Andras Franko,[1,2,3] Peter Huypens,[1,3] Susanne Neschen,[1,3,4] Martin Irmler,[1] Jan Rozman,[1,3,4] Birgit Rathkolb,[1,4,5] Frauke Neff,[1,6] Cornelia Prehn,[7] Guillaume Dubois,[1] Martina Baumann,[1] Rebecca Massinger,[1] Daniel Gradinger,[1,3] Gerhard K.H. Przemeck,[1,3] Birgit Repp,[8] Michaela Aichler,[9] Annette Feuchtinger,[9] Philipp Schommers,[10,11] Oliver Stöhr,[12] Carmen Sanchez-Lasheras,[13] Jerzy Adamski,[3,7,14] Andreas Peter,[2,3,15] Holger Prokisch,[8] Johannes Beckers,[1,3,16] Axel K. Walch,[9] Helmut Fuchs,[1,3,4] Eckhard Wolf,[5] Markus Schubert,[12,17] Rudolf J. Wiesner,[10,18,19] and Martin Hrabě de Angelis[1,3,4,16]

Bezafibrate Improves Insulin Sensitivity and Metabolic Flexibility in STZ-Induced Diabetic Mice

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Bezafibrate (BEZ), a pan activator of peroxisome proliferator–activated receptors (PPARs), has been generally used to treat hyperlipidemia for decades. Clinical trials with type 2 diabetes patients indicated that BEZ also has beneficial effects on glucose metabolism, although the underlying mechanisms of these effects remain elusive. Even less is known about a potential role for BEZ in treating type 1 diabetes. Here we show that BEZ markedly improves hyperglycemia and glucose and insulin tolerance in mice with streptozotocin (STZ)-induced diabetes, an insulin-deficient mouse model of type 1 diabetes. BEZ treatment of STZ mice significantly suppressed the hepatic expression of genes that are annotated in inflammatory processes, whereas the expression of PPAR and insulin target gene transcripts was increased. Furthermore, BEZ-treated mice also exhibited improved metabolic flexibility as well as an enhanced mitochondrial mass and function in the liver. Finally, we show that the number of pancreatic islets and the area of insulin-positive cells tended to be higher in BEZ-treated mice. Our data suggest that BEZ may improve impaired glucose metabolism by augmenting hepatic mitochondrial performance, suppressing hepatic inflammatory pathways, and improving insulin sensitivity and metabolic flexibility. Thus, BEZ treatment might also be useful for patients with impaired glucose tolerance or diabetes.

[1]Institute of Experimental Genetics, Helmholtz Zentrum München, Neuherberg, Germany
[2]Division of Endocrinology, Diabetology, Angiology, Nephrology and Clinical Chemistry, Department of Internal Medicine, University Hospital Tübingen, Tübingen, Germany
[3]German Center for Diabetes Research (DZD e.V.), Neuherberg, Germany
[4]German Mouse Clinic, Helmholtz Zentrum München, Neuherberg, Germany
[5]Institute of Molecular Animal Breeding and Biotechnology, Ludwig-Maximilians-Universität-München, Munich, Germany
[6]Institute of Pathology, Helmholtz Zentrum München, Neuherberg, Germany
[7]Genome Analysis Center, Institute of Experimental Genetics, Helmholtz Zentrum München, Neuherberg, Germany
[8]Institute of Human Genetics, Helmholtz Zentrum München, Neuherberg, Germany
[9]Research Unit Analytical Pathology, Helmholtz Zentrum München, Neuherberg, Germany
[10]Institute of Vegetative Physiology, University of Köln, Cologne, Germany
[11]Department I of Internal Medicine, University Hospital Cologne, Cologne, Germany
[12]Center for Endocrinology, Diabetes and Preventive Medicine, University of Köln, Cologne, Germany
[13]Institute of Genetics, University of Köln, Cologne, Germany
[14]Lehrstuhl für Experimentelle Genetik, Technische Universität München, Freising-Weihenstephan, Germany
[15]Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Zentrum München at the University of Tübingen, Tübingen, Germany
[16]Center of Life and Food Sciences Weihenstephan, Technische Universität München, Freising, Germany
[17]Internal Medicine, SCIVIAS Hospital St. Josef, Rüdesheim am Rhein, Germany
[18]Center for Molecular Medicine Cologne (CMMC), University of Köln, Cologne, Germany
[19]Cologne Excellence Cluster on Cellular Stress Responses in Ageing-associated Diseases (CECAD), University of Köln, Cologne, Germany

Corresponding author: Martin Hrabě de Angelis, hrabe@helmholtz-muenchen.de.

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S.N. is currently affiliated with Sanofi Deutschland GmbH, R&D Diabetes Research and Translational Medicine, Industriepark Hoechst, Frankfurt am Main, Germany.

Bezafibrate (BEZ) has been used to treat patients with high lipid levels (1–3). The beneficial effect of BEZ on lipid metabolism is attributed to the activation of the transcription factor family of peroxisome proliferator–activated receptors (PPARs; PPARα, PPARβ/δ, and PPARγ) (4). BEZ treatment is also associated with a lower prevalence of type 2 diabetes in high-risk populations (5) and decreased blood glucose levels in patients with established type 2 diabetes (6). Since lipid and glucose metabolism are closely linked (7), the decreased prevalence of type 2 diabetes in BEZ-treated patients is not completely unexpected, although the underlying molecular mechanisms of BEZ action remain unclear.

In addition to its beneficial effects on lipid and glucose metabolism, BEZ is also reported to directly improve skeletal muscle mitochondrial function in patients and mice with mitochondrial dysfunction (8,9). We previously demonstrated that mitochondrial capacity is impaired in skeletal muscle of streptozotocin (STZ) mice (10), and, considering that mitochondria play a pivotal role in both glucose and lipid metabolism (7), we postulated that BEZ could ameliorate impaired glucose/lipid metabolism by stimulating mitochondrial performance in metabolic organs.

RESEARCH DESIGN AND METHODS

Materials

All chemicals were purchased from Sigma-Aldrich (Germany) unless otherwise stated.

Animal Studies

Male C57BL/6N mice were purchased from Charles River Laboratories. Twelve-week-old mice were injected with 60 mg/kg STZ on 5 consecutive days (10). In an initial experiment, aiming at studying muscle, BEZ treatment started 8 weeks after STZ injection (Supplementary Fig. 1*A–C*). For all other experiments, BEZ treatment began 2 weeks after STZ injection. Mice received a standard diet (R/M-H; ssniff Spezialdiäten GmbH, Soest, Germany), which was supplemented with 0.25% or 0.5% (w/w) BEZ (B7273; Sigma-Aldrich) for the BEZ groups. Animals were killed by isoflurane overdose, and dissected tissues were immediately frozen in liquid nitrogen, unless otherwise stated. All mouse studies were approved by local government authorities and performed according to GV-SOLAS (Society of Laboratory Animal Science) in accordance with the German Animal Welfare Act.

Plasma triglyceride (TG), nonesterified fatty acid (NEFA), creatinine, creatine kinase, urea, and C-reactive peptide levels were quantified by routine clinical chemistry using an AU480 analyzer (Beckman Coulter, Krefeld, Germany) (11). Blood glucose and hemoglobin A_{1c} levels were measured in tail blood samples using a glucometer (Contour, Bayer, Germany) and the Clover A1c Self system (Obelis, Belgium). Insulin levels were determined by ELISA (Mercodia, Uppsala, Sweden) or a Bio-Plex Pro mouse diabetes immunoassay (Bio-Rad, Munich, Germany). Intraperitoneal glucose and insulin tolerance tests were performed with 1 g/kg glucose or 0.5 units/kg insulin. Quantitative insulin sensitivity check index (QUICKI) values were calculated as follows: 1/(log[fasting insulin μU/mL] + log[fasting glucose mg/dL]). Body composition and energy balance were determined by quantitative nuclear magnetic resonance (MiniSpec LF60; Bruker Optics, Ettlingen, Germany). Indirect calorimetry (PhenoMaster System; TSE Systems, Bad Homburg, Germany) was conducted as described previously (11). A linear regression model, including body weight as a covariate, was used to adjust for body weight–dependent differences in energy metabolism parameters (TIBCO Spotfire S+ 8.1 for Windows).

Human Cells

Patients with a possible complex I deficiency according to the clinical phenotype were analyzed by muscle biopsy. Those who showed deficient complex I–driven respiration in skeletal muscle and mutations in mitochondrial genes revealed by genomic DNA sequencing were selected for the study. Skin samples were taken for fibroblast isolation (12–14). The NHDF-neo cell line (Lonza, Cologne, Germany) was used as a healthy control. Cells were grown under standard conditions (DMEM, 4.5 g/L glucose supplemented with 10% FBS) and treated with 400 μmol/L BEZ for 72 h.

Oxygen Consumption Measurements

Mitochondrial respiration was determined in soleus muscles, as described previously (10). Mitochondrial respiration was determined in digitonin-permeabilized fibroblasts using high-resolution respirometry (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria). In brief, we measured Complex I respiration using glutamate/malate (10 mmol/L/5 mmol/L), ADP (1 mmol/L), and rotenone (0.5 μmol/L), followed by measurement of Complex II respiration using succinate (10 mmol/L) and antimycin A (2.5 μmol/L) followed by Complex IV respiration after the addition of tetramethylphenylendiamin (TMPD) (2 mmol/L) and ascorbate (0.5 mmol/L). Respiration rates were normalized to cell number, and the lowest values for control fibroblasts were set as 100%.

Mouse mitochondria were freshly isolated from 100- to 150-mg liver samples with a gentleMACS Dissociator and the Mitochondria Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Oxygen consumption was determined using an XF Extracellular Flux Analyzer (XF96; Agilent Technologies, Santa Clara, CA), as recently described (15). Mitochondrial protein (2 μg) was added to individual wells of 96-well plates for the following protocols: *1*) electron flow assay, 10 mmol/L pyruvate and 2 mmol/L malate were given as substrates, and 2 μmol/L rotenone (Port A), 10 mmol/L succinate (Port B), 4 μmol/L antimycin A (Port C), 100 μmol/L TMPD, and 10 mmol/L ascorbate (Port D) were used; and *2*) coupling assay, 10 mmol/L succinate was given as a substrate in combination with 2 μmol/L rotenone, and 4 mmol/L ADP (Port A), 2 μmol/L oligomycin (Port B), 4 μmol/L FCCP (Port C), and 4 μmol/L antimycin A (Port D) were applied. All values represent final concentrations in the assay buffer (Mitochondrial Assay Solution).

Real-Time PCR

Tibialis cranialis muscles were homogenized in liquid nitrogen, total RNA was prepared, and expression of mitochondrial genes was studied by real-time PCR, as described (10). Mouse livers were pulverized in liquid nitrogen, and total RNA was prepared using an miRNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was prepared by reverse transcription (Invitrogen, Karlsruhe, Germany), and real-time PCR assays were carried out with a LC480 Light Cycler (Roche, Mannheim, Germany) using the applied primer sequences listed in Supplementary Table 1.

Microarrays

Amplification of total RNA and array processing (mouse Affymetrix Gene 1.0 ST) were performed as previously described (16). Array data were submitted to the National Center for Biotechnology Information GEO (Gene Expression Omnibus) database (GSE39752, GSE79008). Statistical analysis was performed in MultiExperiment Viewer (MeV, version 4.9.0) using the Significance Analysis of Microarrays algorithm with 1,000 permutations and a local false discovery rate (FDR) of <10%. Pathway enrichment analysis was performed with g:Profiler (http://biit.cs.ut.ee/gprofiler). Upstream regulator analysis was performed with the Ingenuity Pathway Analysis tool (Qiagen, Valencia, CA).

Metabolomics

The targeted metabolomics approach was based on liquid chromatography-electrospray ionization–tandem mass spectrometry and flow injection analysis-electrospray ionization-tandem mass spectrometry measurements acquired using an Absolute*IDQ* p180 kit (BIOCRATES Life Sciences AG, Innsbruck, Austria). Metabolites were quantified from 10-μL plasma samples, and the assay was performed as previously described (17). Statistical analysis was performed on 176 metabolites, as well as 50 ratios, and were calculated in MeV version 4.9.0 using a Wilcoxon-Mann-Whitney test with an FDR <10% by the Benjamini-Hochberg method.

Histochemistry

Gastrocnemius muscles were frozen, and 9-μm cryosections were stained for complex II and IV activities, as described previously (18). Pancreata were fixed in 4% paraformaldehyde and embedded in paraffin blocks. Consecutive 7-μm sections were stained with hematoxylin-eosin using standard procedures. Imaging was performed using an Axioplan 2 microscope (Zeiss, Oberkochen, Germany).

Immunofluorescence Staining

Pancreata were fixed in 4% paraformaldehyde and incubated in solutions with increasing sucrose concentrations (9–30%) before embedding in Tissue Tek (VWR, Darmstadt, Germany). Cryosections (7-μm thickness) were cut with 300-μm distances between sections, and three independent areas/animal were stained with anti-insulin (Invitrogen catalog #180067) or anti-glucagon (Invitrogen catalog #180064) antibodies and with corresponding fluorescent-labeled secondary antibodies. Antibodies were purchased from Invitrogen (Karlsruhe, Germany), except for anti-GLUT2 (Abcam, Cambridge, UK); DAPI was used to visualize cell nuclei. Slides were scanned using a NanoZoomer 2.0 HT Fluorescence Scanner (Hamamatsu, Hamamatsu City, Japan); pancreatic islets (11–92 islets/animal) were evaluated using Definiens Developer XD 2 image analysis software (Definiens AG, Munich, Germany).

Transmission Electron Microscopy

Quadriceps muscles or liver samples were analyzed, as described previously (15).

Western Blot

Quadriceps muscles were pulverized in liquid nitrogen and dissolved in HEPES buffer supplemented with protease and phosphatase inhibitors. Antibodies were purchased from Cell Signaling Technology (Danvers, MA), and Western blots were performed as described previously (19).

Statistics

Statistical evaluations were performed using GraphPad Prism version 6.07. Unless otherwise indicated, ANOVA with post hoc Holm-Šídák multiple comparison test was used to calculate statistical significance, which was assumed as $P < 0.05$.

RESULTS

BEZ Does Not Change Mitochondrial Function in Skeletal Muscle

We previously found an impairment of mitochondrial respiration in skeletal muscle from STZ mice (10). To restore mitochondrial dysfunction, STZ mice were treated with 0.5% BEZ before mitochondrial capacity was analyzed in skeletal muscle. BEZ treatment of STZ mice did not enhance mitochondrial gene expression or complex II and IV staining, and mitochondrial oxygen consumption in muscle was not affected (Supplementary Fig. 1*A–C*). We also assessed whether BEZ treatment of mice could induce rhabdomyolysis (20), a rare side effect described in human studies. Measurement of rhabdomyolysis-related factors showed no effect of BEZ treatment (Supplementary Fig. 1*D–F*), suggesting that BEZ did not induce rhabdomyolysis.

BEZ Ameliorates Impaired Glucose Metabolism by Improving Systemic Insulin Sensitivity

As expected, 0.5% BEZ treatment caused a significant decrease in plasma TG and NEFA levels in STZ mice (Fig. 1*A* and *B*). Interestingly, relative to untreated STZ mice, 0.5% BEZ also significantly reduced blood glucose concentrations by 45% and 40% in fasted (Fig. 1*C*) and randomly fed conditions (Fig. 1*D*), respectively. A reduced BEZ dose (0.25%) could also significantly reduce hyperglycemia, and plasma TG and NEFA levels (Supplementary Table 2). In all later experiments 0.5% BEZ was used. The improved glycemic control in BEZ-treated versus untreated STZ mice was reflected by a 28% reduction in HbA_{1c} levels (Fig. 1*E*). Meanwhile, BEZ treatment had no effect on the

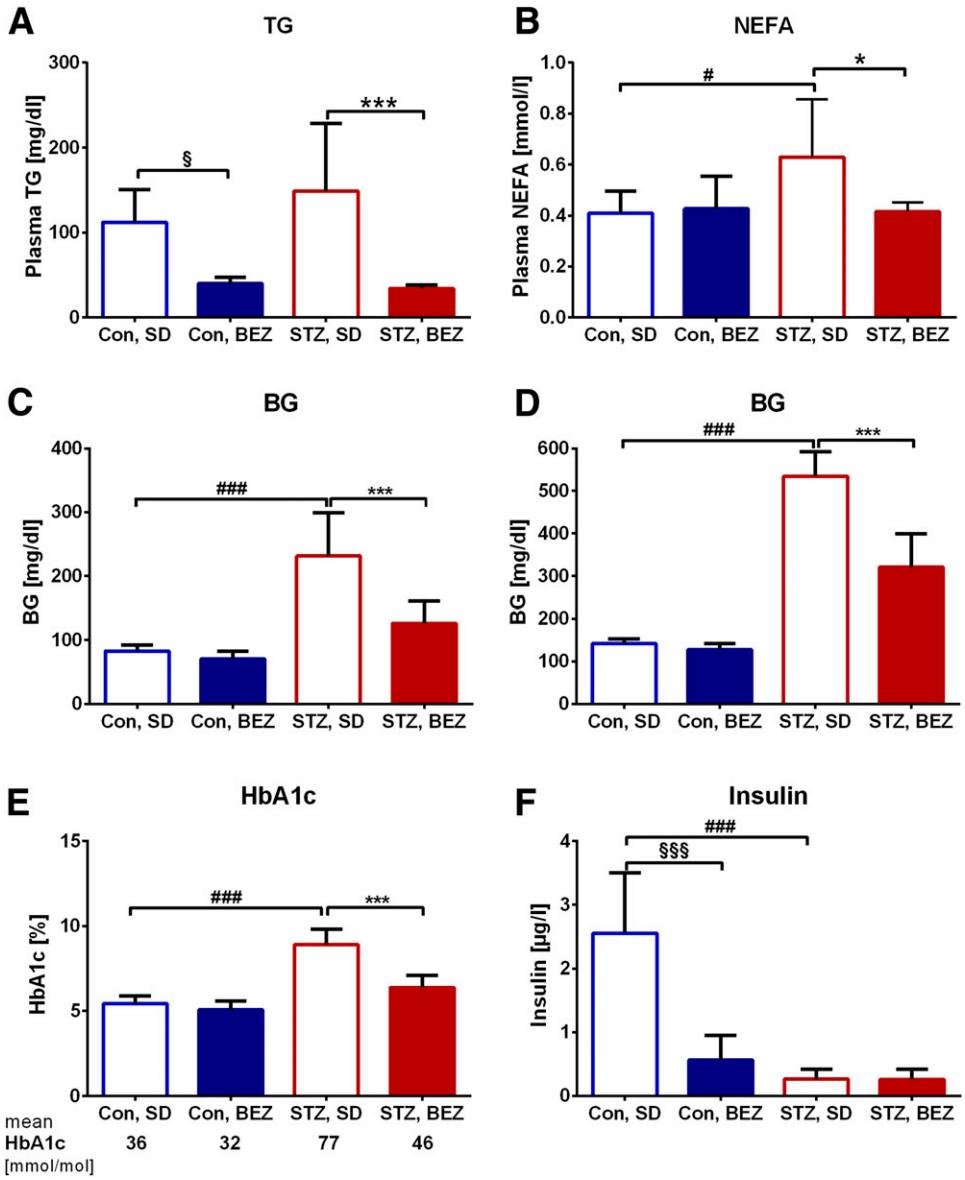

Figure 1—Levels of plasma lipid, insulin, blood glucose, and HbA_{1c} in STZ mice. Plasma TGs (*A*) and NEFAs (*B*). Fasted blood glucose (BG) (*C*), randomly fed blood glucose (*D*), and HbA_{1c} (*E*) levels. Values under the panels represent International Federation of Clinical Chemistry and Laboratory Medicine–recommended mean HbA_{1c} data in mmol/mol. *F*: Plasma insulin levels. Columns represent averages ± standard deviation; n = 5–8. *$P < 0.05$ and ***$P < 0.001$ between STZ, BEZ and STZ, SD; #$P < 0.05$ and ###$P < 0.001$ between STZ, SD and Con, SD; §$P < 0.05$ and §§§$P < 0.001$ between Con, BEZ and Con, SD. Con, control; SD, standard diet.

low insulin levels in STZ mice fed a standard diet but decreased circulating insulin levels in control mice (Fig. 1*F*).

The QUICKI index, a well-established marker for insulin sensitivity, was increased in both BEZ-treated groups (Fig. 2*A*). Since these findings suggest that BEZ treatment may improve systemic insulin sensitivity, we next determined glucose and insulin tolerance. BEZ treatment significantly improved glucose and insulin tolerance in both control and STZ mice (Fig. 2*B*–*E*), indicating that the insulin-sensitizing actions of BEZ are independent of hyperglycemia. In quadriceps muscle, BEZ treatment elevated levels of phosphorylated AKT, a key component of insulin signaling (Fig. 2*F* and Supplementary Fig. 2*A* and *B*). These data suggest that BEZ treatment improves both muscle and systemic insulin sensitivity, which in turn ameliorates the impaired glucose metabolism of STZ mice.

BEZ Treatment Improves Islet Morphology and Increases Islet Number

Since pancreatic endocrine cells play a central role in glucose metabolism, we analyzed pancreata sections from STZ mice. Hematoxylin-eosin staining showed that islet structure was severely damaged in STZ mice, whereas BEZ treatment exhibited a protective effect on islet morphology (Fig. 3*A*).

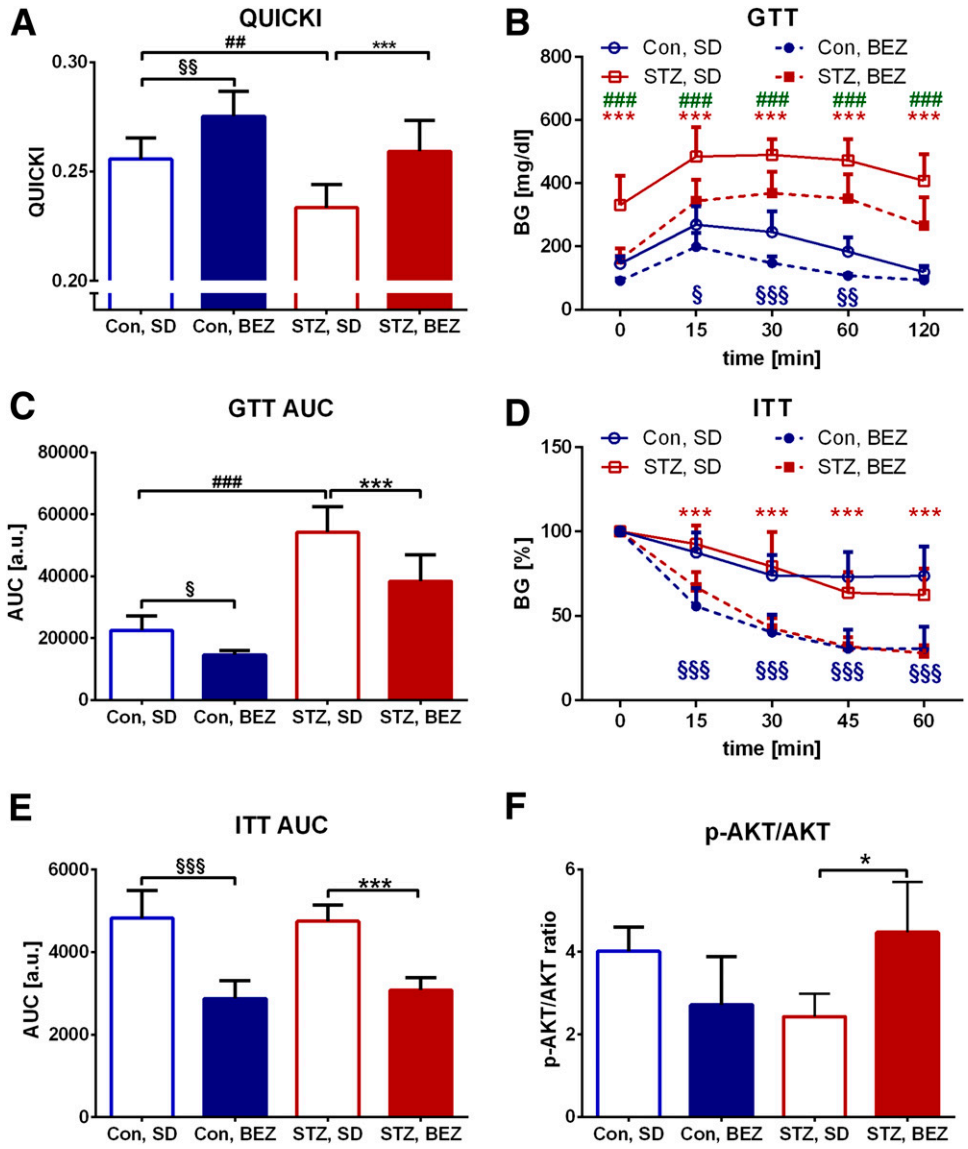

Figure 2—QUICKI, glucose tolerance tests (GTTs), insulin tolerance tests (ITTs), and muscle p-AKT/AKT ratio in STZ mice. *A*: QUICKI. Glucose tolerance test (*B*) and area under the curve (AUC) (*C*) evaluation. Insulin tolerance tests normalized for basal blood glucose (BG) (*D*) and area under the curve (*E*) evaluation. *F*: Anti-AKT and anti–phospho-473-AKT antibodies were used in Western blotting to visualize protein levels from quadriceps muscles. Values were densitometrically evaluated (Supplementary Fig. 2*A* and *B*), and p-AKT/AKT ratios were built. Columns represent averages ± standard deviation; n = 4–8 animals. *$P < 0.05$ and ***$P < 0.001$ between STZ, BEZ and STZ, SD; ##$P < 0.01$ and ###$P < 0.001$ between STZ, SD and Con, SD; §$P < 0.05$, §§$P < 0.01$, and §§§$P < 0.001$ between Con, BEZ and Con, SD. a.u., arbitrary units; Con, control; SD, standard diet.

STZ mice had fewer β-cells compared with healthy controls, and, relative to untreated STZ mice, BEZ-treated mice tended to increase β-cell area by 29%, as well as islet numbers by 56% (Fig. 3*B–E*). BEZ also elevated the diminished GLUT2 staining, which is another important β-cell marker (Supplementary Fig. 3). To assess whether BEZ suppresses apoptosis in β-cells, we used staining with anti–caspase-3 as an apoptotic marker; however, BEZ treatment did not decrease the number of caspase-3–positive β-cells (data not shown). Taken together, these data, although not statistically significant, suggest that BEZ exerts a beneficial effect on islets, especially on β-cell areas. Anti-CD45R staining revealed no elevated levels of immune cells in pancreata from STZ mice (data not shown), suggesting that inflammation in the pancreas did not occur.

BEZ Treatment Enhances Metabolic Flexibility in STZ Mice

In order to assess whether improved insulin sensitivity after BEZ treatment was due to altered body composition, the body weight and fat mass were determined. In healthy mice, BEZ treatment reduced the body weight and fat mass (Fig. 4*A–C* and Supplementary Fig. 4*A–C*). However, in STZ mice, BEZ-mediated effects were not caused by changes in body composition or body weight

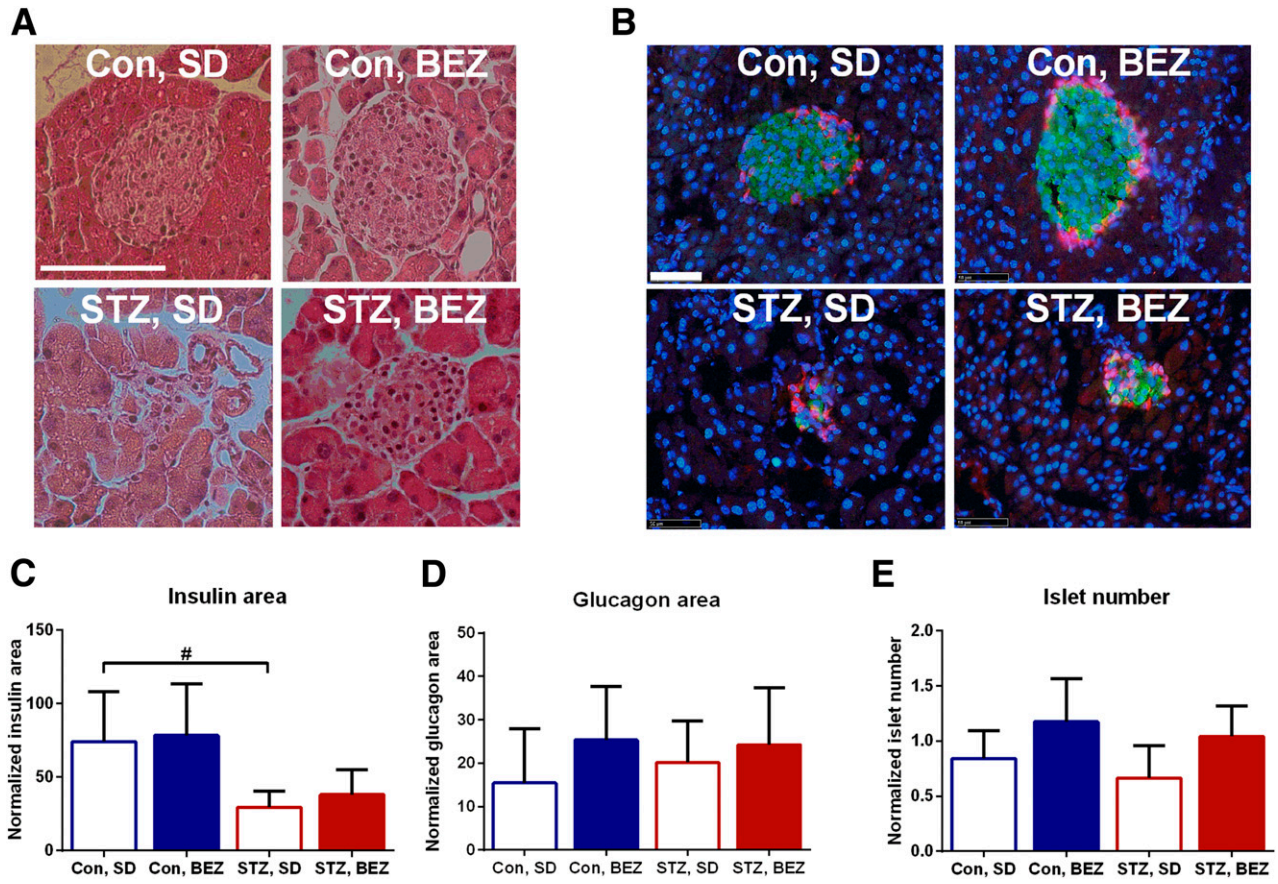

Figure 3—Pancreas architecture in STZ mice. *A*: Pancreas sections were stained with hematoxylin-eosin and visualized by light microscopy. White bar, 100 μm. *B*: Pancreas sections were stained with anti-insulin (green) and anti-glucagon (red) antibodies and were visualized by fluorescence microscopy. Cell nuclei were stained with DAPI (blue). White bar, 50 μm. Representative areas are shown. Insulin (*C*) and glucagon (*D*) areas were calculated using Architect software, and values were normalized to the total pancreas area. *E*: Islet number was manually counted, and values were normalized to the total pancreas area. Columns represent averages ± standard deviation. *A* represents $n = 2$; *B–E* represent $n = 7–8$ animals. #$P < 0.05$ between STZ, SD and Con, SD. Con, control; SD, standard diet.

(Fig. 4*A–C* and Supplementary Fig. 4*A–C*). Body weight–normalized linear regression of fat or lean mass did not differ among the analyzed groups (Supplementary Fig. 4*D* and *E*).

Since low metabolic flexibility is a hallmark of disturbed glucose homeostasis (21), we next performed indirect calorimetry (Fig. 4*D–F*). BEZ-treated STZ mice displayed a significantly lower respiratory exchange ratio (RER) during the first half of the dark phase and an elevated RER in the second light phase when compared with untreated STZ mice (Fig. 4*D*). Cumulative food intake curves (Fig. 4*E*) outlined that the lower RER observed during the first half of the dark phase resulted from a marked delay in food intake in BEZ-treated STZ mice. In contrast, the marked increments in RER in BEZ-treated STZ mice during the second light phase could not be attributed to a difference in food intake when compared with untreated STZ mice (Fig. 4*E* and Supplementary Fig. 4*F* and *G*). Metabolic flexibility, determined by calculating the change in RER (21) between dark and light phases, was lower in STZ mice fed a standard diet but increased upon BEZ treatment in both groups (Fig. 4*F*). BEZ did not significantly change the average oxygen consumption, carbon dioxide production, running distance, and rearing activity (Supplementary Fig. 5), suggesting that BEZ did not affect the energy expenditure or activity of STZ mice.

BEZ Induces PPAR-Regulated, Peroxisomal, and Mitochondrial Gene Expression Programs

To examine the activation of PPAR transcription factors by BEZ treatment in the liver, microarray analysis was performed. BEZ treatment of STZ mice significantly changed the expression of 3,603 genes (FDR <10%) that displayed at least a ±20% change compared with STZ animals fed a standard diet. Among the 1,808 genes that were significantly upregulated, genes in the "PPAR signaling pathway" ($P = 2.7 \times 10^{-15}$) and genes related to peroxisomes ($P = 4.6 \times 10^{-25}$) were significantly enriched. Notably, genes related to mitochondria ($P = 1.8 \times 10^{-49}$) were also enriched, indicating that BEZ treatment may promote both peroxisomal and mitochondrial proliferation.

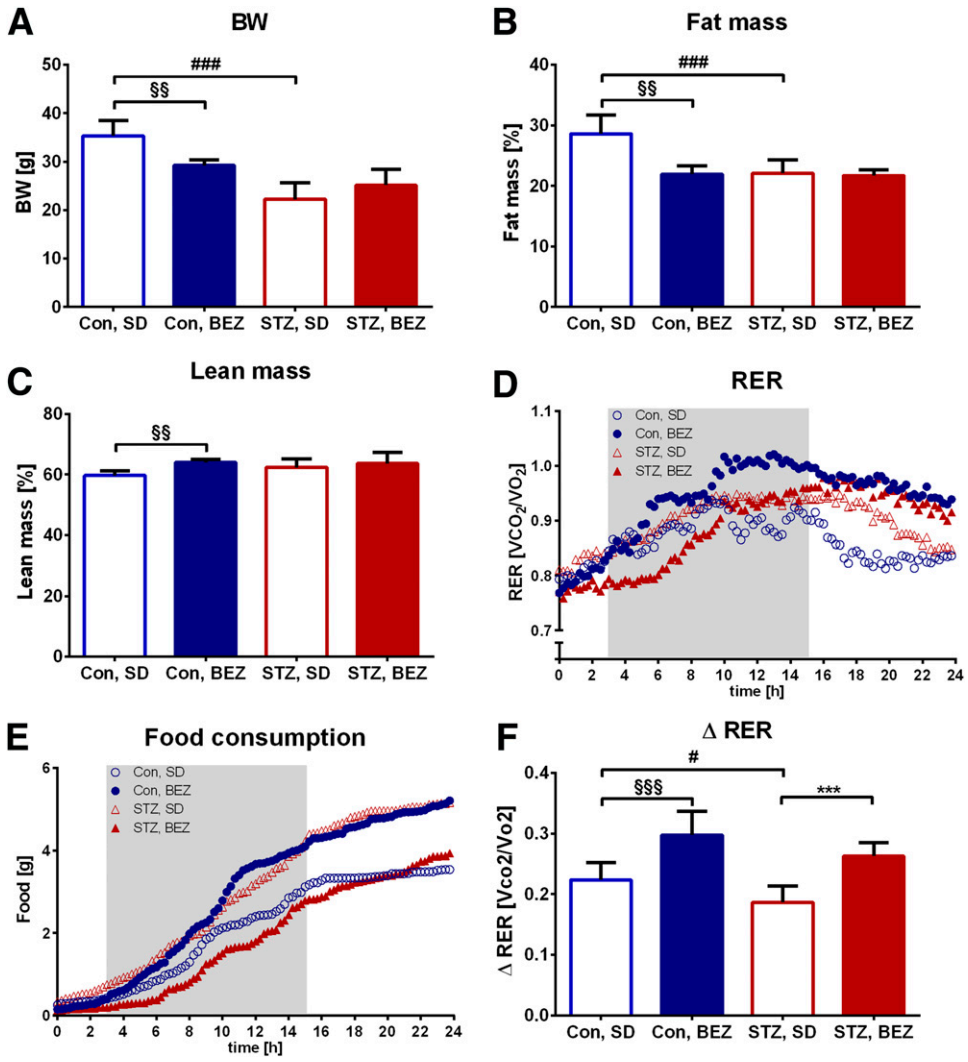

Figure 4—Body composition and indirect calorimetry in STZ mice. *A*: Body weight. Fat mass (*B*) and lean mass (*C*) were measured by quantitative nuclear magnetic resonance (Supplementary Fig. 4*B* and *C*) and normalized to body weights (in %). *D*: RERs were calculated by dividing carbon dioxide production (VCO_2) by oxygen consumption (VO_2) (Supplementary Fig. 5*A*–*D*). The gray rectangle represents the 12-h dark phase (0 time point represents 3 P.M.). *E*: Cumulative food consumption. *F*: ΔRER was calculated as $RER_{max} - RER_{min}$. Columns represent averages ± standard deviation; n = 5–8 animals. ***$P < 0.001$ between STZ, BEZ and STZ, SD; #$P < 0.05$ and ###$P < 0.001$ between STZ, SD and Con, SD; §§$P < 0.01$ and §§§$P < 0.001$ for Con, BEZ and Con, SD. Con, control; SD, standard diet.

STZ Treatment Suppresses Insulin Target Genes and Induces Inflammatory Genes, Whereas Both Pathways Are Normalized Upon BEZ Treatment

The expression of genes regulating fatty acid oxidation in the liver was significantly elevated by BEZ treatment (Supplementary Table 3), suggesting elevated fatty acid oxidation via PPAR activation. To determine which PPAR was activated by BEZ treatment in STZ mouse livers (STZ, BEZ vs. STZ, standard diet), the 3,603 genes that showed significantly altered expression were analyzed by upstream regulator analysis using Ingenuity Pathway Analysis. The positive *z* scores calculated by this analysis suggested that all PPARs were induced upon BEZ treatment (Table 1). To analyze the effect of STZ on hepatic gene regulation, microarray data from our previous study (16) were re-evaluated and compared with healthy control animals. STZ treatment significantly changed the expression of 1,771 genes (FDR <10%) that displayed at least a ±20% change compared with healthy control animals. Upstream regulator analysis using these genes suggested a suppression of all PPARs in STZ mice, as represented by negative *z* scores (Table 1). STZ treatment was also associated with reduced expression of insulin target genes and elevated expression of inflammatory genes in the liver, whereas BEZ treatment counteracted these STZ-mediated effects (Table 1). These results were supported by real-time PCR data (Supplementary Fig. 6*A* and *B*). Furthermore, plasma concentrations of the proinflammatory marker C-reactive peptide were significantly lower in BEZ-treated STZ animals compared with the STZ animals being fed the standard diet (1.38 ± 0.40

Table 1—Ingenuity upstream regulator analysis predicts that BEZ activates PPARs as well as insulin target genes and reduces mediators of inflammation in the liver

Category	Upstream regulator	STZ, SD/Con, SD (*z* score)	STZ, BEZ/STZ, SD (*z* score)
PPAR	PPARα	−1.59	8.30
PPAR	PPARβ/δ	−2.03	2.52
PPAR	PPARγ	−2.85	4.30
Insulin sig	Insulin	−1.31	1.35
Insulin sig	INSR	−0.96	4.15
Insulin sig	IRS1	−1.82	1.52
Insulin sig	IRS2	−0.77	1.37
Inflammation	TNF	5.62	−3.29
Inflammation	IL-1β	4.62	−2.29
Inflammation	IFN-γ	5.34	−5.87
Inflammation	IL-6	4.98	−3.34
Inflammation	OSM	2.41	−3.01
Inflammation	Proinfl. cytokines	2.95	−2.16
Inflammation	TLR3	4.32	−2.91
Inflammation	TLR4	4.21	−2.60

STZ treatment significantly altered the expression of 1,771 genes compared with control (Con), standard diet (SD) animals. On the other hand, BEZ treatment of STZ mice significantly altered the expression of 3,603 genes compared with STZ, SD mice. These two significantly altered gene groups were analyzed with Ingenuity Pathway Analysis, and common, significantly enriched upstream regulators with inverse regulation were identified. The *z* scores >0 indicate activation; *z* scores <0 indicate inhibition. Values >2 or <2 indicate significant results, whereas values between −2 and 2 represent trends. IFN-γ, interferon-γ; IL, interleukin; INSR, insulin receptor; IRS, insulin receptor substrate; OSM, oncostatin M; Proinfl., proinflammatory; Sig, signaling; TLR, Toll-like receptor; TNF, tumor necrosis factor.

Table 2—Fold changes of plasma metabolite levels (19 of 52), which show inverse regulation between STZ, BEZ and STZ, SD, and between STZ, SD and Con, SD comparisons

Metabolites	STZ, SD/Con, SD	STZ, BEZ/STZ, SD
C0	−1.8	2.3
C10	1.6	−1.4*
C14:2	1.7	−2.4*
C18	1.6	−2.2*
C18:2	2.4	−2.5
C4:1	2.0	−1.5*
H1	2.4	−1.7
LPC a C14:0	−1.8	1.3*
LPC a C16:0	−1.7	1.7
LPC a C16:1	−8.5	12.0
LPC a C17:0	1.5	−1.7
LPC a C18:1	−4.0	5.2
LPC a C20:3	−4.6	6.9
LPC a C20:4	−1.5	1.3
MUFA (PC)	−3.2	3.5*
MUFA (PC)/SFA (PC)	−2.3	2.8*
MUFA (LPC)	−4.0	5.3*
MUFA (LPC)/SFA (LPC)	−2.9	4.0*
Total LPC	−1.4	1.5*

STZ treatment significantly altered the level of 85 metabolites and metabolite ratios compared with control (Con), standard diet (SD) animals (left column). On the other hand, BEZ treatment of STZ mice significantly altered 78 metabolites and metabolite ratios compared with STZ, SD mice (right column). Among these comparisons, there were 54 common metabolites and metabolite ratios, with 52 of them showing inverse regulation. Numbers denote fold changes calculated by dividing the appropriate means of groups. Nineteen chosen ratios are shown; the other 33 ratios are given in Supplementary Table 4. The original data are shown in Supplementary Table 5. C, acylcarnitine; C0, free carnitine (numbers after C denote the length of the carbon chain; the numbers after the colon denote the number of double bounds); H1, hexose; PC, phosphatidylcholine. *Values that were less than three times the zero values.

vs. 4.97 ± 1.17 mg/L, respectively; $P = 0.0003$). Taken together, our data suggest that induced expression of PPAR and insulin target genes in parallel with the suppression of inflammatory gene expression in the liver could promote BEZ-dependent improvements in hyperglycemia.

BEZ Ameliorates Dyslipidemia in STZ Mice

Since lipids act as mediators of insulin resistance to play crucial roles in cellular metabolism (7), we assessed a comprehensive plasma lipid profile in BEZ-treated animals (Supplementary Table 5). STZ treatment significantly changed levels of 85 metabolites and metabolite ratios compared with those in untreated controls. Moreover, BEZ treatment of STZ mice significantly altered 78 metabolites and ratios compared with STZ mice fed a standard diet, whereas 54 metabolites and ratios were common in both comparisons, and 52 showed inverse fold-changes (Table 2 and Supplementary Table 4). STZ mice displayed higher acylcarnitine and lower lysophosphatidylcholine (LPC) plasma levels compared with healthy controls, whereas BEZ reduced acylcarnitine levels and elevated LPC levels compared with untreated STZ mice (Table 2). In STZ mice, the ratios of monounsaturated fatty acids (MUFAs) to saturated fatty acids (SFAs) and total LPC levels were lower, whereas BEZ treatment normalized these ratios (Table 2). Furthermore, hexose, which mainly represents glucose in the plasma, was significantly higher in STZ mice and decreased after BEZ treatment, which was consistent with blood glucose measurements (Fig. 1*C* and *D*). Many changes in the levels of phosphatidylcholines, sphingomyelines, and amino acids were also significantly reverted upon BEZ treatment (Supplementary Table 4).

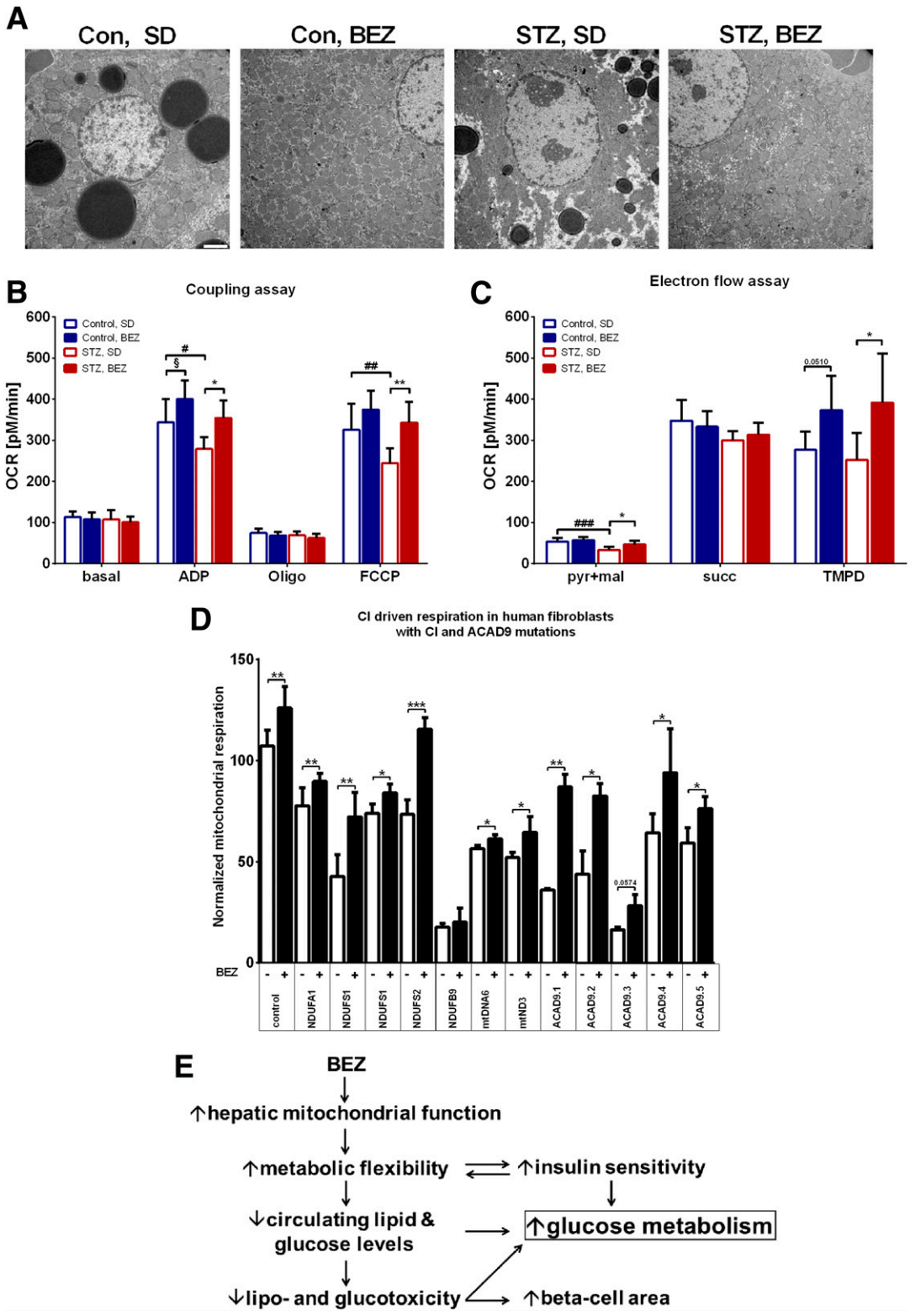

Figure 5—Mitochondrial mass in STZ mouse livers and mitochondrial function in BEZ-treated STZ mice and human fibroblasts. *A*: Liver mitochondrial mass and architecture were assessed by transmission electron microscope. Pictures were taken at ×1,600 magnification. White bar, 2 μm. Representative areas are shown. *B* and *C*: Oxygen consumption rate (OCR) was measured from isolated liver mitochondria with an XF Extracellular Flux Analyzer. *B*: Coupling assay, starting with succinate as a substrate for complex II in the absence of ADP, basal; the indicated substrates/inhibitors were used. Oligo, oligomycin; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone. *C*: Electron flow assay with the indicated substrates. pyr, pyruvate; mal, malate serving as substrates for complex I; succ, succinate, a substrate for complex II. TMPD, tetramethylphenylendiamin, a substrate for complex IV. *D*: Mitochondrial respiration was determined with glutamate and malate as complex I (CI) substrates in fibroblasts of patients with complex I deficiency (the loci of the mutation for the appropriate subunits of complex I are indicated under the figure) or ACAD9 mutations. Data are normalized to the lowest value of untreated

BEZ Increases Mitochondrial Mass and Capacity in Liver

BEZ treatment increased the number of mitochondria and decreased the number of lipid droplets in livers of control and STZ mice compared with their respective untreated controls (Fig. 5*A*). Furthermore, BEZ treatment elevated mitochondrial gene expression compared with STZ mice fed a standard diet (Supplementary Fig. 6*C*), but did not affect mitochondrial mass in skeletal muscle (Supplementary Fig. 2*C*). BEZ treatment also increased oxygen consumption in livers from both healthy and STZ mice (Fig. 5*B* and *C*). Since equal amounts of mitochondrial protein were used in the oxygen consumption experiments, these findings suggest that BEZ treatment not only promoted elevated mitochondrial mass in the liver by increasing the expression of genes encoding mitochondrial proteins, but also enhanced mitochondrial function.

Next, we analyzed whether BEZ could ameliorate the disturbed mitochondrial function in human fibroblasts isolated from patients with mitochondrial complex I deficiency resulting from mutations in genes encoding different complex I subunits or acyl-CoA dehydrogenase 9 (ACAD9). BEZ treatment significantly elevated complex I–driven respiration in almost all fibroblast samples (Fig. 5*D*), and also enhanced mitochondrial respiration via complex II and IV substrates (Supplementary Fig. 7).

DISCUSSION

Several studies suggest that BEZ decreases blood glucose levels in rodent models of type 1 and type 2 diabetes (22,23). In combination with other antidiabetes medications, BEZ has already been shown in 1978 (24) to improve hyperglycemia in patients with type 2 diabetes, which was confirmed by several independent studies later (6,25). Two studies (26,27) reported significantly lower blood glucose levels in type 1 diabetes patients treated with BEZ, which was not obvious later (28). Consistent with the above-mentioned studies we observed here a marked reduction in blood glucose levels in insulin-deficient STZ mice (Fig. 1*C* and *D*), suggesting that BEZ indeed has an antidiabetic effect in rodents. Furthermore, BEZ treatment reduced high HbA_{1c} levels in STZ mice (Fig. 1*E*), which was also shown in type 2 diabetes patients (25), and indicates a long-term benefit of BEZ treatment on glycemic control. BEZ treatment also elevated QUICKI in both treatment groups (Fig. 2*A*), suggesting that it can enhance insulin sensitivity. A similar improvement in the insulin sensitivity index was reported in patients with high lipid levels (29). Furthermore, BEZ treatment improved glucose tolerance in patients with type 2 diabetes or high lipid levels (6,25,29). BEZ treatment also improved glucose and insulin tolerance in STZ mice (Fig. 2*B*–*E*), which was previously reported for rodent models of type 2 diabetes (22). Moreover, we observed a higher phosphorylated (p)-AKT/AKT ratio in skeletal muscle upon BEZ treatment (Fig. 2*F*), indicating improved muscle insulin sensitivity. The induction of inflammatory pathways was postulated to induce diabetes by attenuating insulin sensitivity, and several anti-inflammatory therapies were demonstrated to efficiently improve diabetes (30). BEZ treatment counteracted the elevated expression of inflammatory genes and at the same time the suppressed expression of insulin target genes in the liver of STZ mice (Table 1). These data suggest that BEZ could indeed have anti-inflammatory effects, as was previously reported by others (31,32), and may thereby improve insulin sensitivity.

Decreased plasma acylcarnitine levels were found in BEZ-treated STZ animals, which could also reflect improved β-oxidation (33). Since BEZ was recently shown to affect plasma LPC levels (34), and LPCs can, in turn, regulate glucose uptake (35), the enhanced LPC levels observed after BEZ treatment could increase glucose uptake in the treated mice. In addition, the increased MUFA/SFA ratios could be associated with the improved insulin sensitivity (36). Taken together, we assume that the enhanced whole-body insulin sensitivity contributes to improved glucose tolerance.

BEZ belongs to the group of fibrates, which are known activators of PPARα (37) and decrease cardiovascular risk in diabetes patients (3). BEZ is unique among fibrates as it is the only known pan-PPAR activator (37,38). Indeed, our data indicate that all PPARs, but particularly PPARα, were upregulated in the BEZ-treated animals (Table 1). PPARα induction could be involved in ameliorated glucose metabolism and insulin sensitivity by decreasing levels of circulating lipids through higher expression of genes involved in fatty acid oxidation in the liver. Meanwhile, PPARγ activation is known to increase glucose uptake in muscle and fat tissues, and also has anti-inflammatory properties and the ability to reduce glucose synthesis in the liver (38). PPARγ pathways could participate in the ameliorated glucose metabolism in our mice and are consistent with the increased muscle p-AKT/AKT ratio, as well as with the decreased expression of inflammatory genes and increased expression of insulin target genes in the liver. However, less is known about PPARδ function,

control samples. *E*: Our data demonstrated that BEZ improves glucose metabolism in STZ mice. In this scheme, the possible underlying mechanisms, which are probably involved in the beneficial effects of BEZ, are depicted. Columns represent averages ± standard deviation; *A*–*C* represent n = 5–8 animals, *D* represents values measured from individual fibroblasts. *B* and *C*: *$P < 0.05$ and **$P < 0.01$ between STZ, BEZ and STZ, SD; #$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$ between STZ, SD and Con, SD; §$P < 0.05$ between Con, BEZ and Con, SD. For calculating P values for *B* and *C*, ANOVA and Holm-Šídák tests were applied; for control fibroblasts (n = 7–33) and patient fibroblasts (n = 3–11) in *D*, replicates were applied and Student t tests were used to calculate P values. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ between BEZ-treated and untreated fibroblasts. Con, control; SD, standard diet.

the activation of which is implicated in higher fatty acid oxidation, increased energy consumption, and adaptive thermogenesis (37). Our data suggest that, in addition to PPARα, PPARγ and PPARδ were also activated in the liver after BEZ treatment of STZ mice, thus verifying the pan-activator activity of BEZ and highlighting the clear benefit of BEZ relative to other fibrates that activate PPARα. Since all three PPARs were downregulated in STZ mice, the activation of all of them is likely needed to provide a beneficial effect of BEZ on glucose metabolism in STZ mice.

To investigate whether BEZ causes rhabdomyolysis, a rare side effect found in human studies (1), rhabdomyolysis-associated factors (20) were measured in plasma from BEZ-treated mice. None of the tested factors was significantly changed in the BEZ-treated animals (Supplementary Fig. 1*D–F*), suggesting that rhabdomyolysis did not occur. This outcome is consistent with those of previous studies (9,20,39) that showed no rhabdomyolysis after BEZ treatment at the same dosage. We found that BEZ, like other fibrates, can induce hepatomegaly in mice (data not shown), which is consistent with previous studies (40), and may be attributable to its capacity to induce peroxisome proliferation (41). Peroxisomal proliferation is species specific (42,43) because fibrate treatment does not cause hepatomegaly and peroxisomal proliferation in primates and humans (44–46). The 0.5% BEZ dose used in our study was chosen according to three previous, independent mouse studies (9,20,39). On the other hand, the applied dose in mice is higher than that given to humans in clinical studies, wherein BEZ dosages were normalized to body weight (42). Since mice have higher metabolic turnover and a much smaller body surface area than humans (47), the human equivalent dose was calculated for the applied BEZ dose. According to this calculation (47), 0.5% BEZ is equivalent to 67 mg/kg in humans, which is higher but still in the range of routine doses used in human studies (10 mg/kg) (6,8). We also confirmed that a 0.25% BEZ dose could significantly decrease hyperglycemia in STZ mice (Supplementary Table 2).

Insulin resistance and mitochondrial dysfunction are intimately related (48), and mitochondrial dysfunction can occur in insulin-resistant states (10,49). Improving mitochondrial function seems to play a role in ameliorating insulin resistance (50). After BEZ treatment of STZ mice, we observed a marked increase in hepatic mitochondrial mass and respiration capacity (Fig. 5*A–C*), which was accompanied by improved insulin sensitivity (Fig. 2*D–F* and Table 1).

Furthermore, BEZ treatment of fibroblasts from patients having complex I or ACAD9 mutations elevated mitochondrial respiration (Fig. 5*D* and Supplementary Fig. 7), suggesting that BEZ could reverse diminished mitochondrial function, which was reported for other mitochondrial mutations (8) and is shown for the first time in our study for patients with ACAD9 mutations. Myoblasts isolated from patients with mitochondrial *CPT2* gene mutations who were treated with BEZ showed elevated *CPT1b* and *CPT2* mRNA levels, whereas mitochondria isolated from these patients displayed higher palmitate respiration rates (8,51), which is in line with the elevated mitochondrial oxygen consumption seen in our fibroblast cell lines (Fig. 5*D* and Supplementary Fig. 7). The liver microarray and real-time PCR data showed higher mRNA levels for *CPT1b* and *CPT2* in STZ mice upon BEZ treatment (Supplementary Table 3 and Supplementary Fig. 6*C*). Bastin et al. (52) showed elevated mitochondrial gene expression in fibroblasts isolated from patients with deficiencies in mitochondrial complexes after BEZ treatment as we have also observed higher mRNA levels of genes encoding mitochondrial complexes in the livers of BEZ-treated STZ mice (Supplementary Fig. 6*C*). These data suggest that BEZ treatment likely enhances the gene expression of mitochondrial proteins and oxygen consumption through similar pathways in humans and mice.

Since mitochondria play a central role in cellular metabolism by oxidizing different available substrates for ATP production (53), mitochondrial dysfunction is likely involved in metabolic inflexibility (21). BEZ treatment improved metabolic flexibility (Fig. 4*F*) probably by increasing fatty acid (Supplementary Table 3) and glucose oxidation (Fig. 4*D*). Improved metabolic flexibility in turn could decrease both NEFA and blood glucose levels in STZ mice (Fig. 1*B* and *D*). BEZ-treated patients carrying the *PNPLA2* mutation also show ameliorated metabolic flexibility in parallel with improved mitochondrial function and insulin sensitivity (54), suggesting that enhanced metabolic flexibility could have clinical relevance in BEZ therapies.

Our data suggest that BEZ enhances hepatic mitochondrial performance and insulin sensitivity, which could ameliorate metabolic inflexibility and insulin resistance that, in turn, preserves β-cell area and insulin action to improve glucose metabolism and hyperglycemia in STZ mice (Fig. 5*E*). Our study and previous studies also indicate that glucose metabolism and the underlying molecular pathways involving mitochondrial function are comparable between mice and humans, and could be similarly regulated by BEZ application. The beneficial actions of BEZ on glucose metabolism in addition to its effects in hyperlipidemia patients, suggest that BEZ could also be useful for treating patients with impaired glucose tolerance or diabetes.

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