Pancreatic β-Cell Failure Mediated by mTORC1 Hyperactivity and Autophagic Impairment

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Hyperactivation of the mammalian target of rapamycin complex 1 (mTORC1) in β-cells is usually found as a consequence of increased metabolic load. Although it plays an essential role in β-cell compensatory mechanisms, mTORC1 negatively regulates autophagy. Using a mouse model with β-cell-specific deletion of Tsc2 (βTsc2/−/−) and, consequently, mTORC1 hyperactivation, we focused on the role that chronic mTORC1 hyperactivation might have on β-cell failure. mTORC1 hyperactivation drove an early increase in β-cell mass that later declined, triggering hyperglycemia. Apoptosis and endoplasmic reticulum stress markers were found in islets of older βTsc2/−/− mice as well as accumulation of p62/SQSTM1 and an impaired autophagic response. Mitochondrial mass was increased in β-cells of βTsc2/−/− mice, but mitophagy was also impaired under these circumstances. We provide evidence of β-cell autophagy impairment as a link between mTORC1 hyperactivation and mitochondrial dysfunction that probably contributes to β-cell failure.

Nutrient overload is one of the main causes of insulin resistance. This triggers the compensatory mechanisms leading to β-cell mass increase and hyperinsulinemia. Hyperactivation of the mammalian target of rapamycin complex 1 (mTORC1) is elicited under nutrient overload conditions (1–3). mTORC1 plays a positive role in β-cell mass expansion (3–6), and rapamycin treatment impairs β-cell mass adaptation (7,8). On the other hand, mTORC1 hyperactivation is also a cause of insulin resistance (1) and endoplasmic reticulum (ER) stress (9), conditions linked with β-cell dysfunction and diabetes progression (10,11). We previously described how Tsc2 deletion in β-cells (βTsc2/−/−) results in chronic mTORC1 hyperactivation leading to a biphasic phenotype with early β-cell mass increase, hyperinsulinemia, and hypoglycemia. This was followed by β-cell failure and hyperglycemia in older mice (3). We also found how the first phase in βTsc2/−/− mice is characterized by both an increase of mitochondrial mass and enhanced glucose-stimulated insulin secretion (12). Autophagy is a cytoprotective mechanism also found essential for β-cell homeostasis (13,14). Autophagy plays a protective role under stress conditions, such as ER stress (15), and we and others have described its positive role in β-cells under these conditions (16,17). Autophagy is also responsible for the turnover of mitochondria by the specific elimination of defective or damaged organelles. mTORC1 is a critical negative regulator of autophagy (18), and studies have shown how TSC deficiency leads to impaired autophagy in human tumors and cell lines (19,20).

In this study, we explored the intriguing possibility that mTORC1 hyperactivity in β-cells, apart from being essential for β-cell compensatory mechanisms, might...
trigger β-cell failure when chronically elicited. This could be the consequence of the dysregulation of cytoprotective mechanisms, such as autophagy and mitochondrial turnover (mitophagy), and the development of insulin resistance and ER stress.

RESEARCH DESIGN AND METHODS

Animals

βTsc2fl/fl mice were obtained by crossing Tsc2fl/fl mice with those expressing Cre recombinase under rat insulin 2 gene promoter, as described before (3). Tsc2fl/fl were used as control animals. Animals were maintained as described before (3,12). For rapamycin treatment, βTsc2fl/fl mice 20–24 weeks of age were subjected to intraperitoneal injections of 2 mg/kg body weight rapamycin (LC Laboratories) or vehicle every other day. A 10 mg/mL stock solution of rapamycin was made in 100% ethanol, stored at 20°C, diluted to 0.5 mg/mL vehicle (5% Tween 80 and 5% polyethylene glycol), and used within 24 h. Only male mice were used, and all experiments were performed following the guidelines from the Animal Ethics Committee of Kobe University Graduate School of Medicine.

Cell Culture and Immunofluorescence of Cultured Cells

Cell culture of insulinoma-derived cell lines MIN6 and INS-1E was performed as described before (16). Reagents used for cell culture were rapamycin (LC Laboratories), tunicamycin, chloroquine, insulin, and IGF-I (Sigma-Aldrich). For immunofluorescence assays, cells were grown on glass

Cy3 were excited with a 555-nm laser and visualized with a 576- to 640-nm BP filter. Alexa Fluor 647 was excited with 639-nm laser and visualized with a long pass 640-nm filter. Appropriate controls were used for each experiment to confirm specific signals.

All images were obtained in a 1,024 × 1,024–pixel format, excitation speed was 0.5–5 μs/pixel, and pinhole size set to ~1 airy unit. Z-stacks were collected with 2.5-μm spacing (40× objective) or 1-μm spacing (63× objective). For microscope operation and image gathering, ZEN 2011 (Zeiss) software was used.

Selective Imaging of Active Mitochondrial Membrane Potential

After overnight incubation of islets in RPMI supplemented with 10% FBS, islets were further incubated for 30 min at 37°C in serum-free medium supplemented with 500 nmol/L MitoTracker Orange CMTRos (Life Technologies). Islets were washed twice with cold PBS and fixed as described before (21). MitoTracker CMTRos is only incorporated by actively respiring mitochondria and is irreversibly bound to mitochondrial proteins, allowing its use with antibodies for multiple staining after aldehyde fixation.

Immunohistochemistry and Morphometric Analysis

Immunofluorescence images of pancreatic sections stained with insulin and glucagon were gathered with a Biorzero BZ-8000 microscope (Keyence) and B2 Analyzer software. Images of five different sections per pancreas (spaced at least 200 μm) were analyze to allow stereologic determination of β-cell mass. At least five mice per age and genotype were used for immunohistochemical and β-cell mass determination assays.

Image Processing and Quantification

Image analysis was performed with NIH ImageJ software (http://rsb.info.nih.gov/ij/), ZEN 2011 software (Zeiss), and Amira (Visage Imaging). NIH ImageJ was used for densitometric quantification of Western blot results, islet diameter calculation of isolated islets, and determination of β-cell mass. ZEN 2011 was used for analysis of confocal microscope images. Amira was used to construct three-dimensional models from z-stacks and volumetric measurements.

Quantitative PCR

RNA was isolated from islets from separate control or βTsc2fl/fl mice using the RNeasy kit (Qiagen). cDNA synthesis and PCR amplification was performed in one step with SYBR Green PCR Master Mix (Life Technologies) and quantified with an ABI 7900 sequencer (Life Technologies) with the specific primers detailed in Supplementary Table 1.

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coverslips, and fixation and antibody incubation were performed as previously described (16).

**Lentivirus Production**

Human embryonic kidney 293T cells were cotransfected using Lipofectamine 2000 (Life Technologies) with lentiviral packaging plasmid pMD2.G (Addgene #12259) and psPAX2 (Addgene #12260) along with lentiviral vector pLKO.1-neo for short hairpin RNA (shRNA) production (Addgene #13425). Tsc2 and scrambled sequences (indicated in Supplementary Table 2) were cloned according to recommendations from Addgene as previously described (16).

**Electron Microscopy and Immunogold Labeling**

Pancreatic tissue was obtained from two mice per age and genotype and processed as previously described (12). Thin sections (60–70 nm) were obtained with an Ultracut E (Leica) ultramicrotome, stained with lead citrate, and examined under a JEM-1010 transmission electron microscope (JEOL).

Immunogold labeling of epoxy resin sections was performed according to standard methods. Briefly, ultrathin sections of pancreas embedded in epoxy resin were mounted onto nickel grids. To increase resin hydrophilicity, sections were incubated in drops of 4% (w/v) sodium metaperiodate and then 1% (w/v) periodic acid. This was followed by incubation in PBSG (PBS containing 50 mmol/L glycine), and nonspecific binding sites were blocked by incubation of sections in PBSG containing 5% (w/v) BSA. Antibodies were diluted in PBSG containing 1% BSA, guinea pig anti-p62/SQSTM1 (1:200) or guinea pig anti-insulin (1:200), the latter used as a positive control for the technique. Twelve-nanometer colloidal-gold donkey anti–guinea pig secondary antibody (1:200 in PBSG containing 1% BSA) was used (Jackson ImmunoResearch). Further staining and observation was performed as previously described (12).

**Mitochondrial Fractionation and Protein Oxidation Assays**

For detection of mitochondrial protein oxidation, 2 × 10^7 cells per condition were used. After trypsinization, centrifugation (3 min, 110g, 4°C), and PBS washing, mitochondria were isolated using the Mitochondria Isolation Kit for Cultured Cells (Thermo Scientific) according to manufacturer instructions. All buffers were enriched with protease inhibitors and 1% (volume/volume) 2-mercaptoethanol to avoid further oxidation of proteins after cell lysis. Mitochondrial pelleting was performed at low gravitational force to avoid peroxisome contamination of the mitochondrial fraction (15 min, 3,000g, 4°C). Mitochondrial pellets were lysed in 30 μL buffer containing 2% (w/v) CHAPS (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate), 10 mmol/L Tris-HCl, 150 mmol/L NaCl, and 1% (w/v) 2-mercaptoethanol, pH 7.5. Protein concentration was determined, and 10 μg were used for either direct Western blot analysis or derivatization with OxyBlot kit (Merck Millipore).

**Statistical Analysis**

Statistically significant differences between mean values were determined using paired Student t test for paired comparisons. Differences were considered statistically significant at P < 0.05.

**RESULTS**

**Tsc2 Ablation in β-Cells Results in a Biphasic Phenotype**

Consistent with our previous study (3), young βTsc2−/− mice became hypoglycemic as a consequence of increased β-cell mass. However, after 40 weeks, β-cell mass declined, and hyperglycemia appeared (Fig. 1A and B). Islet morphology analysis showed diminished β-cell composition in older βTsc2−/− islets (Fig. 1C and Supplementary Fig. 1A) as well as the presence of cleaved caspase-3 in pancreatic slides and islet lysates (Fig. 1D and E). We sought to discern the mechanisms responsible of β-cell failure in older βTsc2−/− mice.

**mTORC1 Hyperactivation in β-Cells Causes Cell Hypertrophy and Insulin Resistance**

Tsc2 ablation led to mTORC1 hyperactivation in β-cells; protein lysates from isolated βTsc2−/− islets showed increased phosphorylation of key proteins downstream of mTORC1, such as S6 kinase (S6K) and the ribosomal S6 protein (Fig. 2A). Volumetric analyses in 24-week-old islets showed a fivefold increase in β-cell volume as well as a doubled β-cell nuclei volume in βTsc2−/− islets (Fig. 2B and Supplementary Movies 1 and 2). α-Cell volumes were also slightly increased, probably as a consequence of hyperinsulinemia or other compensatory mechanisms (22). Distribution of islet sizes is highly shifted in young βTsc2−/− mice, with a mean diameter in control mice of 201 ± 10 μm and 281 ± 20 μm for βTsc2−/− islets (Supplementary Fig. 1B–D).

Tsc2 ablation and the consequent mTORC1/S6K hyperactivation induces insulin resistance by Ser/Thr phosphorylation of insulin receptor substrates (1,2,23). MIN6 cells stably expressing Tsc2-shRNA showed impaired Akt phosphorylation in response to insulin or IGF-I, which was not fully recovered by rapamycin treatment (Fig. 2C), consistent with other reports (24). Insulin and IGF-I signaling are necessary for the correct functioning and maintaining of β-cell mass (11). FoxO1 is an important player in β-cell proliferation and stress response, being normally excluded from the nucleus in β-cells by autocrine/paracrine insulin signaling, Akt directly phosphorylates FoxO1, driving its nuclear exclusion (25). In βTsc2−/− islets, increased mTORC1 activity impaired FoxO1 nuclear exclusion and partly recovered after rapamycin treatment (Fig. 2D); these observations were consistent in islets from βTsc2−/− mice of 8, 24, and 40 weeks of age (data not shown). Similar observations were performed in MIN6 Tsc2-shRNA cells. Nuclear localization and impaired phosphorylation of FoxO1 Thr21 was observed and partly recovered after rapamycin treatment (Supplementary Fig. 2A and B). Insulin signaling and FoxO1 in β-cells also modulated Pdx1 (25); immunofluorescence of pancreatic islets showed decreased Pdx1-positive nuclei in βTsc2−/− throughout life but recovered after rapamycin treatment (Fig. 2E).
Tsc2 Ablation in β-Cells Triggers the Unfolded Protein Response

Previous reports have shown how Tsc2- or Tsc1-deficient fibroblasts or human tumors display increased activation of the unfolded protein response (9). Islets from 40-week-old βTsc2−/− mice showed increased eukaryotic initiation factor 2a kinase 3 (Eif2ak3/PERK) phosphorylation as well as Eif2a Ser51 phosphorylation and activating transcription factor 4 (ATF4) expression (Fig. 3A). The ER chaperone heat shock protein 5 (Hspa5/BiP) was also increased together with some transcription factors associated with apoptotic outcome after ER stress, such as the DNA damage-inducible transcript 3 (Ddit3/CHOP) or the CCAAT/enhancer-binding protein β (Cebpβ) (Fig. 3A). Transcripts of Hspa5, Ddit3, and Cebpβ were increasingly expressed in βTsc2−/− islets in an age-dependent manner (Fig. 3B). Ddit3 expression was only found in islet protein extracts from older βTsc2−/− mice, whereas p62/SQSTM1 was increased in both, as discussed in the next section (Fig. 3C). Ddit3 was found specifically in β-cell nuclei of older βTsc2−/− islets (Fig. 3D) as was the phosphorylated form of Eif2a (Fig. 3E). Increased ER stress in β-cells has been related to accumulation of polyubiquitinated proteins (26). βTsc2−/− islets showed an increased profile of protein ubiquitination by Western blot or immunohistochemical analyses of pancreatic sections (Supplementary Fig. 3A and B).

Impaired Autophagy in βTsc2−/− Islets

mTORC1 is a negative regulator of autophagy (18); thus, chronic upregulation of mTORC1 due to Tsc2 ablation is expected to impair autophagy. βTsc2−/− islet protein extracts showed no differences in basal lipidation of LC3B; however, basal levels of the adaptor protein p62/SQSTM1 were markedly increased, indicating a defect in the clearance of autophagic substrates (Fig. 4A). Immunofluorescence analysis of isolated islets revealed an increased number and diameter of p62/SQSTM1-positive puncta in βTsc2−/− islets, specifically in β-cells (Fig. 4B and E). Ex vivo challenge of isolated islets to ER stress-induced autophagy showed impaired response in βTsc2−/− islets. mTORC1 activity, seen as phosphorylation of S6, was not downregulated after tunicamycin treatment, and lipidation of LC3B was not increased as observed in control islets (Fig. 4C). Chloroquine was used as a tool to estimate autophagic flux, which was not increased in βTsc2−/− after

Figure 1—Biphasic response of β-cell mass and blood glucose levels in βTsc2−/− mice. A: Blood glucose concentration at the indicated ages. B: β-Cell mass calculated from pancreatic sections immunostained with anti-insulin antibodies. Positive insulin area divided by the total area of the pancreas. C: Representative images of islets from pancreatic sections stained with anti-insulin antibodies for β-cells (red), and antiglucagon for α-cells (green). Scale bars, 50 μm. D: Pancreatic sections of 35-week-old mice were subjected to immunohistochemistry with an antibody detecting the cleaved form of caspase-3. E: Protein extracts of isolated islets from 40-week-old animals were subjected to Western blot analysis and incubated with specific antibodies. Densitometric quantification of cleaved caspase-3 levels is shown (n = 3). Data are mean ± SD (n = 3–8 per age and model). *P < 0.05, **P < 0.01, ***P < 0.001. KO, knockout.
Figure 2—mTORC1 hyperactivation in β-cells induces cell hypertrophy and insulin resistance. A: Islet protein extracts were subjected to Western blot analysis with specific antibodies. B: Isolated islets from 24-week-old mice were incubated with specific antibodies for identification of β-cells (insulin, red), α-cells (glucagon, green), and membranes (β-catenin, blue). Nuclei were stained with DAPI and visualized in white. Multiple z-stack images were collected, which allowed for three-dimensional reconstruction and volume measurements, as indicated in the graphs, and expressed in cubic micrometers as mean ± SD. C: MIN6 cells stably expressing Tsc2-shRNA or control shRNA (scrambled) were serum starved for 3 h, with the addition of rapamycin 20 nmol/L where indicated, and subsequently stimulated with insulin or IGF-I for 5 min. D: Isolated islets were cultured for 24 h in RPMI containing 10% FBS and subjected to
acute ER stress induction. Similar results were observed in the MIN6 Tsc2-shRNA cell line (Supplementary Fig. 4A). Immunofluorescence analysis of ER stress–challenged islets also showed impairment of LC3B puncta upregulation in βTsc2−/− islets (Fig. 4E). Additionally, glucose deprivation was used as a proautophagic stimulus. In
Figure 4—Autophagy is impaired in βTsc2⁻/⁻ islets. A: Protein extracts from 24-week-old isolated islets were subjected to Western blot analysis. p62/SQSTM1 levels are represented in the graph as mean ± SD (n = 3). B: Isolated islets from 24-week-old mice were subjected to immunostaining with anti-p62/SQSTM1 antibody. Representative confocal microscopy images are shown. Representation of the number and diameter of puncta per islet is mean ± SD. C: Isolated islets were stabilized overnight in RPMI containing 10% FBS and treated ex vivo with tunicamycin 2 μg/mL for 20 h in the presence or absence of chloroquine 10 μmol/L. Protein extracts were subjected to Western blot analysis, and images from a representative experiment are shown (n = 3). D: Representative confocal microscopy image showing specific accumulation of p62/SQSTM1 puncta in β-cells of βTsc2⁻/⁻ islets. E: Representative confocal microscopy images of the same
pancreatic islets, both energy stress and loss of autocrine insulin signaling may trigger autophagy after glucose withdrawal. Glucose starvation did not properly trigger an increase of LC3B-positive puncta in \( \beta Tsc2^{2/2} \) islets compared with controls (Supplementary Fig. 4B). Of note, \( \alpha \)-cells within \( \beta Tsc2^{2/2} \)-starved islets showed a marked upregulation of LC3B puncta, meaning that autophagy is only impaired in \( \beta \)-cells within \( \beta Tsc2^{2/2} \) islets (Supplementary Fig. 4C).

**Increased Colocalization of p62/SQSTM1 With Mitochondria in \( \beta Tsc2^{2/2} \) Islets**

Our previous work showed how mTORC1 hyperactivation causes an increase in mitochondrial biogenesis in \( \beta \)-cells of 10-week-old \( \beta Tsc2^{2/2} \) mice (12). Immunohistochemical analyses showed how mitochondrial markers are also increased in 35-week-old \( \beta Tsc2^{2/2} \) mice (Supplementary Fig. 5A). The finding of impaired autophagy prompted us to investigate a possible defect in autophagic clearance of mitochondria (mitophagy). Colocalization of mitochondrial structures with the adaptor molecule p62/SQSTM1 was evident in \( \beta \)-cells of \( \beta Tsc2^{2/2} \) mice (Supplementary Fig. 5B). Tsc2 downregulation in MIN6 cells also increased the number of p62/SQSTM1-positive mitochondria but not in the presence of rapamycin (Supplementary Fig. 5C). Moreover, the findings for these mitochondrial structures in \( \beta Tsc2^{2/2} \) islets were negative for a mitochondrial membrane potential (\( \Delta \psi _{m} \))–sensitive probe (Fig. 5A and Supplementary Movies 3 and 4). Quantification of these mitochondrial structures (Tom20 positive) with collapsed \( \Delta \psi _{m} \) (MitoTracker negative) showed an age-dependent increase of p62/SQSTM1 colocalization in \( \beta \)-cells of \( \beta Tsc2^{2/2} \) islets.

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**Figure 5** — Accumulation of low \( \Delta \psi _{m} \) and p62/SQSTM1-positive mitochondria in \( \beta Tsc2^{2/2} \) islets. A: Isolated islets were stabilized overnight in RPMI containing 10% FBS and then incubated for 30 min in medium containing MitoTracker (MTR) Orange CMTMRos 500 nmol/L and subsequently subjected to immunofluorescence assays with specific antibodies. Representative images of isolated islets from 40-week-old mice are shown. Arrows indicate collapsed \( \Delta \psi _{m} \) (MTR-negative) and p62/SQSTM1-positive mitochondria. B: Quantification of the number of MTR-negative and p62/SQSTM1-positive mitochondrial structures (Tom20 positive). Number per cell is mean per islet ± SD (several islets from 3–5 mice per age and genotype). C: Isolated islets from mice treated with rapamycin or vehicle were subjected to staining with Cox4 and p62/SQSTM1, as shown in Supplementary Fig. 5D. Bars show the average number of colocalizing spots per cell expressed as mean per islet ± SD. \(* P < 0.05, ** P < 0.01, *** P < 0.001. KO, knockout.*
mice (Fig. 5B), whereas the number of positive \( \Delta \psi_m \) mitochondrial structures (MitoTracker and Tom20 positive) colocalizing with p62/SQSTM1 was anecdotic and age and model independent (data not shown). \( \beta Tsc2^{-/-} \) mice that were subjected to rapamycin treatment showed a great reduction of p62/SQSTM1-positive mitochondria (Fig. 5C and Supplementary Fig. 5D). p62/SQSTM1 can bind to protein aggregates or damaged organelle, allowing recognition by the autophagic machinery (27), and its accumulation is a marker of impaired autophagy (13,14). Such differences observed between control and \( \beta Tsc2^{-/-} \) islets could only be explained by assuming deficient mitophagy in \( \beta \)-cells due to mTORC1 hyperactivation.

Moreover, electron micrographs from 35-week-old mice showed mitochondrial abnormalities in \( \beta \)-cells from \( \beta Tsc2^{-/-} \) mice. Mitochondrial mass was clearly higher in 35-week-old mice compared with controls, as previously reported in 10-week-old mice (12). However, mitochondria of \( \beta \)-cells in older \( \beta Tsc2^{-/-} \) mice showed a higher degree of complexity as well as highly dilated cristae, which were frequently found with abnormal orientation (Fig. 6). Signs of degenerated mitochondria were evident in electron micrographs of \( \beta \)-cells from 35-week-old \( \beta Tsc2^{-/-} \) mice and hardly found in either control or 10-week-old \( \beta Tsc2^{-/-} \) mice (Fig. 6 and Supplementary Fig. 6). In addition, immunogold labeling assays revealed p62/SQSTM1-positive mitochondria in \( \beta \)-cells of 35-week-old \( \beta Tsc2^{-/-} \) mice (Fig. 6F and G and Supplementary Fig. 7).

**Inefficient Mitophagy Caused by Tsc2 Ablation Produces the Accumulation of Oxidized Mitochondrial Proteins**

To assess consequences of impaired mitophagy in \( \beta Tsc2^{-/-} \) islets, pancreatic sections were probed with an antibody recognizing oxidized proteins (nitrotyrosine); \( \beta Tsc2^{-/-} \) islets showed increased immunoreactivity compared with reactivity in exocrine pancreas or control islets (Fig. 7A). Moreover, mitochondrial protein extracts were used for the detection of oxidized proteins. Both MIN6 and INS-1E stably expressing Tsc2-shRNA showed increased protein oxidation compared with controls (Fig. 7B). Mitochondrial protein oxidation was reduced by rapamycin treatment (Fig. 7C). In addition, autophagy was found to play a role in the clearance of oxidized mitochondrial proteins. An increased pattern of oxidized proteins was only observed after chemical inhibition of autophagy in control MIN6 cells, not in Tsc2-shRNA cells, indicating basal impairment of autophagic mitochondrial clearance in Tsc2-shRNA MIN6 cells (Fig. 7D). Immunofluorescence assays also showed increased colocalization of

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**Figure 6**—Electron micrographs of \( \beta \)-cells from 35-week-old mice. A and B: Control mice, normal mitochondrial mass, complexity and morphology. C–H: \( \beta Tsc2^{-/-} \) mice. C and D: Increased mitochondrial mass and complexity, degeneration of mitochondria. E: Degenerated mitochondria, highly dilated cristae. F: Aberrant orientation of cristae in fused mitochondria, indicated by arrow. G: Immunogold labeling using anti-p62/SQSTM1 antibody; boxed region is magnified in H, arrows indicate gold particles.
DISCUSSION

mTORC1 signaling in β-cells is essential for the adaptive mechanisms that allow β-cell mass increase and compensatory hyperinsulinemia. In β-cells, TSC2/mTORC1 integrates glucose and growth factor signaling to control protein synthesis and cell growth and proliferation (23). mTORC1 hyperactivation occurs as a consequence of increased metabolic load, being a feature of obesity-associated type 2 diabetes progression (1–3). Therefore, we aimed to study the consequences of chronic mTORC1 hyperactivation in β-cells due to Tsc2 ablation.

In young βTsc2−/− mice, increased β-cell mass due to striking hypertrophy was found. However, as previously reported, β-cell mass failure and hyperglycemia became evident from 40 weeks (3). Of note, other authors using another strain of βTsc2−/− mice did not observe this biphasic behavior (4). Rapamycin treatment of βTsc2−/− mice from 20 weeks of age abolished β-cell failure (3).

On the other hand, moderate hyperactivation of mTORC1 in β-cell–specific Rheb-overexpressing mice was shown to play a protective role against diabetes development (5). Although disruption of signaling downstream of mTORC1 in β-cells classically leads to diminished β-cell mass and glucose intolerance (28), increased β-cell apoptosis is also observed in mice expressing a constitutively active form of S6k1 (29).

In the current study, the fact that β-cell failure and hyperglycemia are only apparent in old βTsc2−/− mice (9 months old) is consistent with other studies linking mTORC1 hyperactivity to late-onset pathologies or deficiencies (30,31). Other reports described mTORC1 as a key regulator of aging (32,33); thus, increased mTORC1 signaling might accelerate aging and the development of aging-related pathologies.

The current results point to diverse deleterious consequences due to mTORC1 hyperactivation. First, in β-cells of βTsc2−/− mice, insulin resistance is observed throughout life, leading to impaired FoxO1 nuclear exclusion and decreased Pdx1-positive nuclei. These events are
linked to β-cell dysfunction (25,34) and increased β-cell susceptibility to ER stress (35,36). Additionally, ER stress in βTsc2−/− islets becomes evident with aging. Several reports linked the increased insulin demand in insulin-resistant states, with ER stress development resulting from the large load placed in the β-cell ER. This finally causes the expression of Ddit3 and Cebpb, leading to β-cell failure (10,37).

Hyperactivation of mTORC1 in β-cells impairs basal autophagy, as p62/SQSTM1 levels were found increased in β-cells within islets. Moreover, autophagic response to ER stress challenge was also impaired in βTsc2−/− islets. Reports have indicated how autophagy is an important protective mechanism in β-cells under ER stress (16,17). Autophagic impairment might be the underlying cause of ER stress development (17), which becomes evident with aging in βTsc2−/− mice or in other TSC-deficient models (9).

mTORC1 hyperactivation leads to enhanced mitochondrial mass and oxidative function (12,38), but in the current study, we also found impaired mitophagy due to Tsc2 ablation. Accumulation of mitochondrial structures with collapsed mitochondrial membrane potential and positive for the adaptor protein p62/SQSTM1 was evident, and those structures were found in higher numbers in an age-dependent manner and decreased by rapamycin treatment. p62/SQSTM1 plays an important role in the recognition and grouping of mitochondria before their incorporation into the autophagic machinery (39,40). Antiautophagic activity of mTORC1 is conducted though interaction with ULK1 complex (41,42), which is consistent with similar observations between βTsc2−/− islets and Ulk1−/− fibroblasts (43) where increased mitochondrial mass together with accumulation of p62/SQSTM1 is attributed to inefficient mitophagy.

Aberrant and degenerated mitochondria were found in β-cells from older βTsc2−/− mice, and atypical cristae and highly interconnected mitochondria were frequently seen on electron micrographs. Increased fusion of mitochondria has been proposed as a defense mechanism against cellular aging (44) and deleterious conditions that might trigger apoptosis (45), also enabling mitochondrial function under these conditions (46). Chronic mTORC1 hyperactivity leading to impaired mitophagy is likely the underlying cause of increased mitochondrial degeneration and elongation observed in β-cells from older βTsc2−/− mice. Although mTORC1 plays a well-described role in mitochondrial biogenesis (12,38), its particular impact on mitochondrial dynamics is understudied. Consistent with the results shown here, another report showed how mTORC1 inhibition is also required for mitophagy (47).

Other reports have shown how impaired mitophagy leads to the accumulation of dysfunctional mitochondria and excess production of reactive oxygen species (48,49). βTsc2−/− islets also showed increased oxidation of proteins, and by using a cellular model of mTORC1 hyperactivation in β-cells, we confirmed the finding of increased oxidation of mitochondrial proteins. Mitochondrial dysfunction is a characteristic feature of β-cell failure (50). Other reports have also linked insulin resistance and FoxO1 activity with mitochondrial dysfunction (51).

We cannot fully determine one specific cause responsible for β-cell failure under mTORC1 hyperactivity. Still, the experimental evidence points to autophagic impairment as the major cause. Rapamycin treatment prevents β-cell failure in this model (3), and we show how it ameliorates autophagic deficiency. However, rapamycin cannot fully restore insulin signaling toward mTORC2/Akt, consistent with other reports (24,52). Therefore, we believe that other deleterious events overshadowed by autophagic impairment are the leading causes of β-cell failure in aged βTsc2−/− mice.

In conclusion, mTORC1 hyperactivation may, in a first stage, play a positive role in β-cell compensatory mechanisms leading to hypertrophy, increased mitochondrial mass, and hyperinsulinemia (Fig. 8). However, we report
on how chronic activation of these compensatory mechanisms driven by mTORC1 may result in insulin resistance in β-cells, ER stress, autophagic impairment, and accumulation of dysfunctional mitochondria that could lead to β-cell failure and diabetes onset.

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