The physiological mechanisms underlying the compensatory growth of β-cell mass in insulin-resistant states are poorly understood. Using the insulin-resistant Zucker fatty (fa/fa) (ZF) rat and the corresponding Zucker lean control (ZLC) rat, we investigated the factors contributing to the age-/obesity-related enhancement of β-cell mass. A 3.8-fold β-cell mass increase was observed in ZF rats as early as 5 weeks of age, an age that precedes severe insulin resistance by several weeks. Closer investigation showed that ZF rat pups were not born with heightened β-cell mass but developed a modest increase over ZLC rats by 20 days that preceded weight gain or hyperinsulinemia that first developed at 24 days of age. In these ZF rats, an augmented survival potential of β-cells of ZF pups was observed by enhanced activated (phospho-) Akt, phospho-BAD, and Bcl-2 immunoreactivity in the postweaning period. However, increased β-cell proliferation in the ZF rats was only detected at 31 days of age, a period preceding massive β-cell growth. During this phase, we also detected an increase in the numbers of small β-cell clusters among ducts and acini, increased duct pancreatic-duodenal homeobox-1 (PDX-1) immunoreactivity, and an increase in islet number in the ZF rats suggesting duct- and acini-mediated heightened β-cell neogenesis. Interestingly, in young ZF rats, specific cells associated with ducts, acini, and islets exhibited an increased frequency of PDX-1+/phospho-Akt+ staining, indicating a potential role for Akt in β-cell differentiation. Thus, several adaptive mechanisms account for the compensatory growth of β-cells in ZF rats, a combination of enhanced survival and neogenesis with a transient rise in proliferation before 5 weeks of age, with Akt serving as a potential mediator in these processes. Diabetes 54: 2294–2304, 2005

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leptin receptor gene (25–30). These rats physiologically adapt to the insulin resistance through increased β-cell mass and enhanced β-cell function such that hyperglycemia is largely prevented. As a result, this model has been used extensively to investigate the biochemical and molecular bases for β-cell compensation. A study of adult ZF rats has reported a striking increase of β-cell mass in response to their insulin resistance attributable to enhanced β-cell hypertrophy and neogenesis (1). This study did not address the mechanisms that initiate or mediate these β-cell mass–augmenting processes. Furthermore, the temporal relationship between the growth of β-cells and the onset of fully established insulin resistance in these rats has never been determined.

The present study has investigated the time of onset and origin of the cells that mediate β-cell expansion in ZF rats. We have also screened for potential signaling intermediates. Importantly, we observed that β-cell mass expansion preceded the onset of obesity and marked insulin resistance. Our results have uncovered potential regulatory mechanisms whereby Akt/PKB signaling promotes enhanced β-cell mass in an animal that develops severe insulin resistance in adulthood.

**RESEARCH DESIGN AND METHODS**

**Tissue processing.** Adult male ZF and ZLC (+/+ or +/+ ) rats at 5, 10, and 13 weeks of age (n = 5 for each group) were obtained from Harlan and housed in the UVM Animal Facility at least 5 days before use. Young male and female 10-, 20-, 28-, and 31-day-old (+/+ rats were obtained from litters derived from pregnant (+/+ mothers (also acquired from Harlan) that had been mated to +/+ studs (see genotyping details below)). The guidelines set forth by the UVM IACUC Committee were strictly adhered to for these studies. Six hours before they were killed, adult rats were given 100 mg/kg BrdU by intraperitoneal injection. Pancreata were removed from the abdomen immediately after death and stained with guinea pig anti-insulin IgG (Linco Research) followed by donkey anti–guinea pig IgG-alkaline phosphatase (Jackson Immunoresearch). Following development with Vector Red substrate (Vector Laboratories), sections were counterstained with hematoxylin, cleared, and mounted in Permount (Fisher Scientific).

The proportion of islet β-cell surface area versus surface area of the whole pancreas was determined planimetrically by digitally imaging 14–20 (young rats) or 50–60 (adult rats) islet sections from each rat on a Zeiss Universal microscope coupled to a Spot RT color charge-coupled device (Diagnostics Instruments). Single and small clusters of β-cells were presumed to be neogenic and were below the threshold for accurate surface area measurements; hence, we measured these cells in different assays (see below). Therefore, only islets >5 cell diameters were included for islet β-cell surface area measurements. Image files were analyzed using NIH Image version 1.62 with the “Area-Measure” tool, then tabulated (in pixel units) by integration into MExcel and entered for statistical analysis (determined by Student’s t test where P ≤ 0.05 was considered significant). This planimetric method also allowed us to estimate relative islet numbers and compare islet sizes. The possibility of measuring large islets twice was minimized due to the wide sampling interval between sections. β-Cell mass was estimated for each animal by determining the average β-cell surface area per animal multiplied by their pancreatic weight.

**β-Cell and duct proliferation.** At least two islets per adult pancreas were sectioned and stained histochemically with ethanols and embedded in paraffin. Hydroxytissue sections were subjected to pretreatment with 0.125% trypsin solution (Zymed) for 10 min at 37°C or a 10-min boil in 10 mmol/l citrate buffer, pH 6.0, followed by denaturation in 2N HCl for 20 min. After blocking, sections were incubated overnight in mouse anti-BrdU-biotin (1:20; Zymed), washed, and then incubated in Z-avidin–horseradish peroxidase (1:300; Zymed Laboratories) for 1 h. In other experiments examining young ZF β-cell and duct proliferation, slides were immunostained for the proliferation marker Ki-67 and insulin. This alternative strategy was used due to inconsistencies in BrdU labeling in these young rats that are known to occur in certain situations (31), including paraformaldehyde fixation (32). Since BrdU labeling marks the “S” phase of the cell cycle and Ki-67 marks a cycling cell, these two markers are not interchangeable but are related. Therefore, in young Zucker rats, the values for proliferation were expressed as Ki-67-β-cells (%) and were much higher than the values for BrdU incorporation. Hydroxytissue sections were subjected to pretreatment as above and then incubated overnight in mouse anti–Ki-67 (1:500; Transduction Laboratories), washed, and then incubated in donkey anti-mouse-IgG-biotin (Jackson Immunoresearch) followed by streptavidin–horseradish peroxidase (1:300; Zymed). Following development in DAB/H2O2 sections were then stained for insulin as described above and finally counterstained with hematoxylin. 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.

**β-Cell size measurements.** Islet β-cell size was determined by double immunofluorescence labeling and confocal imaging of insulin and either GLUT2 (Alpha Diagnostics; young rats and 5-week groups) or pan-cadherin (Zymed) (10-week group) to mark β-cell surface boundaries. A nuclear counterstain was also used in an effort to maximize the number of cells studied. Sections treated with anti-insulin and anti-C- or P-cell antibodies with clearly defined nucleus were measured. Pseudocolored images were merged using Adobe Photoshop, and β-cell boundaries were outlined and the surface area measured (μm²) using NIH Image.

**Islet number and size measurements.** Using the primary data obtained for the β-cell mass measurements, the relative islet number for each animal (calculated as average number per microscopic field in 400-μm intervals) was determined. With this same primary data set, we also determined the relative islet sizes, or cross-sectional area of individual islets, by converting pixel values to micrometers squared and tabulating for each animal.

**Analysis of neogenesis.** Single and small clusters of cells too small to be considered definitive islets and thus not accounted for in the islet mass measurements for the 10-, 20-, 28-, and 31-day and 10-week-old rats were tallied in each group (n = 4–5 each group). Using the same insulin-stained sections that were used for islet β-cell mass measurements, the total number of β-cells as singles and clusters as doublets, triplets, quadruplets, and up to five cell diameters (very small islets) were counted for each animal. Single and clustered β-cells, regardless of their location, show no signs of replication and therefore were considered neogenic and may aggregate to form new islets.

**Multiple-labeling immunofluorescence.** Hydrides sections of pancreas were blocked in 5% normal donkey serum + 1% BSA and then incubated overnight at 4°C in a mixture of sheep anti-insulin, guinea pig anti-insulin or C-peptide (Linco), rabbit or guinea pig anti-glucagon (Linco), sheep anti-somatostatin (Cortex), and rabbit or guinea pig anti–pancreatic polypeptide (Linco). For staining of PDX-1, a rabbit antisem was used at 1:1,000 (gift from Dr. Chris Wright, Vanderbilt University). Staining for
PDX-1 required antigen retrieval as detailed previously. Labeling of phospho-Akt and phospho-BAD required rapidly fixed and processed frozen sections that were stained with antisera specific for activated Akt using a mouse monoclonal anti-phospho-S473Akt (Cell Signaling Technology) or a sheep anti-phospho-S73Akt (Upstate), and mouse anti-phospho-BAD (Cell Signaling Technology). Polyclonal antibody to Bcl-2 was obtained from Santa Cruz Biotechnology.

Following washing, sections were incubated in a secondary antibody mixture that contained “ML” grade donkey anti-species-specific IgG conjugated to CY2 (1:300), CY3 (1:2000), or CY5 (1:500; all from Jackson ImmunoResearch), counterstained in 0.5 μg/ml Hoechst 33342, and mounted in Aqua Polymount (Polysciences). Alternate sections were stained with nonimmune serum from the appropriate species. Controls for secondary antibody specificity were performed as previously detailed (10). Images were acquired using the aforementioned color charge-coupled device camera for conventional epifluorescence or by confocal microscopy.

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 PMSE, and protease inhibitors. The lysate was sonicated, spun, and the protein concentration determined. Protein was separated by SDS-PAGE, transferred to polyvinylidine fluoride membranes, and probed 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. Bcl-2 antibody was purchased from Santa Cruz Biotechnology. These were the same antibodies used for the immunostaining experiments.

Analysis of apoptosis for β-cells in situ and in vitro. For determination of the apoptotic β-cells in young rats (10–35 days), a modified TUNEL staining protocol was used to fluorescently mark cells with fragmented DNA strands (33). A Dead-End TUNEL Kit (Promega) was used with the initial steps of the protocol followed by the manufacturer’s suggestions. Modification of the kit (TdT-based end labeling with biotin-UTP as the labeled nucleotide) included incubation with streptavidin-CY3 (1:2,000, Jackson ImmunoResearch) in the final labeling step. Pancreas or islet sections were immunofluorescently stained for insulin (CY2) and finally counterstained with 0.5 μg/ml Hoechst. Verification of TUNEL labeling included staining adjacent slides with rabbit anti-cleaved caspase 3 (R&D Systems) or propidium iodide to label apoptotic nuclei. TUNEL labeling of short-term cultured islets was accomplished by isolating islets from 4.5-week-old rats (ZLC, n = 3; ZF, n = 6; mean islets per rat, 15) using standard methods and incubating in RPMI-1640 + 10% fetal bovine serum at 37°C for 1 h before fixing and processing for frozen sections.

Confocal imaging. Sections were imaged using a Bio-Rad MRC 1024 scanning confocal head coupled to an upright Olympus BX50 microscope with an Ar-Kr laser utilizing the 488-, 568-, and 647-nm excitation lines (UVM Microscopy Imaging Facility). Images were acquired with the Bio-Rad LaserSharp software merged and formatted on a Power Macintosh running Adobe Photoshop.

Quantitative imaging for Bcl-2 and PDX-1. Semiquantitative comparisons of Bcl-2 immunofluorescence intensity for islet β-cells from young pups were accomplished by batch staining and sampling by confocal microscopy for Bcl-2 (CY3), insulin (CY5), and a nuclear counterstain (YO-PRO1; Molecular Probes). The approach allowed us to measure the relative levels of immunoreactive Bcl-2 within individual β-cells without potential signal from the non-β-cells, an inherent pitfall in whole-islet analyses. Samples were imaged with the confocal microscope using a 60× PlanApo objective lens (N.A. = 1.4) and the 568-, 488-, and 647-nm excitation lines, respectively, of an ArKr laser. For each field, the microscope was focused to maximize the number of cells optically sectioned through the middle of the nucleus. All confocal imaging parameters were identical for each imaged field with four nonoverlapping fields of 170 × 170 μm captured for each animal analyzed. Grayscale images (512 × 512 pixels) were transferred to a Power Macintosh running NIH Image for analysis. For each field studied, the Area-Measure tool was used to determine the mean pixel intensity (range 0–255 grayscale levels) of Bcl-2 immunofluorescence in randomly picked 20-pixel areas of the perinuclear region of β-cells. The final corrected intensity values were calculated for each cell after subtraction of the field background fluorescence values.

Using an imaging strategy similar to method above, semiquantitative comparisons of duct nuclear PDX-1 immunofluorescence intensity were obtained from sections of the common pancreatic duct epithelium from 10-week-old ZF and ZLC rats (n = 5 per group).

RESULTS

β-Cell mass of adult ZF rats. These studies were initiated to pinpoint both the age at which the increased β-cell mass occurs in ZF rats and to study the underlying mechanisms. We first quantified β-cell mass of ZF and ZLC rats at several time points from 5 to 13 weeks of age (Fig. 1A). Unexpectedly, an ∼3.5-fold increase was observed in the ZF rats at 5 weeks of age (P = 0.005), which is well before marked insulin resistance that occurs at ∼7–10 weeks (25,34,35). Very similar and proportional β-cell mass differences between the ZF and ZLC rats were seen at 10 and 13 weeks of age, which suggested that the compensatory β-cell expansion was completed before 5 weeks of age and maintained into adulthood. Examining the morphological bases for the increased β-cell mass in 5- and 10-week-old ZF rats revealed enhancement in the size of islets (Fig. 1B and C, respectively). These data initially suggested that the mass augmentation effect was principally due to β-cell hyperplasia. Surprisingly, however, the β-cell proliferation rate of ZF rats was not increased at 5,
10, or 13 weeks (Fig. 2A). We also quantified the relative number of islets by assessing their prevalence in sections at 400-μm intervals: 5-week-old ZF rats had an 18% increase, and 10-week-old ZF rats exhibited a >40% increase (P < 0.01) in the islet number over age-matched ZLC rats (Fig. 1B and C). In addition, no detectable differences in β-cell apoptosis as judged by TUNEL, Hoechst nuclear staining, and cleaved caspase-3 immunohistochemistry were found between the ZF and ZLC rats (data not shown). There was a 33–38% increase in the size (by surface area) of individual β-cells of 5- and 10-week-old ZF rats versus the lean controls, respectively (Fig. 2B; P < 0.001 for both time points), but this degree of hypertrophy alone could account for only a portion of the increased β-cell mass. Since there was also an increase in the number of islets in ZF rats, we reasoned that another source for the β-cell mass increase might be accelerated islet neogenesis. Accordingly, we quantified single β-cells and small β-cell clusters and observed significant increases in 10-week-old ZF rats (Fig. 2C), thus suggesting the possibility of increased β-cell neogenesis. Importantly, these cells never showed signs of proliferation (data not shown). Thus, these scattered β-cells probably differentiate directly or indirectly (postmitotically) from a non–β precursor cell type. Increased ductal proliferation and PDX-1 expression in the adult has been correlated with enhanced neogenic potential (rev. in 2). Although we did not detect significant differences in proliferation among the epithelium of the common or main ducts in 10-week-old ZF rats (data not shown), we found an ~50% increase in nuclear PDX-1 immunofluorescence intensity in the common duct of ZF rats (Fig. 2D).

Collectively, these results suggested that the increased β-cell mass in adult ZF rats occurs from both β-cell hypertrophy and enhanced neogenesis but not increased proliferation. However, the most dramatic observation was that the mass augmentation was essentially completed by 5 weeks of age. As such, resolving the underlying mechanisms for this differential growth response required us to study younger rats.

**β-Cell mass studies of ZF pups.** Before this study, β-cell mass measurements and growth parameters of ZF rats <5 weeks of age had not been reported. We investigated 10-, 20-, 28-, and 31-day-old ZLC and ZF pups and observed a subtle increase in β-cell mass of the ZF pups at 20 days of age (1.4-fold over lean littermate controls; P = 0.029) (Fig. 3A). This early β-cell growth preceded any differences in body weight or insulinemia compared with the ZLC pups (Fig. 3B and C and Table 1). Also, blood glucose levels were not different between the ZF and ZLC pups at any time point (Fig. 3D).

The β-cell mass increase in the ZF pups at 20 and 28 days old did not appear to be the result of enhanced β-cell proliferation (Fig. 3E). Interestingly, however, at 31 days of age, ZF pups exhibited a twofold enhancement (P = 0.004) in β-cell proliferation over the ZLC pups (Fig. 3E). There were no differences among the two groups in the size of β-cells, nor were there detectable differences in β-cell apoptosis (as determined by a sensitive TUNEL fluorescence assay, activated [cleaved] caspase-3 immunostaining, and propidium iodide/Hoechst staining), in 10-, 20-, 28-, or 31-day-old pups (data not shown). In fact,
apoptotic cells were quite rare in all animals examined (e.g., <1 in 1,000 β-cells, or 0.1%). Since a wave of apoptosis in the pancreas has been reported to occur in Sprague-Dawley rat pups during the 3rd week of life, presumably to serve a remodeling function (36), we also studied apoptosis in 15-day-old rats. Again, we found too low frequencies of apoptosis in these rats to be useful for comparison.

**Survival potential is enhanced in islets from young ZF rats.** The rarity of TUNEL+ β-cells in tissue sections made cell survival potential determinations between the groups untenable, possibly due to efficient apoptotic clearance mechanisms in Zucker rat pancreata. To circumvent this problem, we performed TUNEL staining on frozen sections of isolated islets from 4.5-week-old rats (ZLC, n = 3; ZF, n = 6) that were then cultured for 1 h before fixation. Accordingly, we found that islets from ZF rats exhibited a significantly reduced fraction (P = 0.009) of TUNEL+ β-cells compared with the ZLC islets (Fig. 3F).

In the absence of finding any detectable differences in islet β-cell death in situ, we predicted that the early β-cell mass expansion in young ZF rats was initially because of enhanced neogenesis. This was supported by our finding of significant increases in the number of small β-cell clusters at 20–28 days of age that, unexpectedly, transiently wane at 31 days (Fig. 4A). Since no differences were seen in the numbers of clusters between the groups at 10 days of age before the β-cell mass increase (Fig. 4A), there appears to be a temporal correlation with the increased β-cell mass and increased prevalence of β-cell clusters from 20 days of age onward. These β-cells consistently lacked any indications of proliferation (not shown). Upon closer analysis, these clusters were composed of insulin+/PDX-1+ cells that were associated with both ducts and acini (Figs. 4B and C), and may serve as the source of new islets. This was followed by a transient rise in proliferation at 31 days that preceded a substantial β-cell mass increase at 35 days of age.

**Enhanced Akt/PKB signaling in ducts of ZF pups.** Our prior study of β-cell regeneration following a 60% pancreatectomy in Sprague-Dawley rats implicated Akt/PKB signaling in duct-related β-cell neogenesis (19). We examined this possibility in 25-day-old ZF and ZLC pups, an age that immediately follows the normal weaning period. We de-
ected activated (phospho-) Akt/PKB staining in noncontiguous cells of the common duct epithelium in both the surface lining and in the evaginations (Fig. 5A and B). While rare in the ZLC pups, these phospho-Akt+ cells were considerably more frequent in the ducts of ZF pups. Further characterization of these cells revealed that they were Ki-67 negative and did not express any of the principal islet hormones. In contrast, they routinely exhibited very high levels of nuclear PDX-1 (Fig. 5A and B). We also observed insulin+/PDX-1+ cells in small ducts presumed to be nascent β-cells that displayed little or no detectable phospho-Akt staining (Fig. 5C). Cells with the same staining profiles were also observed in the centroacinar region (Fig. 5D) of both groups of 25-day-old rats. This latter observation supports the current hypotheses of acinar cell transdifferentiation (37–39) and centroacinar cells (40) as a potential source of β-cells. Collectively, these findings suggested a developmental sequence whereby activation of Akt in exocrine precursors initiates or mediates early events in β-cell differentiation, but its activity is then rapidly suppressed during the progression to fully mature β-cells.

Enhanced Akt/PKB signaling and survival in islet β-cells of ZF pups. In the 25-day-old ZF pups, we detected moderate phospho-Akt immunoreactivity in islet β-cells, whereas in the ZLC rat β-cells, it was generally quite low (compare Fig. 6A with C). In contrast, β-cell total Akt immunostaining (not shown) and immunoblot analysis (Fig. 6I) revealed no differences among islets of ZF and ZLC pups. Immunoblot analyses of phospho-Akt in islets from young ZLC and ZF rats failed to show consistent differences between the groups, possibly due to non–β-cell Akt activation in islets or inadequate stabilization of islet phosphatases during islet isolation. On the other hand, using a rapid fixation and processing method for pancreas cryosections (19), we can reproducibly detect phospho-Akt and other labile phospho-intermediates in β-cell in situ. Since Akt is an established survival factor for many cell types including β-cells (12,21,23,24), we next examined the expression patterns of the genes encoding two related β-cell proteins that function in the mitochondrial regulation of apoptosis, BAD, and Bcl-2, a proapoptotic factor and a survival factor, respectively. BAD activity is inhibited upon phosphorylation and is an established target of Akt/PKB (41). In 4-week-old rats, we found no differences in BAD or Bcl-2 mRNA expression between ZF and ZLC rats by RT-PCR (data not shown), nor did we find consistent differences in their respective protein levels by immunoblot (Fig. 6I). In contrast, immunostaining for phospho-BAD showed that it was markedly elevated in islets of 25-day-old ZF pups compared with the ZLCs (Fig. 6B and D), suggesting functional inhibition of this protein. Although we found no significant differences in Bcl-2 immunoreactivity in β-cells of ZF and ZLC pups at 20 days of age (not shown), at 28 days of age, Bcl-2 immunofluorescence intensity was clearly increased (Fig. 6E–H). Using semiquantitative confocal analysis to measure cytoplasmic Bcl-2 immunofluorescence at the level of individual β-cells, the signal was 2.2-fold enhanced in ZF rats (Fig. 6J) (P = 0.046). This disparity between the whole-islet immunoblot analysis and the β-cell immunofluorescence measurements of Bcl-2 may be due to non–β-cell

| Table 1: Trends in increased body weight gain and progressive hyperinsulinemia in ZF rat pups |
|--------------------------------------------------|------------------|------------------|------------------|
| | Body Weight (g) | Insulin (μU/ml) | Body Weight (g) | Insulin (μU/ml) |
| | 20 day | 22 day | 24 day | 26 day | 28 day | 20 day | 22 day | 24 day | 26 day | 28 day |
| ZF | 37.4 ± 1.1 | 39.4 ± 1.2 | 41.3 ± 1.3 | 43.2 ± 1.4 | 45.1 ± 1.5 | 0.25 ± 0.05 | 0.26 ± 0.06 | 0.28 ± 0.07 | 0.30 ± 0.08 | 0.32 ± 0.09 |
| ZLC | 34.4 ± 1.1 | 36.4 ± 1.2 | 38.4 ± 1.3 | 40.4 ± 1.4 | 42.4 ± 1.5 | 0.22 ± 0.05 | 0.24 ± 0.06 | 0.26 ± 0.07 | 0.28 ± 0.08 | 0.30 ± 0.09 |
| **P** | * < 0.05 | † < 0.01 | ‡ < 0.001 | † < 0.01 | ‡ < 0.001 |

Values represent measurements from the 28-day group comprised of 6 ZF and 11 ZLC rats. Body weight and blood insulin were measured from fed rats at the same time each morning.

Trends in increased body weight gain and progressive hyperinsulinemia in ZF rat pups.
Bcl-2 protein in the whole-islet extracts. Based on these observations of detecting increased phospho-Akt, Bcl-2, and phospho-BAD immunoreactivity in islet β-cells of young ZF rats, we propose that activated Akt in islet β-cells may be targeted to mitochondrial BAD, thus inhibiting its function and thereby promoting β-cell survival. **Enhanced Akt/PKB signaling in non–β-cells of ZF islets.** A closer examination of islets revealed an additional cell type–specific staining pattern of phospho-Akt in the ZF pups at the postweaning age. In addition to the β-cells, we noted an increased prevalence of intensely stained, peripheral cells in the ZF rats compared with the ZLCs (Figs. 6C and 7) that were glucagon and pancreatic polypeptide immunonegative, but stained strongly for PDX-1. About half of these cells lacked a classical islet cell hormonal phenotype, whereas the remainder was found to be somatostatin+ δ-cells (data not shown). Since it has been proposed that somatostatin+/PDX-1+ cells may serve as β-cell progenitors during islet compensatory growth (42), this observation may relate to new β-cell development within the islet periphery of young ZF rats.

**DISCUSSION**

The ZF model has been widely used to study the stepwise functional (2,25,35) and morphological (1,43) adaptations in islet β-cells that occur in response to insulin resistance and obesity. In the current study, we have uncovered a novel mechanism for the β-cell expansion in ZF rats that incorporates enhanced neogenesis, a transient rise in proliferation, and increased β-cell survival potential and provides evidence of potential roles for Akt/PKB kinase in mediating these responses.

At the outset of these experiments, we had anticipated that the β-cell mass of ZF rats would grow in concordance with their progressive insulin resistance and peak when severe insulin resistance is established. We were unable to establish the dominant mechanisms of β-cell mass expansion in adult ZF rats due to the fact that a steady-state increase in mass was already established by the time of adulthood. In fact, we found that the β-cell mass of ZF rats was substantially elevated by 5 weeks of age. Although several studies on ZF rats have considered the 10- to 12-week-old age period when insulin resistance is fully
established, mild insulin resistance in skeletal muscle has been detected as early as 30 days of age (44). This early period of mild insulin resistance temporally correlates well with our finding of increased β-cell mass and proliferation. Our investigation of earlier time points has shown that ZF pups are not born with enhanced β-cell mass; instead, a significant increase was observed just before weaning (at 20 days), which clearly precedes major changes in energy metabolism after 23 days that result in hyperphagia, hyperinsulinemia, and significant weight gain (29). Thus, our results demonstrate an apparent temporal disparity between the β-cell expansion and ensuing metabolic alterations that merits further study.

We thus focused on ZF rat pups before 5 weeks of age and have demonstrated that the key insulin signaling molecule, Akt, is activated in ducts, acini, and β-cells. Downstream of IRS-2/PI3-kinase signaling, Akt, and in particular, the Akt-2 isoform (13) appears to be a crucial element in compensatory β-cell expansion (10,11). Activated (phospho-) Akt staining levels in the duct epithelium were dramatically enhanced in ZF rats in the postweaning period. Furthermore, the most intense PDX-1+ cells consistently contained for phospho-Akt. This observation is significant, since studies of mouse models harboring mutations of genes encoding key insulin signaling intermediates indicate that the link between the insulin signaling pathway and β-cell mass compensation to insulin resistance is through the stimulation of pdx-1 expression via nuclear exclusion of the forkhead protein transcription factor Foxo1 in β-cells, an established target of Akt (14,15). Thus, the phospho-Akt+/PDX-1+ cells we have identified in ducts and centroacinar regions of young ZF rats may represent a novel insulin/IGF-responsive progenitor for β-cell neogenesis, although this remains to be proven.

In addition to the exocrine duct epithelium and centroacinar region, we also observed increased phospho-Akt immunoreactivity in islet β-cells of ZF rats. In β-cells, Akt has been proposed to function in mediating proliferation (10,22,24,45), size (10,11), and survival (11,12,21,23,24). Enhanced β-cell proliferation in ZF rats was limited to a restricted time (31 days of age) that preceded the onset of the largest β-cell mass increase (~3.8-fold at 35 days of age). Since Akt has been ascribed with roles associated with enhanced β-cell proliferation in vivo (10), future studies are necessary to determine whether it may be serving such a role in the young ZF rat.

The Bcl-2 family member BAD, a well-established substrate of Akt that is inhibited upon phosphorylation, functions as a proapoptotic factor by antagonizing Bcl-2 activity, a related prosurvival protein (46). Both BAD and Bcl-2 are expressed in β-cells and have been implicated in the coordinate control of β-cell apoptosis/survival, respectively (46). We detected both increased phospho-BAD and Bcl-2 staining in β-cells of ZF pups by immunostaining that is consistent with the notion of an enhanced survival potential of β-cells of ZF rats. Elevated Bcl-2 staining might also represent increased compartmentalization of the protein to cellular membranes. We speculate from these findings in young rats that activated Akt in β-cells may be targeted to mitochondrial BAD, thus inhibiting its function and promoting β-cell survival. We failed to detect...
decreased apoptosis in the ZF rat pancreas based on in situ apoptosis assays, but the rarity of these profiles (<0.1% in all rats and time points), probably from their rapid engulfment from phagocytes or neighboring β-cells (47), made this approach ineffective to demonstrate changes in apoptosis. However, we quantified apoptosis in β-cells from 4.5-week-old ZF and ZLC pups using another strategy. Hence, we observed increased survival potential in isolated, short-term cultured islets from young ZF rats based on decreased TUNEL staining. Relatedly, a recent study of pdx-1+/−/− haploinsufficient mice has ascribed PDX-1 with a pro-survival role in islets (48). Although the mechanisms involved are unresolved, a similar role for PDX-1 could be operating in the pancreas of young ZF rats, although this has yet to be explored.

Akt/PKB has been proposed to regulate β-cell size (10,11) and may also be playing such a role in the β-cells of ZF rats. Transgenic overexpression of Akt in mouse β-cells results not only in a several-fold enhancement in β-cell mass, but also increased β-cell size, as well as their heightened survival potential (11). A global knockout of the gene encoding for the ribosomal protein p70S6-kinase 1, a downstream target in the Akt-mTOR pathway, results in glucose intolerance and atrophied β-cells (49). Collectively, Akt in β-cells may control size through regulation of p70S6-kinase activation, hence, increasing protein translation and cellular growth. We have determined that β-cell size, as measured by surface area, is enhanced 33% in 5-week-old ZF rats with over a 3.5-fold enhancement in β-cell mass. The magnitude of this growth is actually more
striking when the volume of the β-cell is considered. This augmentation in β-cell size and overall mass is sustained throughout adulthood. Although not studied here, enhanced Akt signaling in adult ZF β-cells likely impacts their growth through S6-kinase activation.

We have also demonstrated that strongly phospho-Akt immunopositive, but insulin-negative cells in the islet periphery of young Zucker rats also stain intensely for nuclear PDX-1. About 50% of these cells lack a typical islet peptide hormone phenotype, but half are somatostatin+ δ-cells. A lineal relationship of δ- and β-cells has been reported during both embryonic development (50) as well as during islet regeneration in the adult (42). Hence, these islet-borne PDX-1+/phospho-Akt+/somatostatin+/- cells may represent a β-cell precursor and might also contribute to the β-cell mass increase in ZF rats.

In summary, we have presented evidence suggesting that at almost all examined time points, β-cell differentiation from progenitor cells (neogenesis) is significantly enhanced in ZF rats, as judged by significantly increased numbers of β-cell clusters and single β-cells associated with the exocrine compartment. Additional support for β-cell neogenesis was a significant increase in ductal nuclear PDX-1 staining. Increased ductal PDX-1 expression in regeneration models has been correlated with the acquisition of a “de-differentiated” phase analogous to an embryonic state that, in turn, may lead to new β-cell development (2). Although these criteria serve as only indirect evidence of neogenesis, we surmise that these cells may migrate and aggregate to form new islets, with phospho-Akt staining (arrow). B: Comparable field of a typical islet from a young ZF rat showing increased prevalence of these strongly PDX+/phospho-Akt+ peripheral cells (arrows). The red channel was underexposed to clearly reveal the strongly Akt+ peripheral cells. These cells were glucagon and pancreatic polypeptide negative, but half were determined to be somatostatin+ δ-cells.

β-cells. Importantly, we have also localized activated (phospho-) Akt to specific PDX+ cells of ducts, acini, and islets of young ZF rats. Thus, in the leptin-signaling deficient ZF rats, mechanisms of β-cell mass expansion are clearly in play early in life to insure a sufficient insulin supply for the greatly increased insulin requirements later in adulthood.

ACKNOWLEDGMENTS

This work was supported by research awards from the American Diabetes Association to J.L. and T.L.J.

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