Original Article

Distinct In Vivo Roles of Caspase-8 in β-Cells in Physiological and Diabetes Models

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Inadequate pancreatic β-cell mass resulting from excessive β-cell apoptosis is a key defect in type 1 and type 2 diabetes. Caspases are the major molecules involved in apoptosis; however, in vivo roles of specific caspases in diabetes are unclear. The purpose of this study is to examine the role of Caspase (Casp)8 in β-cells in vivo. Using the Cre-loxP system, mice lacking Casp8 in β-cells (*RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice) were generated to address the role of Casp8 in β-cells in physiological and diabetes models. We show that islets isolated from *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice were protected from Fas ligand (FasL)– and ceramide-induced cell death. Furthermore, *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice were protected from in vivo models of type 1 and type 2 diabetes. In addition to being the central mediator of apoptosis in diabetes models, we show that Casp8 is critical for maintenance of β-cell mass under physiological conditions. With aging, *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice gradually develop hyperglycemia and a concomitant decline in β-cell mass. Their islets display decreased expression of molecules involved in insulin/IGF-I signaling and show decreased pancreatic duodenal homeobox-1 and cAMP response element binding protein expression. At the level of individual islets, we observed increased insulin secretory capacity associated with increased expression of exocytotic proteins. Our results show distinct context-specific roles of Casp8 in physiological and disease states; Casp8 is essential for β-cell apoptosis in type 1 and type 2 diabetes models and in regulating β-cell mass and insulin secretion under physiological conditions. *Diabetes* 56:2302–2311, 2007

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7-AAD, 7-amino-actinomycin D; Casp, caspase; CREB, cAMP response element binding protein; FACS, fluorescent-activated cell sorting; FasL, Fas ligand; GSIS, glucose-stimulated insulin secretion; GTT, glucose tolerance test; H-E, hematoxylin and eosin; HFD, high-fat diet; IGF-IR, IGF-I receptor; IRS, insulin receptor substrate; PDX-1, pancreatic duodenal homeobox-1; pGSK3β, phospho–glycogen synthase kinase 3β; STZ, streptozotocin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

β-Cell apoptosis is an important pathological mechanism of β-cell loss in type 1 and type 2 diabetes (1–3). Although the instigating factors that lead to β-cell destruction in these two types of diabetes are distinct, a common cell death machinery is likely operational. Caspases are the major components of the cell suicide machinery, and the two major pathways are intrinsic or mitochondrial and extrinsic or death receptor–mediated (4–6). To date, the in vivo roles of specific caspases in β-cell apoptosis in diabetes are not clear.

In addition to apoptosis, caspases have been described in other cellular processes such as proliferation, differentiation, and survival (7–12). The capacity of caspases to participate in various cellular processes is highly cell type and stimulus dependent. Caspase (Casp)8 in particular possesses these complex and versatile functions (7,13,14). Casp8 is a critical component of the death receptor–mediated apoptotic pathway, and deletion of Casp8 in the whole mouse leads to embryonic lethality (13). The Cre-loxP system has allowed us to circumvent this obstacle and to further investigate the physiological and pathological roles of Casp8 in pancreatic β-cells postnatally in vivo. Death receptor–mediated apoptosis has been shown to be important in type 1 and type 2 diabetes (1,15); however, the specific role of Casp8 in pancreatic β-cells was elusive. In this study, we show Casp8 to be essential in β-cell apoptosis in streptozotocin (STZ) and high-fat diet (HFD)–induced diabetes models, demonstrating the potential importance of Casp8 in both types of diabetes. We also show a physiological role of Casp8 in the maintenance of β-cell mass and in insulin secretion.

RESEARCH DESIGN AND METHODS

Mice harboring exons 3 and 4 of the *Casp8* gene flanked by *loxP* sites (*Casp8*$^{fl/fl}$) (7) were crossed to mice expressing *cre* under the rat insulin-2 promoter (TgN(ins2-*cre*)25Mgn [16], referred to as *RIPcre*$^{+}$; The Jackson Laboratories). *RIPcre*$^{+}$*Casp8*$^{+/fl}$ mice were intercrossed to generate *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ and *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice. Mice were maintained on a mixed 129J-C57BL/6 background, and only littermates were used as controls. All genotyping was done using PCR protocols, as previously described (7). Mice were maintained in the pathogen-free animal colony at the Ontario Cancer Institute in accordance with protocols approved by the research institute (Toronto, Canada).

Immunofluorescence staining, immunohistochemistry, and islet morphometry. Pancreatic tissue was fixed and processed as previously described (17). Paraffin sections were stained with hematoxylin and eosin (H-E), stained with antibodies to Casp8 (Alexis), insulin (Dako), glucagon (Sigma), pancreatic duodenal homeobox-1 PDX-1 protein (gift of C. Wright), Ki67 (Dako), and GLUT2 (Chemicon, Temecula, CA) antibodies, as previously described (17).

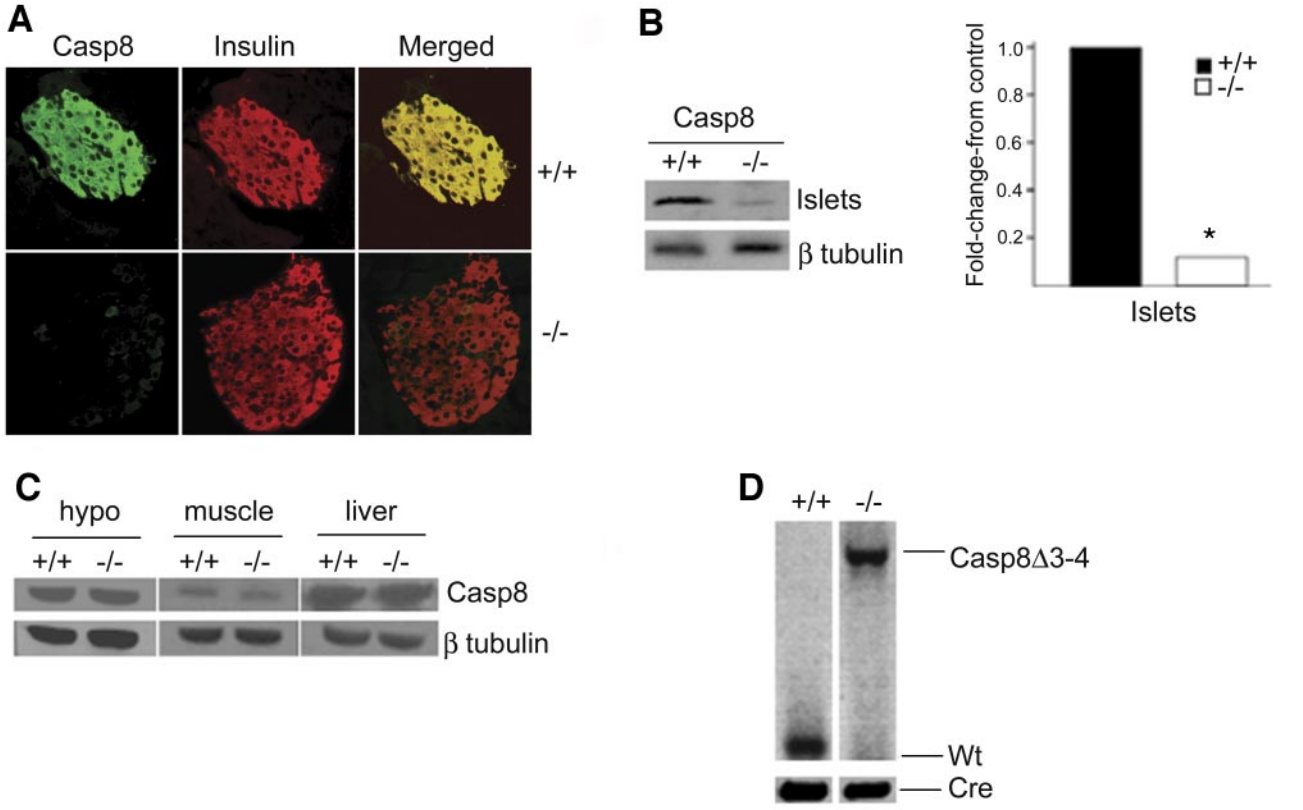

FIG. 1. Deletion of Casp8 in pancreatic β-cells. *A*: Immunofluorescence used to depict expression of Casp8 and costained with insulin in pancreatic sections of *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ (−/−) mice and *RIPcre*$^{+}$*Casp8*$^{+/+}$ (+/+) mice (magnification ×20). *B*: Western blots (*left*) and quantification of Western blot signal (*right*) showing decreased expression of Casp8 (55 kDa) in isolated islets, **P* < 0.05. *C*: Western blotting showing no difference in the expression of Casp8 in the hypothalamus (hypo), muscle, and liver. *D*: PCR analysis of Cre-mediated recombination of the *Casp8* locus (Δ3–4) in sorted β-cells. The results are representative of three independent experiments performed on mice >5 months of age. (Please see http://dx.doi.org/10.2337/db06-1771 for a high-quality digital representation of this figure.)

Total islet area and total pancreatic area were determined from sections immunostained for insulin as previously described (17). To examine apoptotic cells, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL; Roche Biochemicals) was performed as described previously (17,18).

Islet isolation and β-cell sorting. For islet isolation, 2 ml of 2 mg/ml collagenase (Sigma) was injected into the common bile duct for pancreatic digestion, as previously described (17). Isolated islets were maintained in suspension culture in RPMI-1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and incubated at 37°C with 5% CO_2. For β-cell sorting, freshly isolated islets were dispersed and subjected to flow cytometry based on high autofluorescence, as previously described (19).

In vitro killing assay. Twenty to thirty isolated islets were added to a single well of a 96-well tissue culture plate with RPMI-1640 (described above). Islets were incubated in 100 ng/ml interleukin-1β for 48 h, and then Fas ligand (FasL; 10 μg/ml) was added to culture for 6 days. For the killing assay with ceramide, isolated islets were incubated in 50 μmol/l ceramide for 24 h.

Islet viability and flow cytometry analysis. For assessment of viability, isolated islets were trypsinized to generate single cell suspensions, and the Annexin V/7-amino-actinomycin D (7-AAD) binding assay was performed as previously described (17).

Induction of diabetes by STZ and glucose monitoring. The mice were injected intraperitoneally with STZ (40 mg/kg body wt) for 5 consecutive days, as previously described (17). Tail vein glucose was monitored weekly using One Touch Ultra (Life Scan; Johnson & Johnson).

HFD-induced diabetes. Four- to 6-month-old *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ and *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice were fed for 8–12 months on an HFD (45% calories from fat; product no. 03560; Harlan Teklad). Their body weight and fasting glucose was measured monthly. Blood glucose levels were determined from tail venous blood.

Blood glucose determination and metabolic studies. Mice were fasted overnight for 14–16 h before experiments. Glucose tolerance tests (GTTs) were performed on mice fasted overnight using a glucose dose of 1 g/kg body wt injected intraperitoneally. Blood glucose measurements were made at 0, 15, 30, 60, and 120 min after the injection. Fasting serum insulin levels were measured by enzyme-linked immunosorbent assay using rat insulin standard (Crystal Chem). For glucose-stimulated insulin secretion (GSIS) assays, mice fasted overnight received an intraperitoneal injection of glucose (3 g/kg body wt). Tail vein blood was collected at 0, 2, 10, and 30 min after the injection, and insulin levels were measured by enzyme-linked immunosorbent assay.

Western blotting and RT-PCR. Islets were isolated and protein lysates obtained as previously described (20). Using SDS–10% PAGE, lysates were separated and then immunoblotted with antibodies to Casp8 (Alexis), AKT, phosphor-AKT (Ser473; Cell Signaling Technology), phospho–glycogen synthase kinase 3β (pGSK3β; Cell Signaling Technology), insulin receptor substrate (IRS)-2 (Santa Cruz Biotechnology), insulin receptor (Santa Cruz Biotechnology), IGF-I receptor (IGF-IR) (IGF-IRβ; Santa Cruz Biotechnology), active Casp3 (Cell Signaling Technology), proCasp3 (BD Transduction Laboratories), Casp7 (Cell Signaling Technology), Casp9 (gift of R. Hakem), Casp2 (Pharmingen), poly(ADP-ribose) polymerase (Cell Signaling Technology), GLUT2 (Chemicon), glucokinase (gift from M. Magnusson), cAMP response element binding protein (CREB Cell Signaling Technology), PDX-1, vesicle-associated membrane protein 2 (Stressgen), syntaxin 1A (Sigma), and Munc18a (Transduction Laboratories). mRNA was extracted from fluorescent-activated cell sorting (FACS) sorted β-cells by TRIzol reagent by following the manufacturer's protocol (Invitrogen, Toronto, ON, Canada). Semiquantitative reverse transcription amplification (RT-PCR) amplification was performed with a one-step RT-PCR kit (Invitrogen, Carlsbad, CA; primer sequences available upon request). Western blotting and RT-PCR signal densities were analyzed using National Institutes of Health Image software. Protein and transcript levels were normalized with β-tubulin and expressed as fold change relative to littermate control levels.

Islet perifusion secretory assay and determination of islet insulin. Batches of ~60 islets on average were placed in perifusion chambers with a capacity of ~1.3 ml at 37°C and perifused at a flow rate of ~1 ml/min with a Krebs-Ringer bicarbonate buffer. Islets were first equilibrated for 30 min in Krebs-Ringer bicarbonate HEPES buffer supplemented with 2.8 mmol/l glucose. They were then stimulated during a 10-min period with 2.8 mmol/l glucose followed by a 40-min period with 16.7 mmol/l glucose. Fractions were collected

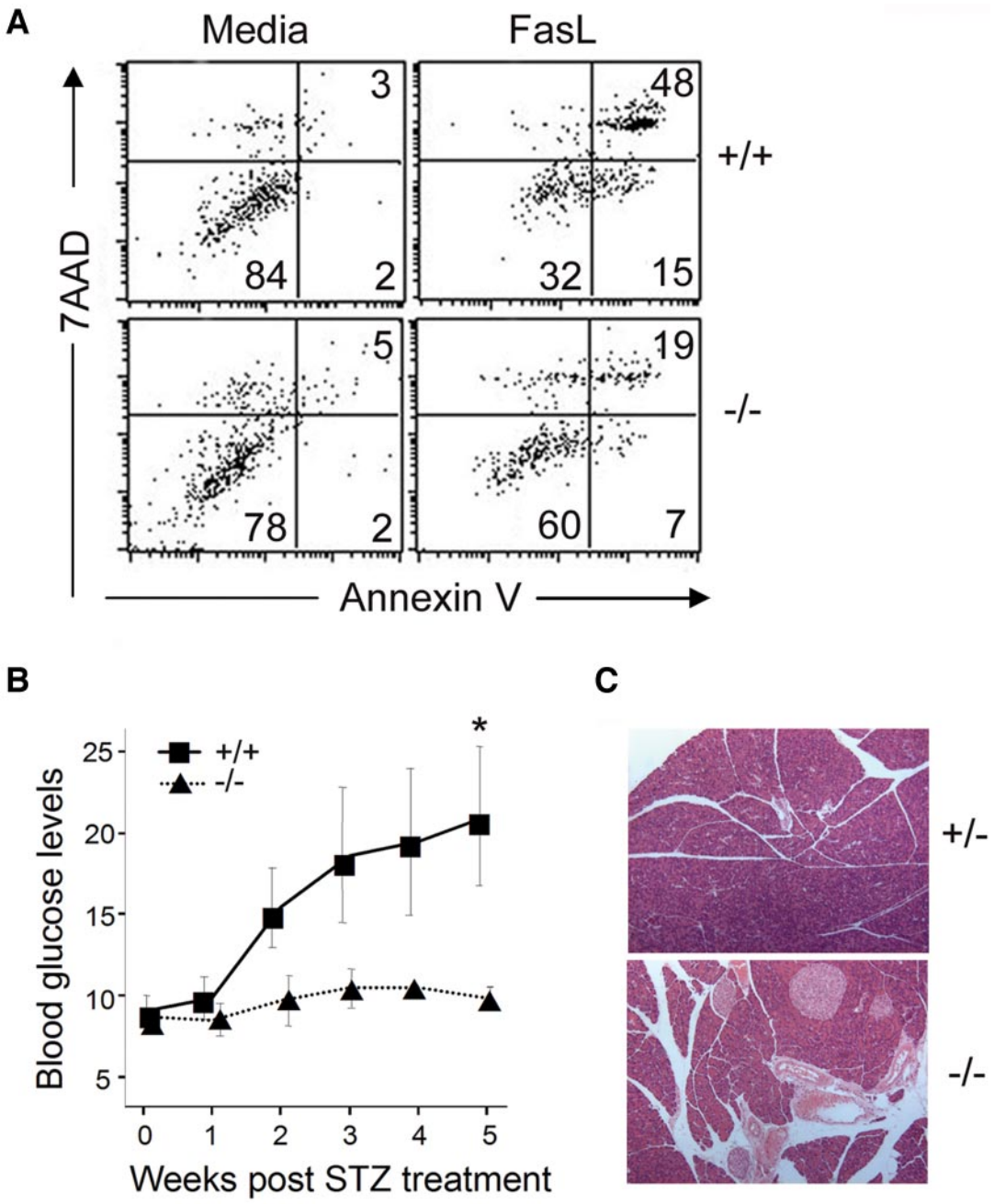

FIG. 2. Deletion of Casp8 in β-cells protects from FasL-induced killing in vitro and STZ-induced diabetes in vivo. *A*: Annexin V/7-AAD profiles of *RIPcre*$^{+}$*Casp8*$^{+/+}$ and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ islets treated with 10 μg/ml FasL. The results are representative of experimental groups containing three mice per genotype. *B*: *RIPcre*$^{+}$*Casp8*$^{+/+}$ (+/+) and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ (−/−) mice were injected with STZ, and tail vein glucose was monitored weekly ($n = 4$ mice/genotype, $^{*}P < 0.05$). *C*: H-E–stained pancreatic sections (magnification ×4) 4 weeks after STZ (two random sections examined per mouse, $n = 4$ mice/genotype). (Please see http://dx.doi.org/10.2337/db06-1771 for a high-quality digital representation of this figure.)

for insulin determination using a radioimmunoassay kit (Linco Research, St. Louis, MO). At the end of each perifusion, islets were collected and lysed in acid ethanol for assessment of insulin content. Results are presented as insulin secreted normalized to 60 islets and to total insulin content.

Membrane capacitance measurement and electrophysiology. Patch electrodes were pulled from borosilicate glass, coated close to the tip with orthodontic wax, and polished to a tip resistance of 2–4 MΩ when filled with intracellular solution, which contained 125 mmol/l potassium glutamate, 10 mmol/l KCl, 10 mmol/l NaCl, 1 mmol/l $MgCl_2$, 5 mmol/l HEPES, 0.05 mmol/l EGTA, 3 mmol/l MgATP, and 0.1 mmol/l cAMP and pH to 7.1. The extracellular solution consisted of 138 mmol/l NaCl, 5.6 mmol/l KCl, 1.2 mmol/l $MgCl_2$, 2.6 mmol/l $CaCl_2$, 5 mmol/l HEPES, and 5 mmol/l D-glucose and pH to 7.4. Cell membrane capacitance was estimated by the Lindau-Neher technique (21), implementing the "Sine + DC" feature of the lock-in module (40 mV peak-to-peak and a frequency of 500 Hz) in the whole-cell configuration. Recordings were conducted using an EPC10 patch-clamp amplifier and Pulse and X-Chart softwares (HEKA Electronik, Lambrecht, Germany). Exocytotic events were elicited by a train of ten 500-ms depolarization pulses (1-Hz stimulation frequency) from −70 to 0 mV. Voltage-dependent calcium (Ca_V) currents from the β-cells were measured in a whole-cell mode as described previously (22).

Statistical analysis. Data were analyzed by the one-sample *t* test and independent samples *t* test where appropriate. The statistical software package used was SPSS version 12.0.1 for PCs.

RESULTS

Deletion of Casp8 in pancreatic β-cells. To study the role of Casp8 in pancreatic β-cells, we generated β-cell–

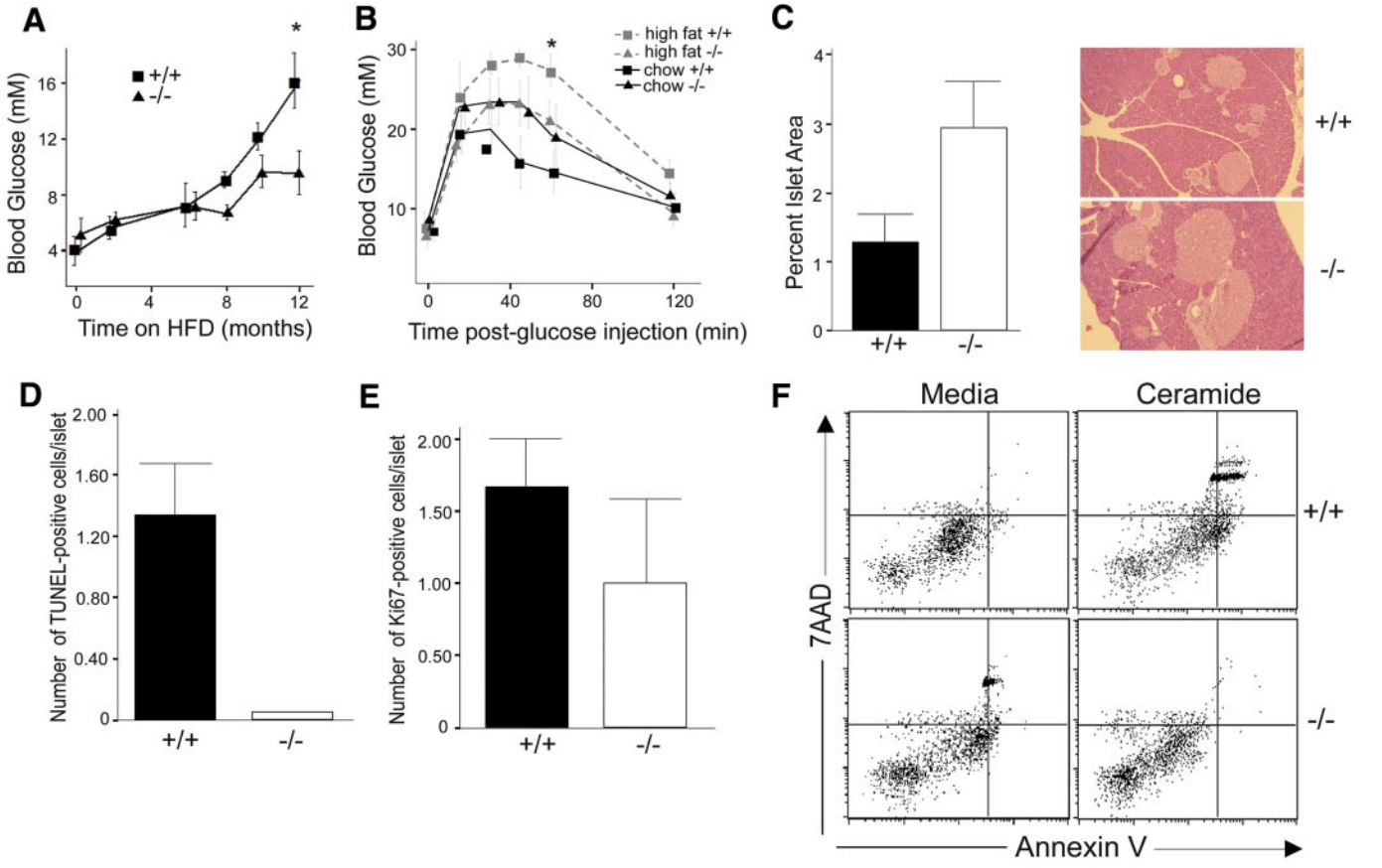

FIG. 3. *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice are protected from type 2 diabetes induced by HFD. *A*: *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice and *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice were fed HFD for 8–12 months. Fasting blood glucose levels were determined from *RIPcre*$^{+}$*Casp8*$^{+/+}$ (+/+) and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ (−/−) mice at 0, 6, and 12 months on HFD (n = 6 mice/genotype, *$P < 0.05$). *B*: At 12 months on HFD, *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ (−/−) mice exhibited improved glucose tolerance after intraperitoneal GTT compared with *RIPcre*$^{+}$*Casp8*$^{+/+}$ (+/+) mice (n = 10 mice/group, *$P < 0.03$). *C*: Quantification of the percent insulin-stained versus total pancreatic area for mice killed after 12 months on HFD (n = 6 mice/genotype, *$P < 0.05$). Representative pancreatic sections stained with H-E (magnification ×10). *D* and *E*: Quantification of the number of apoptotic (TUNEL-positive) cells (*$P < 0.05$) and proliferating cells (Ki67 positive, P = NS) per islet in *RIPcre*$^{+}$*Casp8*$^{+/+}$ (+/+) and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ (−/−) mice after 12 months on an HFD (n = 4 mice/genotype). *F*: Annexin V/7-AAD profiles of *RIPcre*$^{+}$*Casp8*$^{+/+}$ and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ islets treated with 50 μmol/l ceramide. The islets were isolated from 6-month-old mice on normal chow diet. The results are representative of experimental groups containing three mice per genotype. (Please see http://dx.doi.org/10.2337/db06-1771 for a high-quality digital representation of this figure.)

specific Casp8 knockout mice using the Cre-*loxP* system. These mice contained a null mutation for Casp8 in β-cells, as confirmed by immunofluorescence and Western blotting of lysates from isolated islets (Fig. 1*A* and *B*). In addition, we show that Casp8 is specifically deleted in β-cells and not in other tissues, including the hypothalamus (Fig. 1*C*). Consistent with previous reports using these *RIP-Cre* transgenic mice (23), efficient recombination events were observed in β-cells purified by FACS in *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice (Fig. 1*D*). *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice did not exhibit any changes in weight or activity (data not shown) compared with wild-type control mice, suggesting that hypothalamic function was not impacted in the mutant mice.

Deletion of Casp8 in β-cells protects from FasL-induced killing in vitro and STZ-induced type 1 diabetes in vivo. To test whether Casp8 is essential in the death receptor–meditated apoptotic pathway, we first examined *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ islets in a FasL-induced killing assay because Fas is a prototypical death receptor. Fas is not normally expressed in islets under static conditions; therefore, islets were incubated with interleukin-1β to upregulate Fas (24) and then treated with FasL to induce cell death. We showed a greater percentage of viability in islets of *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice (60%) compared with those of *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice (32%) in a FasL-induced killing assay in vitro (Fig. 2*A*). FasL-induced death was not completely abolished in the islets of *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice, perhaps because of the presence of cell death in both β- and non–β-cells. In addition, we examined the role of Casp8 in β-cell apoptosis in an STZ-induced diabetes model. Eight-week-old *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice given multiple low-dose STZ injections were protected from diabetes development (Fig. 2*B*). At baseline, *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice and *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice had similar blood glucose levels. H-E staining of pancreatic sections of *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice showed intact islets at 5 weeks after STZ treatment, whereas those of *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice showed virtually no evidence of islets (Fig. 2*C*).

***RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice are protected from HFD-induced diabetes.** β-Cell apoptosis also plays a significant role in the pathogenesis of type 2 diabetes. Therefore, to further examine the role of Casp8 in a type 2 diabetes model, an HFD was fed to *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ and *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice. The HFD model is characterized by many features of type 2 diabetes, including induction of insulin resistance, compensatory β-cell hyperplasia, and ultimately β-cell apoptosis from increased free fatty acids and glucose, leading to glucolipotoxicity (2,3,25,26). The mice were fed an HFD for 8–12 months. These mice showed a similar increase in body weight, with average body weight being 45 ± 1.5 and 43 ± 2 g for *RIPcre*$^{+}$

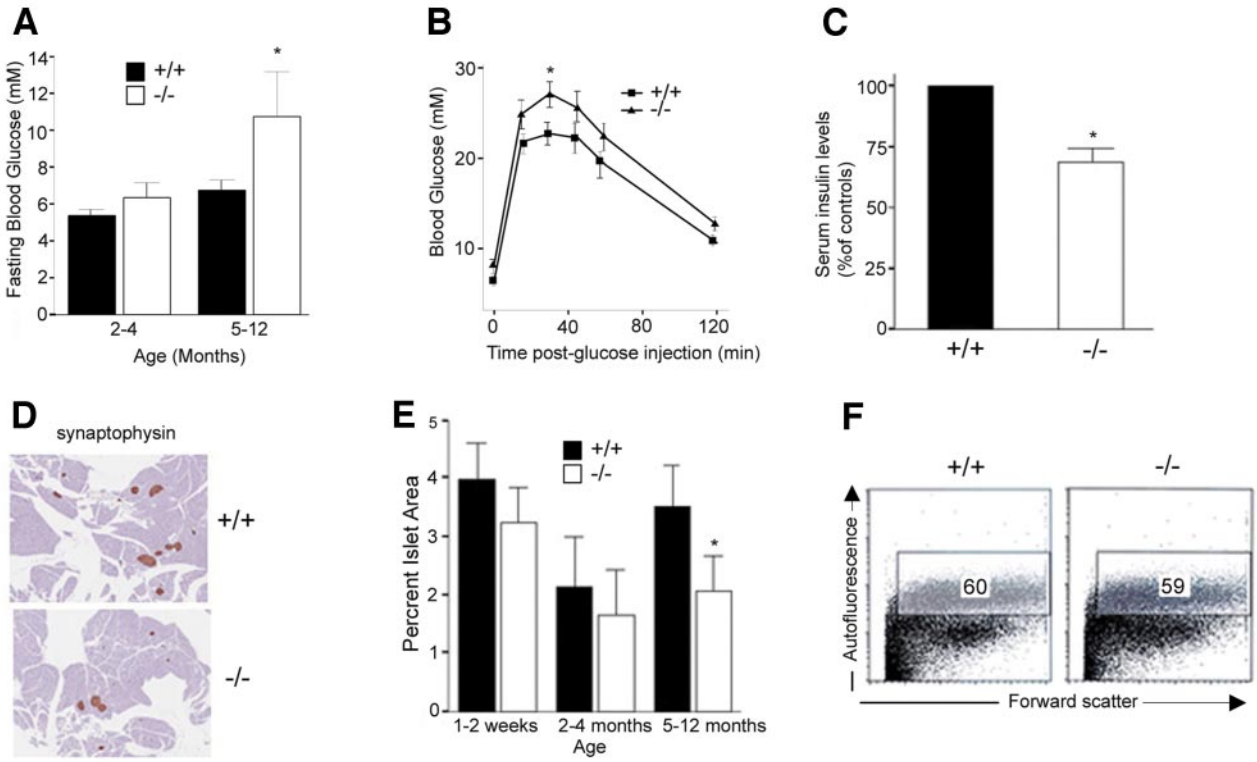

FIG. 4. *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice exhibit defects in glucose homeostasis and islet mass. *A*: At 5–12 months of age, *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ (−/−) mice displayed significantly increased fasting blood glucose levels compared with *RIPcre*$^{+}$*Casp8*$^{+/+}$ (+/+) mice (n = 13–20 mice per genotype, *P = 0.03). *B*: At 7–12 months, *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice exhibit increased glucose excursion after intraperitoneal GTTs compared with *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice (n = 13–22 mice/group, P = 0.04). *C*: Fasting serum insulin levels are lower in *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice compared with *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice >6 months of age (n = 7–10 mice/group, *P < 0.01). *D*: Representative pancreatic sections stained for synaptophysin at 12 months of age (magnification ×4). *E*: Quantification of the percent insulin-stained versus total pancreas area (for 1- to 2-week-old mice, n = 4 mice/genotype; for 2- to 4-month-old mice, n = 5–7 mice/genotype; for 5- to 12-month-old mice, n = 5–6 mice/genotype, *P < 0.01). *F*: FACS analysis of *RIPcre*$^{+}$*Casp8*$^{+/+}$and *RIPcre*$^{+}$*Casp8*$^{fl/f}$ islets. The autofluorescent population representing the β-cells is gated. Error bars indicate SE. (Please see http://dx.doi.org/10.2337/db06-1771 for a high-quality digital representation of this figure.)

Casp8$^{+/+}$ and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice, respectively, at the end of the HFD period. Insulin tolerance tests showed no difference in insulin sensitivity in mice after HFD between *RIPcre*$^{+}$*Casp8*$^{+/+}$ and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice (data not shown). We show that fasting blood glucose levels in *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice increased to a greater degree over the 12-month period compared with *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ on an HFD (Fig. 3*A*). No difference in GTTs was observed in the first 6 months on an HFD (data not shown), however, *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice became more glucose intolerant compared with *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice after 12 months on an HFD (Fig. 3*B*).

To examine whether Casp8 deletion in β-cells affected β-cell mass during the HFD, morphometric analyses were performed on pancreatic sections from mice on an HFD. In keeping with the better glucose homeostasis observed in *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice, there was greater islet area per total pancreatic area from *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice compared with *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice on an HFD for 12 months (Fig. 3*C*). The increased islet area observed in *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice was predominantly due to decreased apoptosis, whereas the difference in proliferation was not statistically significant (Fig. 3*D* and *E*). We further examined the role of Casp8 in ceramide-induced apoptosis because ceramide has been shown to be an inducer of free fatty acid–mediated apoptosis (26). Ceramide has been shown to activate both Fas-mediated and the intrinsic apoptotic pathways (27,28). A greater percentage of viability was observed in islets of *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice (90%) compared with those of *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice (46%) after treatment with ceramide in vitro (Fig. 2*F*).

***RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice display age-dependent defects in glucose homeostasis and β-cell mass.** In addition to examining the role of Casp8 in β-cell apoptosis, we also examined the physiological role of Casp8 in β-cells under physiological conditions. Up to 4 months of age, *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice displayed similar fasting blood glucose levels as *RIPcre*$^{+}$*Casp8*$^{+/+}$ littermate controls (Fig. 4*A*). Similarly, upon intraperitoneal glucose challenge, no differences in glucose excursions were noted between *RIPcre*$^{+}$*Casp8*$^{+/+}$ and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice at 2–4 months of age (data not shown). However, starting at ~5 months of age, *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice developed progressive fasting hyperglycemia (Fig. 4*A*) and worsening glucose tolerance as assessed by GTT compared with *RIPcre*$^{+}$*Casp8*$^{+/+}$ littermates (Fig. 4*B*). These abnormalities in glucose homeostasis in older *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice were accompanied by lower serum insulin levels than control littermates (Fig. 4*C*). To rule out any potential defects in insulin sensitivity that may have accounted for the abnormalities in glucose homeostasis in these older mice, insulin tolerance tests were done. The results showed similar glucose-lowering effects upon insulin challenge between *RIPcre*$^{+}$*Casp8*$^{+/+}$ and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice (data not shown), indicating that the defects in glucose homeostasis are attributed to a β-cell defect and not to changes in insulin sensitivity in *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice.

Defects in glucose homeostasis in *RIPcre*$^{+}$*Casp8*$^{fl/fl}$

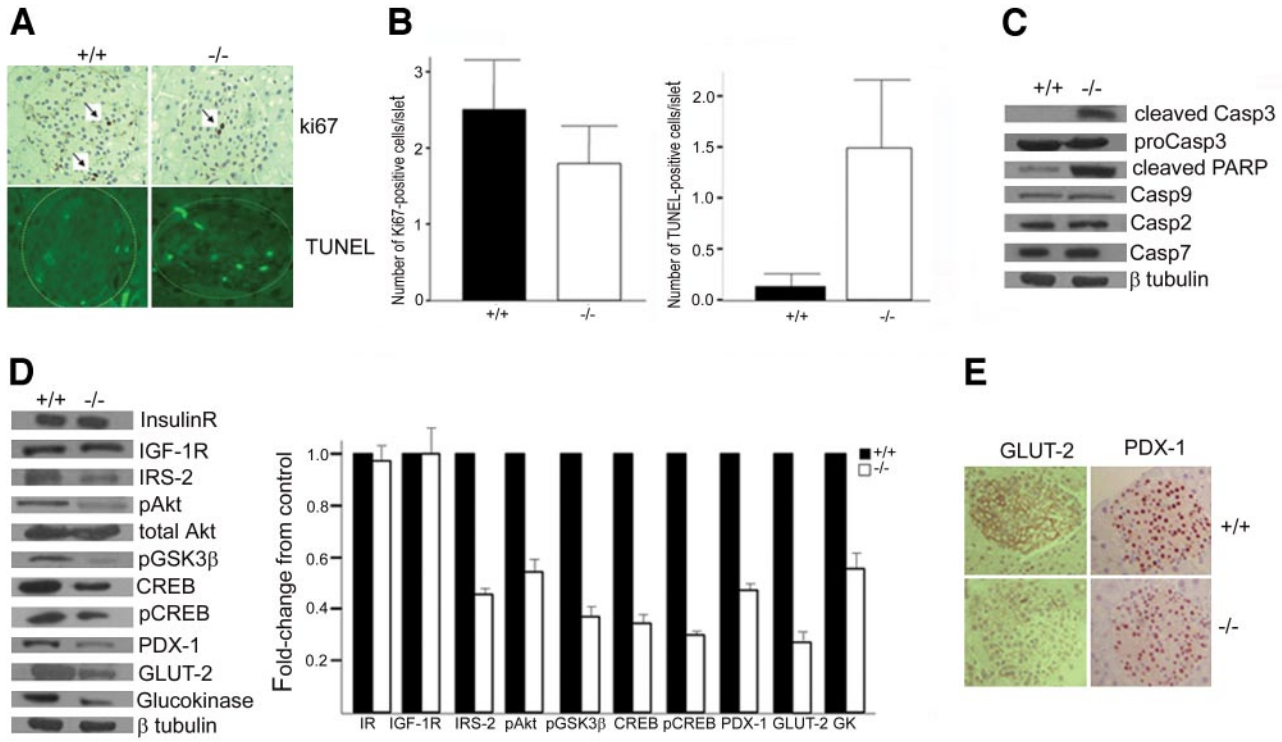

FIG. 5. Deletion of Casp8 in β-cells leads to defects in survival and decreased expression of molecules involved in insulin/IGF-I signaling and transcription factors important in β-cell survival. *A*: Immunohistochemical staining of pancreatic sections for Ki67 (magnification ×25) (*top*). TUNEL staining of pancreatic sections (magnification ×40) (*bottom*). Three random sections examined per mouse (n = 3 mice/genotype). *B*: Quantification of the number of apoptotic (TUNEL-positive) cells (*P < 0.05) and proliferating cells (Ki67-positive, P = NS) per islet in *RIPcre*$^{+}$*Casp8*$^{+/+}$ (+/+) and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ (−/−) mice at 6 months of age (n = 4 mice/genotype). *C*: Western blots of isolated islets from *RIPcre*$^{+}$*Casp8*$^{+/+}$ (+/+) and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ (−/−) mice showing increased cleaved Casp3 (17 kDa), cleaved poly (ADP-ribose) polymerase (70 kDa), proCasp3 (32 kDa), Casp9 (45 kDa), Casp2 (45 kDa), and Casp7 (35 kDa); n = 3 mice/genotype. *D*: Western blots (*left*) and quantification of Western blot signal (*right*) of isolated islets from *RIPcre*$^{+}$*Casp8*$^{+/+}$ (+/+) and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ (−/−) mice showing levels of insulin receptor (90 kDa), IGF-IR (90 kDa), IRS-2 (170 kDa), phospho-AKT (pAKT; 60 kDa), phospho-GSK3β (pGSK3β; 40 kDa), PDX-1 (43 kDa), phospo-CREB (pCREB; 43 kDa), GLUT2 (60 kDa), and glucokinase (47 kDa). *P < 0.05. *E*: Representative pancreatic sections showing decreased levels of PDX-1 and GLUT2 in the islets of *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice compared with *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice (n = 4 mice/genotype). Three independent experiments were performed on mice >5 months of age. (Please see http://dx.doi.org/10.2337/db06-1771 for a high-quality digital representation of this figure.)

mice may be due to either a reduction in islet β-cell mass or β-cell secretory capacity or both. We first examined for changes in β-cell mass in the *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice by morphometric analyses performed on pancreatic sections. There was a decrease in total islet area per total pancreatic area in *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice compared with *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice (Fig. 4*D* and *E*), which became significant at 5 months of age (Fig. 4*E*). There was also a decrease in the number of large islets per pancreatic area in the older *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice compared with *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice (data not shown). Cell sorting of islets by FACS showed that the proportion of β-cells within *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ islets was not perturbed (Fig. 4*F*).

Decreased β-cell mass in *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice is associated with an increase in cell death and a decrease in insulin/IGF-I signaling molecules. To determine whether a defect in proliferation or apoptosis accounted for the observed decrease in β-cell mass, Ki67 and TUNEL staining were performed on pancreatic sections from 6-month-old mice. There was no significant difference in the number of Ki67-positive β-cells between *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice and *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice (Fig. 5*A* and *B*). In contrast, *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice displayed more TUNEL-positive cells in islets compared with *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice (Fig. 5*A* and *B*). In keeping with the increase in TUNEL-positive cells, there was an increased level of active (cleaved) Casp3 and cleaved poly-(ADP-ribose) polymerase, a well-known substrate of Casp3, in islets of *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice compared with those of littermate *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice (Fig. 5*C*). The expression of proCasp3 and other pro-caspases were not increased in islets of *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice compared with those of littermate *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice (Fig. 5*C*).

Given that many molecules in the insulin/IGF-I signaling pathway have been shown to be essential in regulating β-cell mass (23,29–33), we examined the expression of the receptors and intracellular signaling proteins involved in this pathway. There was no difference in the expression of insulin receptor and IGF-IR between islets of *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice (Fig. 5*D*). However, islets of *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice showed a decrease in IRS-2 and phospho-AKT expression as assessed by Western blotting of protein lysates (Fig. 5*D*). We also observed a decrease in expression of pGSK3β, an evolutionarily conserved phosphorylation target of AKT (Fig. 5*D*).

Because PDX-1 is an important transcription factor in promoting β-cell survival (34), at least in part in response to growth factor signaling in β-cells (35), we assessed whether Casp8 deletion would affect expression levels of this transcription factor. PDX-1 expression was decreased in the islets of *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice compared with *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice (Fig. 5*D* and *E*). In addition, CREB, which has also been described as a critical tran-

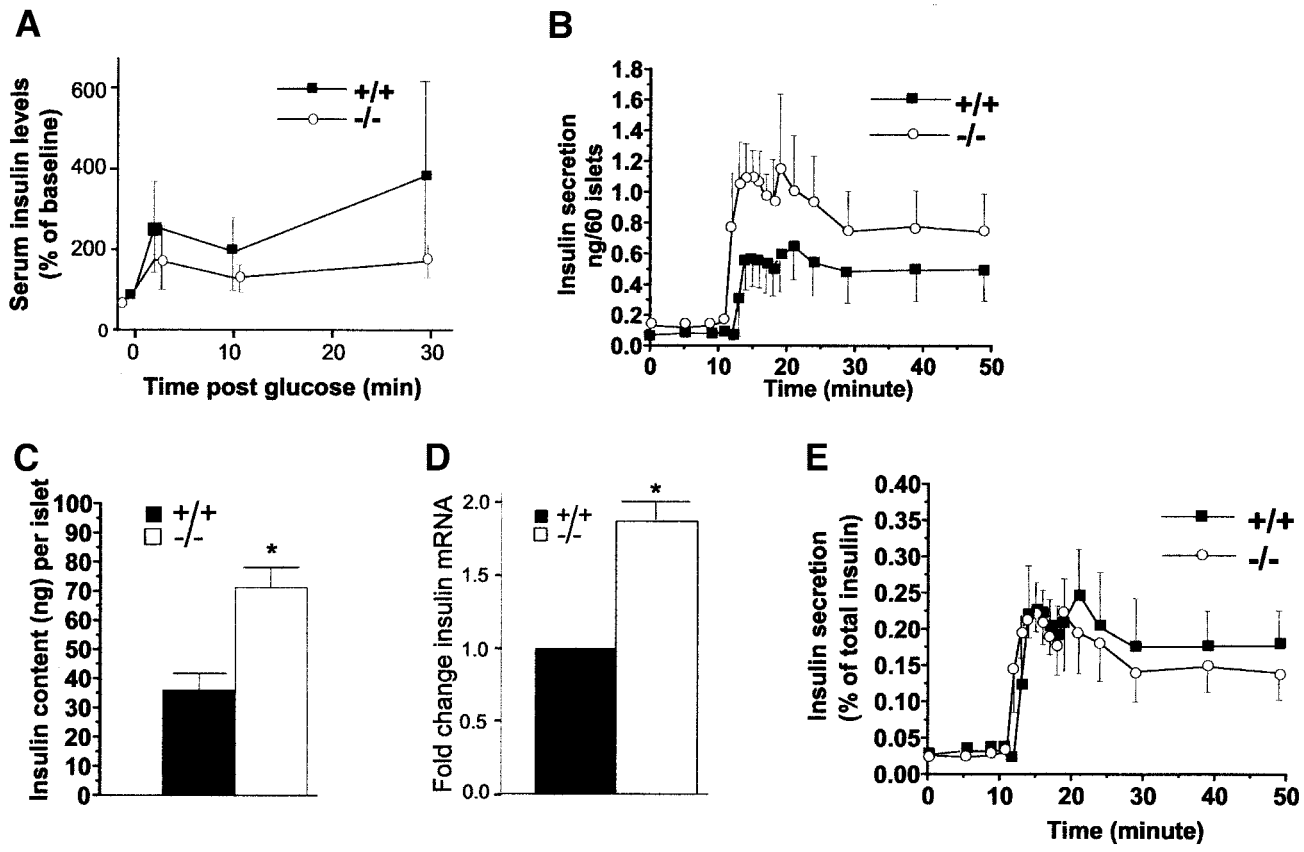

FIG. 6. Increased GSIS and insulin content in isolated islets of *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice. *A*: In vivo GSIS for older *RIPcre*$^{+}$*Casp8*$^{+/+}$ (+/+) and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ (−/−) mice (n = 8 mice/genotype, 8- to 12-month-old mice). *B*: In vitro GSIS was performed on isolated islets from older *RIPcre*$^{+}$*Casp8*$^{+/+}$ (+/+) and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ (−/−) mice by perifusion. Results presented relative to 60 islets (n = 4 mice) from two separate experiments. *C*: Total insulin content in islets of *RIPcre*$^{+}$*Casp8*$^{+/+}$ (+/+) and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ (−/−) mice (for 8- to 12-month-old mice, n = 8, *P = 0.0006). *D*: Quantification by RT-PCR of insulin transcript levels relative to β-tubulin control in isolated islets (n = 3 mice/genotype, 8- to 12-month-old mice, *P = 0.003). *E*: In vitro GSIS results presented relative to total insulin content.

scription factor for β-cell survival (36), was decreased in expression in *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ islets (Fig. 5*D* and *E*).

Islets of *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice exhibit increased secretory function. We first examined the expression of GLUT2 and glucokinase, glucose-sensing molecules that are important in GSIS and are decreased in expression in IGF-IR–deleted β-cells (29). Expression of GLUT2 (Fig. 5*D* and *E*) and glucokinase (Fig. 5*D*) were decreased in the islets of *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice. To further assess the role of Casp8 in β-cell function, we performed GSIS on our *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice. In vivo, there appeared to be a defect in GSIS when comparing *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice with *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice; however, these results were not significantly different and were difficult to interpret in the presence of a defect in β-cell mass (Fig. 6*A*). Therefore, we proceeded to examine individual islets to assess GSIS in vitro by perifusion. Isolated islets from older *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice displayed enhanced GSIS per islet compared with littermate controls (Fig. 6*B*). Furthermore, the islets of these *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice exhibited an increase in total insulin content (Fig. 6*C*) at the level of both protein and mRNA (Fig. 6*D*). When measurement of insulin secretion was normalized to insulin content, there was still a proportional increase in GSIS in *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ compared with *RIPcre*$^{+}$*Casp8*$^{+/+}$ islets (Fig. 6*E*).

Increased exocytosis of insulin in *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ islets. Increased insulin content does not ensure increased secretory capacity but requires an efficient exocytotic machinery to release the insulin. We therefore examined for key exocytosis soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor proteins on insulin granules, vesicle-associated membrane protein 2, plasma membrane syntaxin1A, and exocytosis priming protein Munc18a (37,38). In keeping with the increase in insulin secretion, all of these proteins were increased in the islets of *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice (Fig. 7*A* and *B*). Since these exocytotic proteins are known to directly influence the size and release probability of secretory granules (37), which is best measured by patch-clamp capacitance measurement (39), we used this assay to assess single-islet β-cells (Fig. 7*B–D*). The first two depolarization pulses on *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ β-cells, which determine the initial readily releasable pool of primed and fusion-ready insulin granules, were increased (17.37 ± 5.47 femtofarad/picofarad [fF/pF] at second pulse, n = 16) by ~170% ($P < 0.05$) compared with control cells (6.38 ± 1.27 fF/pF, n = 13). Furthermore, the incremental (after each depolarization) and cumulative increases in cell membrane capacitance between 3rd and 10th pulses (Δcell membrane capacitance$_{3rd–10th\ pulse}$), which measure the rate of insulin granule mobilization to the releasable pool, were similarly increased by ~190% (45.97 ± 11.36 vs. 15.92 ± 3.89 fF/pF in wild-type cells, $P < 0.05$). The increased GSIS of *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ β-cells is associated with enhanced exocytotic machinery and not with Ca^{2+} because Ca^{2+} influx via the L-type Ca^{2+} channels was not different from control littermate β-cells (Fig. 7*E*).

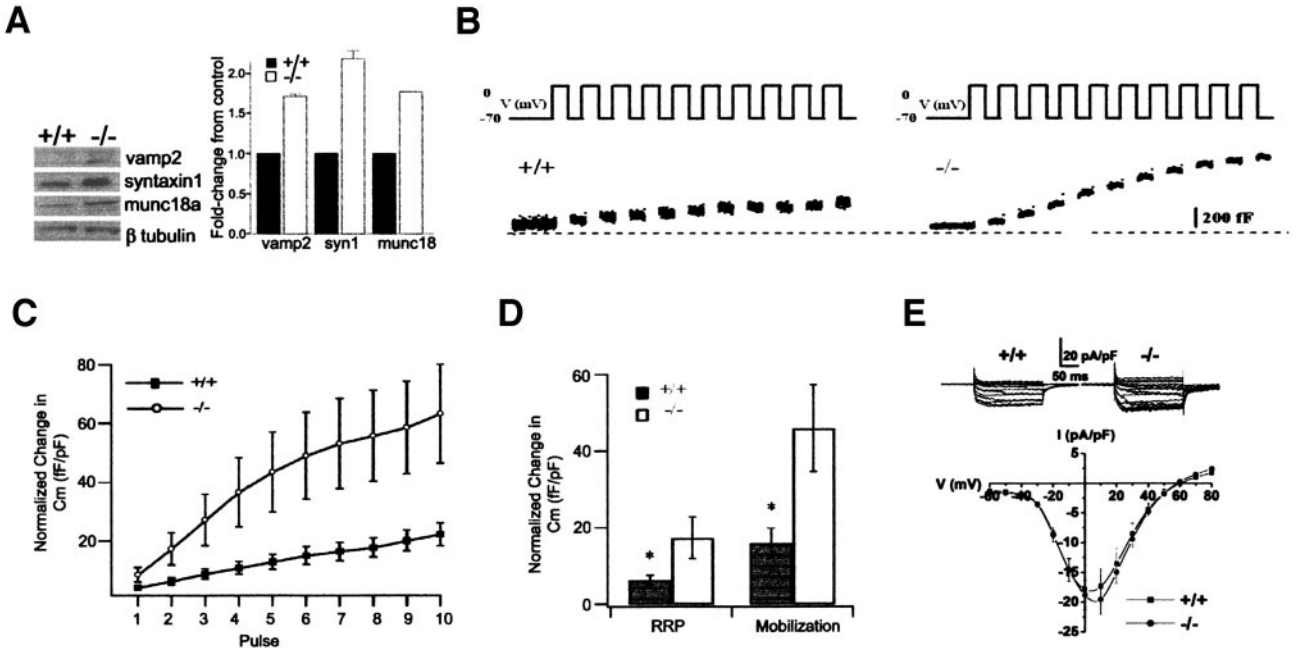

FIG. 7. Absence of Casp8 in β-cells leads to enhanced exocytosis of insulin. *A*: Western blots (*left*) and quantification of Western blot signals (*right*) of isolated islets from *RIPcre*$^{+}$*Casp8*$^{+/+}$ (+/+) and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ (−/−) mice showing increased levels of vesicle-associated membrane protein 2 (18 kDa), syntaxin 1A (syn1; 35 kDa), and munc18a (68 kDa), *$P < 0.05$. *B*: Changes in cell membrane capacitance (Cm) were measured from single β-cells using a train of 10 depolarizing pulses (500 ms in duration) from −70 to 0 mV. Representative recordings of ΔCm from a *RIPcre*$^{+}$*Casp8*$^{+/+}$ β-cell and *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ β-cell. *C*: Corresponding summaries of the changes in ΔCm normalized to basal cell membrane capacitance (fF/pF, = 13–16 cells). *D*: Summary of the size of readily releasable pool of insulin granules ($\Delta Cm_{1st-2nd\ pulse}$) and the rate of granules mobilization ($\Delta Cm_{3rd-10th\ pulse}$) ($n$ = 13–16 cells). Values represent the mean ± SE. *$P < 0.05$ for *RIPcre*$^{+}$*Casp8*$^{+/+}$ (+/+) vs. *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ (−/−) mice. *E*: Representative recorded voltage-dependent calcium currents (*top*) and the current-voltage relationship (*bottom*) (n = 10–12).

DISCUSSION

A tissue-specific gene-targeting approach was used in the current study to provide insights into the biological role of Casp8 in β-cells in vivo. Our results demonstrate the context specificity of Casp8 in β-cells under pathological and physiological conditions. Despite much evidence to support the essential role of β-cell apoptosis in maintenance of β-cell mass and in diabetes (1–3,40), the specific roles of individual caspases have not been comprehensively studied using rigorous genetic approaches in vivo. Much of the current knowledge suggesting the importance of caspase-dependent pathways in β-cell apoptosis are from in vitro studies (24,41). Here, we used the STZ-induced diabetes model to show that loss of Casp8 in β-cells protects mice from diabetes development. In an HFD-induced diabetes model, glucolipotoxicity from HFD feeding induces β-cell apoptosis, which is associated with increased Casp3 and Casp8 activity (25,26). Here, we show that upon prolonged feeding with an HFD, *RIPcre*$^{+}$*Casp8*$^{+/+}$ mice developed diabetes over the 12-month period on an HFD, whereas *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice were protected. These HFD-fed *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice displayed improved glucose tolerance and increased islet mass compared with control HFD-fed mice. Together with our results from STZ-induced diabetes, we show that Casp8 functions as an essential apoptotic molecule in the presence of apoptotic stimuli, and loss of Casp8 in β-cells leads to protection from diabetes in these experimental models.

In addition to apoptosis, caspases have also been shown to possess additional functions (7–12). In this study, we show that mice with deletion of Casp8 in β-cells develop an age-dependent decline in β-cell mass, which is associated with a paradoxical increase in cell death. Previous studies have shown that genes involved in insulin/IGF-I signaling are important in regulating β-cell mass and glucose homeostasis (23,31,32,42). β-Cell–specific insulin receptor knockout mice exhibit age-dependent defects in glucose homeostasis and defects in insulin secretion, which are associated with a decline in β-cell mass (23). Overexpression of AKT and IRS-2 in β-cells leads to increased β-cell mass, thus supporting the critical role of molecules downstream of insulin/IGF-I in regulating β-cell mass (31,42). Here, we show that Casp8 in β-cells regulates β-cell mass under physiological conditions, and these results are associated with decreased levels of molecules involved in insulin/IGF-I signaling, including IRS-2, AKT, and GSK3β. The decrease in these signaling molecules can also be explained by the decrease in the expression of CREB in our *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice because CREB can promote β-cell survival by transcriptionally upregulating IRS-2 (36). PDX-1 was also decreased in *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice, and, interestingly, the phenotype of these mice is similar to mice heterozygous for PDX-1 in β-cells (43). PDX-1$^{+/-}$ mice present with a similar decrease in β-cell mass as *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice, are nondiabetic although their blood glucose levels are in the upper range of normal, and are glucose intolerant. Whether a similar phenotype in insulin secretion is shared by *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ and PDX-1$^{+/-}$ mice is not yet known.

The role of Casp8 in insulin secretion appears to be quite complex. Islets of *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice exhibit decreased levels of glucose-sensing molecules GLUT2 and glucokinase; however, increased insulin transcription, insulin content, and exocytic capacity are observed at the level of individual islets. Interestingly, both IRS-2 and AKT have been previously shown to negatively regulate insulin secretion, while promoting β-cell growth. In

transgenic mice overexpressing AKT in β-cells, reduced insulin content per islet and impaired insulin secretion in response to glucose are observed in the presence of increased islet mass and enhanced glucose tolerance (31). Similarly, mice with β-cell–specific deletion of IRS-2 develop age-dependent glucose intolerance and reduced β-cell mass while exhibiting enhanced GSIS when individual islets were analyzed (44). Our findings are in keeping with these studies, whereby *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice present with defects in β-cell mass in the presence of enhanced insulin secretion. The increase in insulin secretion may be a compensatory mechanism to overcome β-cell mass; however, it is not sufficient to compensate for the reduction in islet mass of *RIPcre*$^{+}$*Casp8*$^{fl/fl}$ mice given their inability to sustain glucose homeostasis. Casp8 may also play a role in selectively removing vulnerable β-cells with aging; thus, in its absence, this vulnerable population of β-cells may accumulate, leading to increased β-cell death. This notion has been demonstrated with Fas (45); therefore, Casp8 may play a similar role in this regard.

Taken together, under physiological conditions, Casp8 is essential in the maintenance of β-cell mass and in regulating insulin secretion, whereas in the presence of apoptotic stimuli, such as in diabetic conditions, Casp8 is required for β-cell apoptosis. Our data highlight the context-specific diverse functions of Casp8 in controlling different facets of β-cell biology and raise the importance of a clear understanding of these apoptotic molecules because these are potential therapeutic targets for prevention of β-cell apoptosis in the treatment of diabetes.

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