, using GLUT2 immunofluorescence and confocal microscopy to estimate β-cell volumes. Although no differences in β-cell size between the groups were detected at 48 h, at 96 h of infusion a modest increase (~22%) in β-cell volume was observed (saline control 849.1 ± 27.3 μm^3 vs. GI 1,075.3 ± 53.0 μm^3; $P < 0.05$). No differences in β-cell TUNEL staining or activated caspase-3 immunoreactivity in islets in situ, or in overnight cultured islets, were observed between the 48-h glucose- and saline-infused groups (not shown). Thus β-cell hypertrophy and/or reduced apoptosis are unlikely to contribute substantially to the BCM enhancement following 48-h GI.

Akt signaling in β-cell growth processes in GI rats. Since glucose is known to have pronounced effects on Irs-2 levels (31, 40) and Akt activation (46), and Akt has multiple roles in β-cell growth (10, 14, 17), we next sought to examine their potential roles in β-cell development and growth in these rats. The largest of the pancreatic ducts, the common duct, is also a site of β-cell neogenesis and exhibits heightened Irs-2 and activated Akt levels in GI rats (Fig. 6, *A–D*), similar to what we previously reported in the regenerating rat pancreas (19). The islet transcription factors Pdx1 and Pax6 have been implicated as having roles during duct-derived neogenesis in rodent regeneration models (29, 30, 45). Consistent among the GI group, increased phosphorylated Akt in the epithelium of the common duct evaginations was observed, with high levels of Pdx1 and Pax6 expression in the same cells (Fig. 6, *D* and *E*), but phospho-Akt did not colocalize with the β-cell-specific factors NeuroD/BETA2 or Nkx6.1 (not shown). In the control rats, phospho-Akt was also detected in Pdx1 (Fig. 6*C*)- and Pax6-positive epithelial cells, but these cells were rare. Hence, Akt signaling may mediate β-cell neogenesis among ducts through regulating the expression or activity of Pdx1 and Pax6.

Irs-2 and Akt both play important roles in BCM regulation by serving as survival and growth mediators. No differences in Irs-2 levels were detected between islets of GI and saline-infused rats (not shown). However, islet β-cell phospho-Akt levels were markedly enhanced in the GI group after 48 h (Fig. 7, *A* and *D*). No consistent differences in islet β-cell Pdx1 immunostaining were observed between the groups. Nonetheless, Bcl-2, a key β-cell survival factor (11) that is transcriptionally regulated by Akt (41), was increased in the GI group (Fig. 7*B*), indicating that β-cell survival potential may be enhanced through Akt activation.

Islet cell clusters and very small islets from both animal groups routinely exhibited moderate to strong phospho-Akt immunoreactivity (Fig. 7, *E* and *F*) in both Pdx1$^+$/insulin$^-$ cells (Fig. 7*E*) and Pdx1$^+$/insulin lightly immunopositive cells

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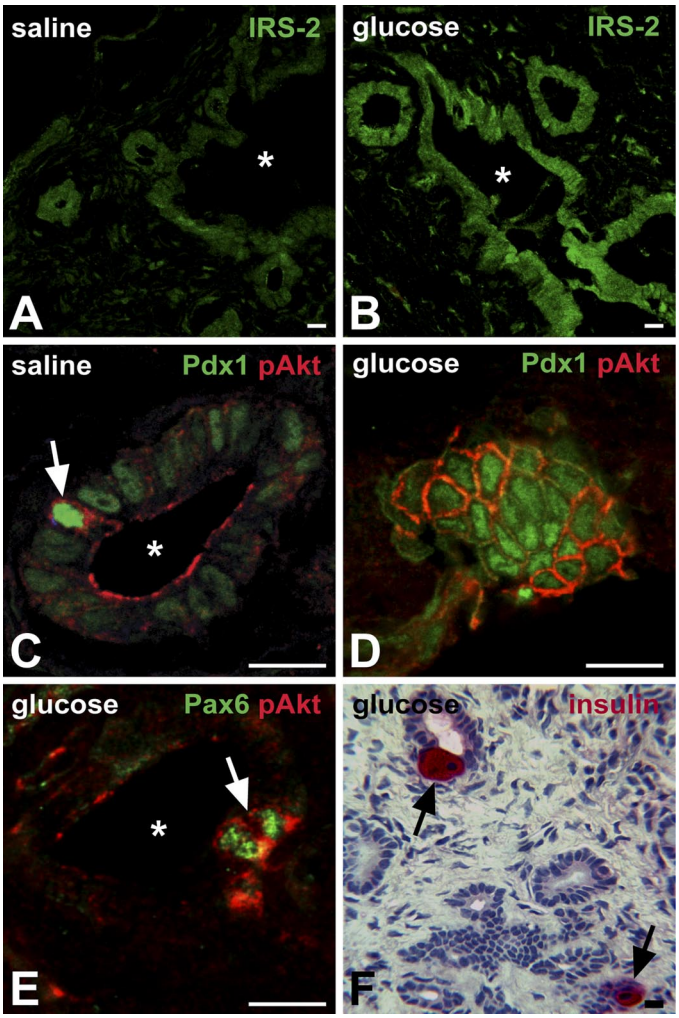

Fig. 6. CPD epithelium exhibits dynamic changes in response to a 20% glucose infusion. Although no changes were detected in insulin receptor substrate-2 (Irs-2) levels in islets, in the CPD epithelium (*A*) Irs-2 immunoreactivity increased in the GI group (*B*). Main lumen is indicated (*). *C*: epithelium of the CPD evaginations of control rats exhibit low levels of Pdx1 staining with occasional cells with intense staining and strong activated (phospho-) Akt (pAkt) staining (arrow). Lumen is indicated (*). *D*: a section through the wall of a CPD evagination from a 20% GI rat shows a generally intense nuclear Pdx1 signal with striking phospho-Akt in those same cells. *E*: the main CPD lining from a 20% GI rat showing a cluster of $Pax6^+$/phospho-Akt^+ cells (arrow). These profiles were rare in the CPD epithelium of control rats. *F*: immunohistochemical staining for insulin (arrows) reveals β-cell clusters emanating from the evaginations. Although these duct-associated clusters appeared to be more abundant in the GI group, their relative paucity precluded a detailed quantitative analysis. Scale bars, 10 μm.

(Fig. 7*F*). Thus Akt activation correlates strongly with Pdx1 expression in putative β-cell precursors.

DISCUSSION

To further address the role of Akt signaling in β-cell growth processes we have characterized a normoglycemic GI rat model in which a rapid compensatory expansion of BCM occurs by 48 h. Akt has been ascribed a central role in the mediation of β-cell growth and function. With respect to mechanisms regulating BCM, Akt has reported roles in individual β-cell growth or hypertrophy, proliferation, survival (reviewed in Ref. 10), and neogenesis (17). This model was developed to examine the mechanisms driving adaptive β-cell growth while circumventing the problems associated with chronic hyperglycemia. Therefore, our model differs markedly from those of previous GI studies in that euglycemia is virtually maintained through 96 h, although a modest but significant rise in glycemia (up ~22 mg/dl) was noted at 24 and 48 h. Notably, our model permits the effects of parenteral glucose on BCM to be studied without confounding influences of the cephalic reflex and normal incretin activation. Unexpectedly, hyperinsulinemia was transient and normoinsulinemia was achieved by 72 h, at which time a greater than twofold increase in BCM was observed. The stimulus for the rapid β-cell growth could therefore be the mild increase in glucose throughout this 48-h period and/or the transient spike in plasma insulin

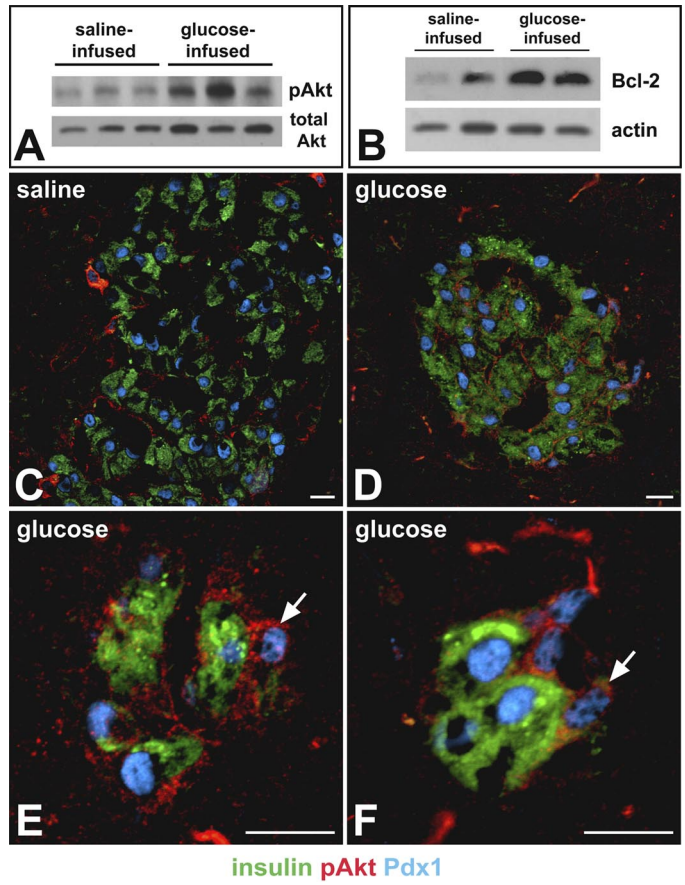

Fig. 7. Increased Akt signaling in islets from glucose-infused rats. *A*: representative immunoblots of isolated islets from 3 saline-infused control rats and 3 20% GI rats demonstrating enhanced phospho-Akt levels in GI rats. Membranes were stripped and reprobed with total Akt antibody for reference. *B*: immunoblot showing enhanced Bcl-2 protein levels in islets from representative GI rats. *C*: representative islet section from a saline-infused control rat demonstrating low to undetectable levels of phospho-Akt immunostaining levels among β-cells (green). Predominant phospho-Akt immunoreactivity within the islet is associated with the capillary endothelium. Some peripheral non-β-cells stain intensely. *D*: a typical islet from a 48-h 20% GI rat showing increased surface phospho-Akt signal in β-cells. No differences in β-cell Pdx1 levels were observed in islets. *E*: an endocrine cluster/small islet composed of $insulin^+/Pdx1^+$ and $insulin^-/Pdx1^+$ (arrow) cells. The peripheral $insulin^-/Pdx1^+$ cell has especially strong phospho-Akt immunoreactivity. *F*: another cluster demonstrating that some of these peripheral $Pdx1^+$/phospho-Akt^+ cells express low levels of insulin (arrow). A subset of these $Pdx1^+$/phospho-Akt^+ cells also express somatostatin (not shown). Endocrine clusters of GI rats were qualitatively similar to those of control rats. Scale bars, 10 μm.

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concentrations in the initial 24-h interval. Experiments are under way to examine these possibilities in our model since glucose and insulin may impact distinct β-cell growth mechanisms (39).

In contrast to most other models of increased β-cell growth, including a recent study in glucose-infused mice (1), increased proliferation of existing β-cells did not play a role in the growth response. Instead, enhanced BCM correlated with increased numbers of β-cell clusters and small islets closely associated with acini. At these sites, we also colocalized activated Akt kinase with the expression of Pdx1 in the same cells, putative β-cell progenitors.

The 20% GI rat model exemplifies the substantial adaptive capacity of the β-cell, demonstrating enhanced function and increased mass. Unexpectedly, in the first 24-h period, increased insulin secretion results in only transient hyperinsulinemia. Although insulin uptake is reportedly reduced during rat GI, yielding frank hyperglycemia (25), the rapid normalization of insulin levels in the present study could be the result of enhanced insulin clearance and/or increased insulin sensitivity under near-euglycemic conditions. The fact that another rat GI study clamping glucose levels at moderate hyperglycemia (170 mg/dl) observed increased tissue insulin sensitivity (24) suggests that this could be occurring in our model. We are currently addressing this possibility. Thus β-cell hypersecretion likely preserves normoglycemia before BCM increases. Our data suggest that in the subsequent 36- to 48-h infusion period, the heightened BCM likely compensates for the increased insulin demand. This correlates well with a significant increase in pancreatic insulin content at the 48-h time point (26).

β-Cell proliferation is generally considered to be the principal mechanism of postnatal β-cell growth (9). In these GI rats, β-cell proliferation was not observed to be enhanced at any time point and was actually reduced at 48 h of infusion. Since the β-cell cycle duration for young rats is ~15 h (42), it is unlikely that we missed a proliferation surge with the proliferation marker Ki-67. Previous studies in frank hyperglycemic GI rats have demonstrated transiently decreased β-cell bromodeoxyuridine incorporation rates at 24 h (2, 39, 40) followed by severalfold increases by 96 h (3, 39). Although the significance of the temporary drop in β-cell proliferation is unknown, unlike β-cells studied in vitro and in the fetal state, it appears that the stimulatory effects of infused glucose on adult β-cell mitogenesis are delayed until several days under hyperglycemic/hyperinsulinemic conditions (3, 39). Accordingly, it was recently shown by Alonso et al. (1) that in glucose-infused B6 mice with a dosage comparable to those in the present study (~2.0 $mg \cdot kg^{-1} \cdot h^{-1}$), β-cell proliferation increases fivefold by 96 h under sustained mild hyperglycemia. Interestingly, however, no changes in BCM or any other growth parameter were observed even when the glucose dosage was reduced by 50%. Thus our morphometric data in GI rats are in diametric contrast to the GI mouse model since we established *1*) no β-cell proliferation differences between GI and saline-infused rats and *2*) a greater than twofold increase in BCM by 48 h of glucose infusion. Furthermore, increased β-cell cyclin D2 nuclear localization reported in GI mice (1) was not observed in the present study, thus confirming our proliferation data. These striking differences in the β-cell response to apparently similar GI conditions between mouse and rat are unexpected and warrant further investigation with different rodent strains and infusion protocols.

We anticipated widespread β-cell proliferation within endocrine clusters since they increase in number and, presumably, grow in size. In fact, proliferation of β-cells of small clusters was rare, and Ki-67^+ single β-cells and doublets were never observed. It appears that these small aggregates exist as a quiescent population that has exited the cell cycle, similar to fetal β-cells following the "secondary transition" (16). Our future studies will address the origin of these endocrine aggregates with lineage-tracing techniques. Whether or not definitive islets form via the growth or aggregation of these clusters remains to be clarified.

The generation of apparently new β-cells appears to be the predominant growth mechanism accounting for the rapid and abrupt increase in BCM in our GI model. In rats, it was previously proposed that GI stimulates β-cell neogenesis from exocrine progenitors (2, 32, 39, 40); however, there is no consensus as to whether the source may be duct- or acinar-derived or both. Our data suggest that β-cell generation may occur from both sources; however, with identification criteria similar to those used by Lipsett and Finegood (32), only the acinar-associated clusters could be quantitatively evaluated (Figs. 4 and 5*A*). Conventional β-cell neogenesis from ducts is thought to involve an initial proliferation event followed by Pdx1 and insulin expression (4). In the present study, no increases in the proliferation of the duct epithelium were observed on GI. However, the frequency of $Pdx1^+$ and $Pax6^+$ cells in the duct epithelium was increased, suggesting that new endocrine cell development may occur without an obligatory replication event. The fact that duct-associated endocrine clusters were less frequently observed compared with acinar-associated clusters could be the result of a rapid mobilization of the former from the periductal zone following their formation. Alternatively, some of these endocrine clusters may have originated from small intercalated duct epithelium or centroacinar cells closely associated with acini, similar to what we observed previously in young Zucker fatty rats (17).

Acinar cells, per se, may serve as a source of β-cells. Acinar-associated endocrine cell clusters were prevalent in the GI rats (~2-fold increased), similar to reports by the Finegood laboratory using a profoundly hyperglycemic GI protocol (32, 48). In tissue culture, $insulin^+$ cells have been shown to transdifferentiate from acinar tissue without an intervening replication (Refs. 34 and 38 and Peshavaria and Jetton, unpublished observation), although this phenomenon has recently been challenged in vivo (7). We also observed binucleate single β-cells in GI rats (Fig. 5*B*), a characteristic of acinar cells. Importantly, this was also observed in transdifferentiation models using lineage tracing (38). If acinar cells serve as a source of β-cells in a neogenic capacity, then it would be expected that they should maintain their numbers through increased proliferation. Although no increase in general acinar cell proliferation was detected in the GI group, we did detect a positive trend in the proliferation frequency of acinar cells that border these clusters. One or both of the daughter cells might give rise to cells of the endocrine compartment. Furthermore, the occasional findings of clustered $amylase^+/insulin^+$ hybrid cells as well as $p48^+/Ki\text{-}67^+/insulin^-$ cells proximal to β-cell clusters suggest a potential acinar source of these cells (Fig. 5, *G* and *H*). Nonetheless, the precise origin, morphogenesis, and

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function of these β-cell clusters in the GI model await detailed lineage-tracing analyses, establishment of an appropriate isolation scheme, and, eventually, their biochemical and functional characterization.

β-Cell hypertrophy and enhanced survival may also contribute to the BCM increase in the GI model. In these GI rats no changes in β-cell size were observed at 48 h of infusion, but a modest increase was detected by 96 h. On the other hand, glucose is known to have profound effects on β-cell survival (2, 31, 46) and has been suggested to counter apoptosis in vivo (2). Because we have found that the TUNEL assay is generally uninformative to derive β-cell apoptotic frequencies in β-cell growth models in young animals, such as those in the present study, a more fruitful approach has been to examine their relative survival potential by assessing the expression of β-cell prosurvival proteins such as Irs-2 and Akt, as well as those of the Bcl-2 family (17). Although here we found no differences in islet Irs-2 or total Akt immunoreactivity, activated Akt levels were increased in the euglycemic GI rats. In contrast, under marked hyperglycemic GI conditions, Paris et al. (40) detected significant increases in islet Irs-2 mRNA expression. Our finding of enhanced activated Akt levels in islets on GI treatment is in accordance with the study by Srinivasan et al. (46) that established a glucose-induced Akt signaling through phosphatidylinositol 3-kinase in β-cell lines. We also detected an increase in islet Bcl-2 protein in the GI group. Since Bcl-2 expression can be regulated by Akt via cAMP response element-binding protein (CREB) (41), it appears likely that glucose and/or the transiently increased insulin levels in the GI rats may enhance β-cell survival potential by the upregulation of Bcl-2 via Akt signaling, possibly through CREB.

Besides playing roles in survival, Akt signaling is likely important in mediating β-cell neogenesis. Insulin itself has been proposed to induce neogenesis when chronically infused into rats (39). We previously showed (17) that in postweanling, profoundly hyperinsulinemic Zucker fatty rats neogenesis is significantly increased. A similar induction of neogenesis by high plasma insulin levels could be occurring in the GI rats at the 24- to 36-h interval, whereby at 48 h, activated Akt is observed both in the duct epithelium and within endocrine cell clusters in the same cells with high levels of nuclear Pdx1. Some of these cells express low levels of insulin, suggesting that they could be differentiating β-cells. Since the downstream Akt target Foxo1 negatively regulates *pdx1* expression (21), these observations suggest that glucose induces an Akt-driven activation of neogenesis through Pdx1, and possibly other factors such as Pax6 (30).

Glucose is the most extensively studied determinant of β-cell growth. Among the islet cell types, it is principally the β-cell population that undergoes accelerated growth in response to hyperglycemia (3). While the 20% GI model does not exhibit hyperglycemia but does experience a very modest but significant rise in blood glucose concentrations with a transient hyperinsulinemia, it remains to be determined whether glucose or insulin could be a principal factor driving the BCM increase. Since glucokinase has recently been shown to impact a β-cell glucose coupling pathway leading to CREB activation, enhanced Irs-2 signaling, and compensatory β-cell growth (46), and glucokinase activity is enhanced in islets from 48-h 20% GI rats (6), glucose likely has a major role in these rapid short-term β-cell growth responses.

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