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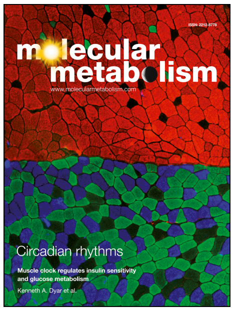

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PII: S2212-8778(16)30286-1

DOI: 10.1016/j.molmet.2016.12.007

Reference: MOLMET 423

To appear in: *Molecular Metabolism*

Received Date: 17 November 2016

Revised Date: 8 December 2016

Accepted Date: 15 December 2016

Please cite this article as: Franko A, Neschen S, Rozman J, Rathkolb B, Aichler M, Feuchtinger A, Brachthäuser L, Neff F, Kovarova M, Wolf E, Fuchs H, Häring H-U, Peter A, de Angelis MH, Bezafibrate ameliorates diabetes via reduced steatosis and improved hepatic insulin sensitivity in diabetic TallyHo mice, *Molecular Metabolism* (2017), doi: 10.1016/j.molmet.2016.12.007.

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Bezafibrate ameliorates diabetes via reduced steatosis and improved hepatic insulin sensitivity in diabetic TallyHo mice

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Abstract

Objective: Recently, we have shown that Bezafibrate (BEZ), the pan-PPAR (peroxisome proliferator-activated receptor) activator, ameliorated diabetes in insulin deficient streptozotocin treated diabetic mice. In order to study whether BEZ can also improve glucose metabolism in a mouse model for fatty liver and type 2 diabetes, the drug was applied to TallyHo mice.

Methods: TallyHo mice were divided into an early (ED) and late (LD) diabetes progression group and both groups were treated with 0.5% BEZ (BEZ group) or standard diet (SD group) for 8 weeks. We analyzed plasma parameters, pancreatic beta-cell morphology, and mass as well as glucose metabolism of the BEZ-treated and control mice. Furthermore, liver fat content and composition as well as hepatic gluconeogenesis and mitochondrial mass were determined.

Results: Plasma lipid and glucose levels were markedly reduced upon BEZ treatment, which was accompanied by elevated insulin sensitivity index as well as glucose tolerance, respectively. BEZ increased islet area in the pancreas. Furthermore, BEZ treatment improved energy expenditure and metabolic flexibility. In the liver, BEZ ameliorated steatosis, modified lipid composition and increased mitochondrial mass, which was accompanied by reduced hepatic gluconeogenesis.

Conclusions: Our data showed that BEZ ameliorates diabetes probably via reduced steatosis, enhanced hepatic mitochondrial mass, improved metabolic flexibility and elevated hepatic insulin sensitivity in TallyHo mice, suggesting that BEZ treatment could be beneficial for patients with NAFLD and impaired glucose metabolism.

Keywords

Bezafibrate; glucose metabolism; insulin resistance; lipid metabolism; NAFLD

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Abbreviations

Bezafibrate (BEZ), blood glucose (BG), early onset of diabetes (ED), electron microscopy (EM), homeostatic model assessment of insulin resistance (HOMA-IR), fatty acid (FA), late onset of diabetes (LD), non-alcoholic fatty liver disease (NAFLD), non-esterified fatty acid (NEFA), peroxisome proliferator-activated receptor (PPAR), quantitative nuclear magnetic resonance (qNMR), respiratory exchange ratios (RER), standard diet (SD), type 2 diabetes (T2D), triglyceride (TG)

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1. Introduction

Bezafibrate (BEZ) is a member of the fibrate group that possesses the unique feature of activating all known peroxisome proliferator-activated receptors (PPARs, PPARα, PPARγ and PPARβ/δ) [1]. PPARs are transcription factors regulating crucial genes involved in fatty acid metabolism and insulin sensitivity [1]. BEZ was primarily used to treat patients with hyperlipidemia [2]; however, it was also shown to improve glucose metabolism in rodents [3] and humans [4]. Recently, we have shown that BEZ improves glucose metabolism and diabetes in the insulin deficient streptozotocin mice [5]. To study whether BEZ could also ameliorate conditions associated with fatty liver and type 2 diabetes, we used the TallyHo mouse model. TallyHo mice were described in 2001 and are characterized by elevated plasma lipid levels and body weight, high fat mass, steatosis, and intermediate to severe diabetes [6, 7]. Compared to the classical monogenic diabetes models like ob/ob or db/db mice, the polygenic nature of TallyHo mice add a clear benefit to these models by better resembling the human disease state of T2D [7]. Recent studies identified several diabetes loci (Tanidd1-4, Tabw3-4), which act in concert to promote diabetes, and male TallyHo mice show reduced peripheral glucose uptake and enlarged pancreatic islets [8]. Our data demonstrated that BEZ ameliorates impaired glucose metabolism in TallyHo mice via decreased hepatic fat content and suppressed hepatic gluconeogenesis in association with increased mitochondrial mass and elevated metabolic flexibility.

2. Materials and methods

2.1 Materials

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

2.2 Animal studies

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TallyHo mice were purchased from Jackson Laboratories and were bred in our animal facility. Only male mice were used in our study, and mice received a standard diet (SD) (R/M-H, Ssniff, Germany), which was supplemented with 0.5% (w/w) Bezafibrate (BEZ, B7273, Sigma-Aldrich) for the BEZ groups for 8 weeks. Animals were killed by isoflurane overdose, and dissected tissues were prepared as stated below. All data represent samples taken after 8 weeks of BEZ (or SD) treatment unless otherwise stated. All animals received human care, and mouse studies were approved by local government authorities and performed according to GV-SOLAS (Society for Laboratory Animal Science) in accordance with the German Animal Welfare Act.

Plasma triglyceride (TG), non-esterified fatty acid (NEFA), glycerol, glucose, and C-reactive protein (CRP) levels were quantified using an AU480 clinical chemistry analyzer (Beckman Coulter, Germany) [9]. Blood glucose levels were measured in tail blood samples using a point of care glucometer (Contour, Bayer, Germany) and plasma insulin levels were determined using ELISA or Multi-Spot electrochemiluminescence Assay System (Mesoscale, Rockville, USA). Intraperitoneal glucose tolerance tests were performed 7 weeks after BEZ treatment with 1 g/kg glucose, and, since most of the tail blood values were higher than the upper limit of glucometer (>600 mg/dl), plasma glucose levels were determined by LabAssay Glucose Kit (Wako, Richmond, USA). Homeostatic model assessment of insulin resistance (HOMA-IR) value was calculated as: ((fasting glucose [mg/dl] x fasting insulin [µIU/ml])/405). Body composition and indirect calorimetry was studied 5 weeks after BEZ treatment as described previously [5].

2.3 Euglycemic-hyperinsulinemic clamps

Euglycemic-hyperinsulinemic clamp studies were performed 6-7 weeks after BEZ treatment as previously published [10]. To initiate the euglycemic-hyperinsulinemic clamp, a continuous insulin infusion (6 mU/kg min^{-1}; Humulin R, Lilly, Indianapolis, USA) was

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started and continued for 120 minutes. Between 90 and 120 min, four blood samples were collected for calculation of insulin-mediated suppression of endogenous glucose appearance rates (EndoRa), a marker of hepatic glucose production. At 120 min, 2-deoxy-D-[1-^{14}C]glucose was injected intravenously (370 kBq), and additional blood samples were collected. Basal EndoRa was calculated as the ratio of [3-^{3}H]glucose infusion rate and plasma [3-^{3}H]glucose specific activity. The EndoRa during insulin-stimulated conditions was determined by subtracting the Glucose Infusion Rate (GINF) from rate of disappearance (Rd). Tissue 2-[^{14}C]deoxyglucose-6-phosphate was extracted, and glucose uptake rates (Rg) were calculated as previously described [11]. Whole body glycolysis rates were calculated from the increase in plasma $^{3}H_2O$ concentration, the latter referring to the difference between ^{3}H counts before and after drying, divided by the specific activity of plasma [3-^{3}H]glucose and the plasma $^{3}H_2O$ concentration.

2.4 Immunofluorescence staining

Pancreata were fixed in 4% paraformaldehyde, and cryosections were stained with anti-insulin or anti-glucagon antibodies as described previously [5].

2.5 Histochemistry

Liver tissues were fixed in 4% paraformaldehyde, and paraffin sections were stained with hematoxylin and eosin as described previously [5].

2.6 Hepatic lipid levels

Liver samples were homogenized in PBS containing 1% Triton X-100 using a TissueLyser (Qiagen, Hilden, Germany). Triglyceride (TG) levels were quantified in the homogenates using the ADVIA XPT clinical chemistry analyzer (Siemens Healthcare Diagnostics, Eschborn, Germany). Trans-esterification of the fatty acids and quantification by gas chromatography with flame ionization detection was performed as described previously [12].

2.7 Real-time PCR

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Mouse livers were pulverized in liquid nitrogen and total RNA was prepared using an RNeasy Mini kit (Qiagen). cDNA was prepared by reverse transcription (Thermo Fischer Scientific, Waltham, USA), and real-time PCR assays were carried out with a LC480 Light Cycler (Roche, Mannheim, Germany) with (*Scd1*, *Scd2*, *Fasn* and *Gapdh)* or without (*CS, Ndufab1, COX19, CPT2*, *Hadha* and *Rps2*) universal probe library (Roche). Calculations were done by a comparative method ($2^{-\Delta\Delta Ct}$) and normalized to *Gapdh* (for *Scd1*, *Scd2* and *Fasn*) or *Rps2* (for *CS, Ndufab1*, *COX19, CPT2* and *Hadha*) as housekeeping genes. The applied primer sequences are shown in Suppl. Table 2.

2.8 Transmission electron microscopy

Liver and quadriceps samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and were analyzed as described previously [13].

2.9 Western blot

Liver homogenates were loaded to an acrylamide gel and western blots were performed as described earlier [5]. α/β-tubulin antibody was purchased from Cell Signaling Technology (Cambridge, UK), citrate synthase antibody from Abcam (Cambridge, UK), and secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, USA).

2.10 Statistics

Statistical evaluations were performed using GraphPad Prism 6.07. ANOVA with post hoc Holm-Šídák's multiple comparison tests were used to calculate statistical significance comparing four groups or two-tailed, unpaired Student´s tests were applied with unequal distribution when two groups were compared. Statistical significance was assumed at $p<0.05$.

3. Results

3.1 TallyHo mice

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TallyHo mice represent a polygenic mouse model for diabetes with mild steatosis and insulin resistance. However, the mice display individual onset of diabetes on standard diet; thus fast and slow progressors can be identified among them. In order to study the effect of Bezafibrate (BEZ) in different stages of diabetes, TallyHo mice were divided into two groups at 9 weeks of age. Mice with fasting blood glucose <200 mg/dl were defined as late onset of diabetes (LD) group, whereas mice with blood glucose values >200 mg/dl were classified as early onset of diabetes (ED) group (Fig. 1A). Thus, LD mice represented a preventive group, in which we studied whether BEZ could protect mice in a prediabetic stage from diabetes progression; whereas ED mice served as a therapeutic group, in which we investigated whether BEZ treatment could revert established diabetes.

3.2 BEZ ameliorates diabetes, reduces plasma lipid levels, and improves glucose tolerance.

In order to study whether BEZ has a beneficial effect to prevent the development of (LD group) or ameliorate T2D (ED group), both TallyHo groups were treated for 8 weeks with the BEZ containing diet (BEZ group) or with standard diet (SD). At 9 weeks of age, ED, SD mice already showed higher BG levels compared to LD, SD mice (Suppl. Fig. 1A), although both untreated groups developed diabetes by the age of 17 weeks (Fig. 1D). Compared to the SD groups, BEZ reduced plasma lipid and glycerol levels (Fig. 1B-C and Suppl. Fig. 1B), suggesting reduced lipolysis. Furthermore, the level of CRP, which is an inflammatory marker, tended to decrease upon BEZ treatment (Suppl. Fig. 1C), indicating a possible amelioration of inflammatory processes. BEZ markedly decreased blood glucose levels measured by a glucometer (Fig. 1D), which was also verified by plasma glucose measurements using glucose assay (Suppl. Fig. 1D). Insulin levels were lower (LD, BEZ vs LD, SD) or remained unchanged (ED, BEZ vs ED, SD) in the BEZ groups (Fig. 1E). As a consequence, HOMA-IR values were normalized in both BEZ groups (Fig. 1F). Furthermore,

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BEZ attenuated the impaired glucose tolerance of TallyHo mice (Fig. 1G-H) without increasing insulin levels during the glucose tolerance test (Suppl. Fig. 1E-F). These results suggest that BEZ improved insulin sensitivity and glucose metabolism, which in turn resulted in the normalization of blood glucose levels independently of plasma insulin levels.

3.3 BEZ increases beta-cell mass.

Next, we studied the pancreatic architecture of BEZ-treated animals. BEZ elevated the content of insulin producing beta-cells in the islets (Fig. 2A-B) as it also increased total insulin area in the pancreas as well as total islet number (Fig. 2C-D). Glucagon area normalized to islets was higher in the ED, SD group compared to LD, SD mice, whereas total glucagon area remained unchanged (Suppl. Fig. 2).

3.4 BEZ elevates energy expenditure and metabolic flexibility.

BEZ treatment resulted in lower body weight in the LD group; however, there was an opposite effect in ED group (Fig. 3A). BEZ increased relative lean and decreased fat mass in LD mice (Fig. 3B-C and Suppl. Fig. 3A-B), the latter remained significant, when normalized to body weight (Suppl. Fig. 3C-D). BEZ increased food consumption in LD group, but water intake remained unaltered upon drug treatment (Suppl. Fig. 3E-F). BEZ elevated carbon dioxide production and oxygen consumption (Suppl. Fig. 4A-D); the latter remained significant when normalized to body weight (Fig. 3D) and used as a marker for energy expenditure. Respiratory exchange ratio (RER) and metabolic flexibility assessed as delta RER were also higher in BEZ-treated animals (Fig. 3E-F). Rearing and run distance were not altered upon BEZ application (Suppl. Fig. 4E-H). These data suggest that improved metabolic flexibility and energy expenditure could be involved in the beneficial role of BEZ.

3.5 BEZ reduces hepatic gluconeogenesis in LD mice.

In order to assess whether BEZ improves hepatic insulin sensitivity, we performed euglycemic-hyperinsulinemic clamps in LD mice. To reach normoglycemia at the applied

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insulin dose (Fig. 4A), a higher glucose infusion rate was needed in the BEZ group (Fig. 4B), suggesting elevated insulin sensitivity. Endogenous glucose production (EGP), which mainly consists of hepatic gluconeogenesis [14], was inhibited in both groups with a significantly stronger effect in the BEZ group (Fig. 4C). Glucose flux analysis showed an elevated whole body glucose uptake in the BEZ group (Fig. 4D), however glucose uptake was not increased in M. quadriceps or epididymal fat tissues (Suppl. Fig. 5A). Glucosuria was normalized under BEZ treatment, suggesting the absence of diabetic urinary glucose loss (Suppl. Fig. 5B). These results suggest that BEZ improved the hepatic insulin sensitivity via reduced gluconeogenesis, which is involved in the amelioration of diabetes.

3.6 BEZ reduces hepatic lipid contents in LD mice and elevates relative MUFA contents in ED mice.

To study the underlying mechanisms of the improved hepatic gluconeogenesis, the hepatic lipid content of BEZ-treated mice was assessed. Histological staining showed reduced steatosis in LD mice upon BEZ treatment (Fig. 5A), which was associated with reduced total hepatic TG content (Fig. 5B). Since fatty liver is a key component of the metabolic syndrome and strongly associated with insulin resistance [15], these data suggest that BEZ improves insulin sensitivity in LD TallyHo mice possibly through reduced hepatic steatosis. Lipotoxic effects of FA and lipid intermediates counteract insulin signaling, and these effects are implicated in the pathogenesis of fatty liver and insulin resistance. Since chain lengths and saturation state have major impact on lipotoxic actions of FA, and monounsaturated FAs (MUFAs) have beneficial effects on patients with T2D [16], we determined the exact FA composition of hepatic triglycerides. As expected from the reduced total hepatic TG content (Fig. 5B), most of the TGs (normalized to liver weight) were lower in LD, BEZ animals compared to LD, SD mice; ED, SD mice also showed lower TGs compared to LD, SD group (Suppl. Table 1). The total TG content is known to influence the relative composition of TGs

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with various FA lengths and saturation state [12]. Therefore, we compared the relative FA composition only between ED, BEZ and ED, SD groups, in which the total TG contents were comparable (Fig. 5B). BEZ increased the relative content of C14:0 and C16:0 FAs and decreased C18:0 and C20:0 FA contents (Fig. 5C, left panel). C16:1 and C18:1 MUFAs were markedly increased upon BEZ treatment (Fig. 5C, middle panel); however, the precursor n-3 and n-6 polyunsaturated FAs (PUFAs) as well as many other PUFAs were decreased (Fig. 5C, right panel and Suppl. Fig. 5C-D). BEZ elevated total MUFA but reduced total PUFA content (Fig. 5D). More importantly compared to the ED, SD group, ED, BEZ mice showed higher MUFA/SFA ratio (0.614±0.176 vs 0.340±0.139, p=0.0121), elevated stearoyl-CoA-desaturase (SCD) activity index (cis-C16:1n-7/C16:0; 0.088±0.032 vs 0.019±0.012, p=0.0003), and increased *de-novo* lipogenesis index (C16:0/C18:2n-6; 4.66±1.95 vs 1.76±0.42, p=0.0011). In order to investigate the role of SCDs and FA synthesis the transcript levels of *Scd1* and *2* as well as the fatty acid synthase (*FASN*) were studied by real-time PCR. BEZ elevated the mRNA level of both SCDs and *FASN* (Fig. 5E). These results suggest that BEZ increases hepatic lipogenesis and SCD activity, which, in turn, elevates the content of MUFAs. On the other hand, the reduced PUFA precursors (C18:3n-3 and C18:2n-6) and PUFAs suggest that BEZ also elevates FA oxidation.

3.7 BEZ increases mitochondrial mass in TallyHo mice.

Since mitochondria play a crucial role in FA oxidation, we studied mitochondrial mass using different approaches. Transmission EM revealed a higher mitochondrial number in the BEZ groups compared to untreated controls (Fig. 6A-B). Citrate synthase (CS) is usually used as a marker for mitochondrial mass, and protein as well as mRNA level of CS were elevated in BEZ-treated animals (Fig. 6C-D and Suppl. Fig. 6A). Higher transcript levels of other mitochondrial genes were also found (Suppl. Fig. 6B-E). These results indicate that BEZ induces mitochondrial biogenesis in the liver of TallyHo mice. The elevated mitochondrial mass and decreased lipid and PUFA precursor content in the liver of LD, BEZ mice suggest increased hepatic fatty acid oxidation, which could be involved in the attenuated hepatic gluconeogenesis. Moreover, BEZ increased hepatic MUFA contents, which is possibly involved in the amelioration of glucose metabolism in ED, BEZ group. Skeletal muscle of BEZ-treated animals showed normal mitochondrial mass (Suppl. Fig. 7).

4. Discussion

The major finding of our study was that BEZ-treated, slow progressor (late onset of diabetes) LD TallyHo mice were protected against diabetes, but, more importantly, the established diabetes in early onset of diabetes (ED) group was reverted upon drug application. The anti-diabetic potential of BEZ was also reported in rodent models for T1D [5] and T2D [3, 17] as well as in patients with T2D [4, 18, 19]. Furthermore, diabetes prevalence in patients with coronary artery disease, who showed impaired glucose tolerance, was also attenuated after BEZ application [20]. These results indicate that BEZ could indeed prevent the progression of a prediabetic state to clinical diabetes and even revert an established diabetic state in rodents and humans; however, the underlying mechanisms are currently poorly understood.

The anti-diabetic effect of the drug is probably attributed to its insulin sensitizing capacity indicated by stronger inhibition of endogenous glucose production and decreased HOMA-IR index. Tenenbaum et al. also reported that BEZ treatment was indeed efficient to prevent the increase of HOMA-IR index during two year follow up in patients with coronary artery disease [21]. Although fenofibrate did not change HOMA-IR in patients with impaired glucose tolerance or diabetes, BEZ did significantly decrease HOMA-IR compared to placebo group after 8 weeks of treatment [22] as other studies also reported lower HOMA-IR in BEZ-treated patients [19, 23].

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In our euglycemic-hyperinsulinemic clamp experiments, insulin stimulus of BEZ-treated animals caused negative endogenous glucose production (EGP) values. Negative or “zero” values for EGP were also reported in rodent models by us [10] and others [14, 24-26] and could be attributed to the high glucose infusion rate [27] in the BEZ group due to the big difference in insulin sensitivity between BEZ and SD groups. Therefore, the negative values of the BEZ-treated mice are assumed to correspond to “zero” as also reported by others [14]. Despite the negative EGP values, our results clearly showed that BEZ treatment led to a pronounced reduction in EGP reflecting improved hepatic insulin sensitivity. There are only a few studies reporting insulin sensitivity from euglycemic-hyperinsulinemic clamp experiments in patients, and BEZ treatment showed no alteration in insulin sensitivity in patients with high lipid levels [28] or diabetes [29], while others found an improved insulin sensitivity [30, 31]. These results indicate that further studies are needed to investigate the role of BEZ in insulin sensitivity in human subjects. However, dual PPARα and PPARδ activation was recently shown to improve hepatic insulin sensitivity in patients with insulin resistance [32].

In contrast to the low insulin levels of BEZ-treated mice, these animals showed an increased beta-cell area compared to untreated controls. BEZ has been shown to improve islet architecture in diabetic mice with type 1 diabetes [5, 33], and, compared to the PPARα activator fenofibrate or PPARγ activator rosiglitazone, it was the only PPAR activator, which prevented the compensatory islet hypertrophy in high sucrose, high fat diet treated mice [34], pointing to its unique attribute. PPAR activation could directly improve beta-cell function [35] or could also occur as a secondary consequence of the amelioration of gluco- and lipotoxicity.

Metabolic flexibility is assumed as the ability to change substrate oxidation from fat to carbohydrate and its malfunction is intimately related to insulin resistance and ectopic lipid

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accumulation [36]. Since mitochondria play a crucial role in substrate oxidation [37], mitochondrial dysfunction was reported in skeletal muscle of insulin resistant mouse models [38] and patients [39] as well as in patients with type 2 diabetes, and it was associated with metabolic inflexibility [40]. Furthermore, impaired mitochondrial beta-oxidation was postulated to contribute to hepatic steatosis [41]. Therefore, the elevated mitochondrial mass found in the liver of BEZ-treated animals could be involved in the improved metabolic flexibility. In TallyHo mice, BEZ elevated respiratory exchange ratio (RER), which reflects higher carbohydrate oxidation, and the higher delta RER upon BEZ treatment indicates better metabolic flexibility. BEZ treatment of patients with *PNPLA2* mutation indeed improved metabolic flexibility, which was associated with better insulin sensitivity [31], suggesting an overall effect of BEZ in mice and humans. In addition, the enhanced energy expenditure observed in BEZ-treated TallyHo mice is postulated to be beneficial in the prevention of lipid accumulation and insulin resistance [42].

Compared to LD, SD animals, ED, SD mice exhibited decreased hepatic fat content, which is probably attributed to the long lasting diabetic and insulin deficient state since the diminished insulin level could impair fat storage. On the other hand, LD, SD mice are a good model for non-alcoholic fatty liver disease (NAFLD), since they showed hepatic steatosis and insulin resistance, which are hallmarks for NAFLD [43]. The precursors of PUFAs, which cannot be endogenously synthesized but only supplied by the food, showed lower hepatic contents in BEZ-treated animals in association with reduced content of other PUFAs. These data indicate the BEZ elevated FA oxidation and as a consequence decreased hepatic lipid levels. The lower hepatic TG level and the increased mitochondrial mass observed in the BEZ-treated LD TallyHo mice suggest an improved FA metabolism, which could lead to less lipid intermediates attenuating insulin resistance and enhancing the inhibitory effect of insulin on endogenous glucose production (Fig. 6E). In addition to reducing lipid levels in

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LD mice, BEZ also changed the fatty acid composition of ED mice. PPARα knock-out mice are characterized by lower C16:1n-7 fatty acid level in hepatic TG fraction compared to wild-type controls [44]. Thus, the 5.7-times higher C16:1n-7 level upon BEZ treatment in ED mice suggests that PPARα plays an important role in elevating MUFAs. A diet enriched in MUFAs was shown to significantly decrease HbA1c, plasma glucose levels, and HOMA-IR index in patients with T2D [16]. Thus, the elevated hepatic MUFAs in the BEZ-treated animals could also participate in ameliorating insulin sensitivity and diabetes. Stearoyl-CoA-desaturase (SCD) is the corresponding enzyme, which is responsible for the production of C16:1n-7 and C18:1n-9. In BEZ-treated ED animals, higher mRNA levels of both SCD isoform and elevated SCD activity index were observed. Since high hepatic SCD1 activity was associated with low hepatic fat content and insulin sensitivity in human subjects [45-47], an increased SCD activity in BEZ-treated mice could contribute to the improved hepatic insulin sensitivity.

Although the combined treatment of NAFLD, obesity, and T2D is intensively studied, currently there are only limited drugs available [48]. In the recent years, novel dual PPARα and PPARγ agonists were studied; however, most of the new candidates showed undesirable effects [49]. In contrast to them, BEZ is already 40 years on the market, it activates all three PPARs, and it has a good safety [20]. Recently, a novel PPARα and PPARδ activator, Elafibranor (GFT505) was shown to have beneficial effects improving endogenous glucose production, blood glucose levels, and steatosis in patients with insulin resistance or NASH, respectively [32, 50]. These observations and our data suggest that the activation of PPARs may represent a good treatment option for subjects with diabetes and NAFLD.

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Figure legends

Fig. 1 Levels of blood glucose, plasma lipid, insulin, and glucose tolerance test

A The figure represents the averages of weekly measured blood glucose values (values for week 9 are also showed as Suppl. Fig. 1A). TallyHo mice were split into two groups according to the 9 weeks old blood glucose values (early onset of diabetes (ED) group:>200 mg/dl, late onset of diabetes (LD) group:<200 mg/dl). BEZ (or SD) feeding was started at 9 weeks of age and lasted for 8 weeks. Standard diet (SD), BEZ diet (BEZ). **B** Plasma non-esterified fatty acids (NEFA) and **C** triglyceride (TG) levels. **D** Fasted blood glucose (BG) values. **E** Plasma insulin levels. **F** Homeostatic model assessment of insulin resistance (HOMA-IR) values. **G** Glucose tolerance test (GTT) and **H** area under the curve (AUC) evaluation. Columns represent averages ± standard deviations; n=6-12. *****denotes significant differences between ED, BEZ vs. ED, SD; **p<0.01, ***p<0.001; #denotes significant differences between ED, SD vs. LD, SD; ##p<0.01, ###p<0.001; **§**denotes significant differences between LD, BEZ vs. LD, SD; §§§p<0.001.

Fig. 2 Pancreas architecture

A Pancreata were stained with anti-insulin (green) and anti-glucagon (red) antibodies and visualized by fluorescence microscopy. Cell nuclei were stained with DAPI (blue). The white bar represents 50 µm. Representative areas are shown. **B** Insulin area normalized to islet area and **C** total insulin area normalized to pancreas area were calculated using Architect software. **D** Islet number was manually counted and values were normalized to total pancreas area. Columns represent averages ± standard deviations; n=5. *****denotes significant differences between ED, BEZ vs. ED, SD; *p<0.05, **p<0.01; **#**denotes significant differences between ED, SD vs. LD, SD; ##p<0.01; **§**denotes significant differences between LD, BEZ vs. LD, SD; §§p<0.01.

Fig. 3 Body composition and indirect calorimetry

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A Body weight. **B** Fat and **C** lean mass were measured by qNMR (Suppl. Fig. 3A-B) and normalized to body weights in %. **D** Average energy expenditure normalized to body weights. **E** Respiratory exchange ratios (RERs) were calculated by dividing carbon dioxide production (VCO_2) by oxygen consumption (VO_2) (Suppl. Fig. 4A-D). The gray rectangle represents 12-hour dark phase (0-time point represents 1 p.m.). **F** ΔRER was calculated as RER_{max}-RER_{min}. Columns represent averages ± standard deviations; n=8-12. *****denotes significant differences between ED, BEZ vs. ED, SD; *$p<0.05$, **$p<0.01$, ***$p<0.001$; #denotes significant differences between ED, SD vs. LD, SD; #$p<0.05$, ###$p<0.001$; **§**denotes significant differences between LD, BEZ vs. LD, SD; §§$p<0.01$, §§§$p<0.001$.

Fig. 4 Euglycemic-hyperinsulinemic clamp

A Steady state BG levels during the clamp. **B** Glucose infusion rate (GINF). **C** Endogenous glucose production (EGP). **D** Whole body glucose uptake. Columns represent averages ± standard deviations; n=8 animals. **§**denotes significant differences between LD, BEZ vs. LD, SD; §$p<0.05$, §§§$p<0.001$.

Fig. 5 Hepatic lipid content

A Hematoxylin and eosin staining of the liver, the black bar represents 50 µm. Representative areas are shown. **B** Liver total TG levels and **C** relative liver TG fatty acid (FA) composition. n- "number" denotes the position of double bounds counted from the omega carbon. Saturated FA (SFA), monounsaturated FA (MUFA) and polyunsaturated FA (PUFA), pre:precursor. **D** The relative content of total SFA, MUFA and PUFA in TG fraction denoted as % of total FA. **E** ED, SD group normalized relative mRNA levels of the indicated transcripts. *Scd*: Stearoyl-CoA-desaturase, *Fasn*: fatty acid synthase. Columns represent averages ± standard deviations; A, C, D and E represent n=4-7; B represents n=8-9 animals. *denotes significant differences between ED, BEZ vs. ED, D; *$p<0.05$, **$p<0.01$,

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***p<0.001. #denotes significant differences between ED, SD vs. LD, SD; ###p<0.001; **§**denotes significant differences between LD, BEZ vs. LD, SD; §§§p<0.001.

Fig. 6 Hepatic mitochondrial mass

A Liver mitochondrial mass and architecture were assessed by transmission EM, the black bar denotes 2 µm. Representative areas are shown. **B** Mitochondrial number was quantified in five independent regions and normalized to the analyzed area (μm^2). **C** Hepatic citrate synthase (CS) protein level was analyzed using western blot, and the intensity of the bands was normalized to tubulin and depicted as ratio to LD, SD group. Representative pictures are shown in Suppl. Fig. 6A. **D** Hepatic gene expression was studied using real-time PCR and depicted as ratio to LD, SD group. *CS*: citrate synthase **E** Our data demonstrated that BEZ improves glucose metabolism in TallyHo mice. In this scheme, the possible underlying mechanisms observed in LD mice are depicted, which are probably involved in the beneficial effects of BEZ. Columns represent averages ± standard deviations; A-B represent n=4; C-D represent n=7-9 animals. *denotes significant differences between ED, BEZ vs. ED, SD; **p<0.01, ***p<0.001; **§**denotes significant differences between LD, BEZ vs. LD, SD; §p<0.05, §§§p<0.001.

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Acknowledgements

This work has been funded by the German Federal Ministry of Education and Research to the German Mouse Clinic (Infrafrontier grant 01KX1012) and the German Center for Diabetes Research (DZD e.V.). We thank Michael Schulz, Moya Wu, Anett Seelig, and Jürgen Schultheiß for excellent technical assistance.

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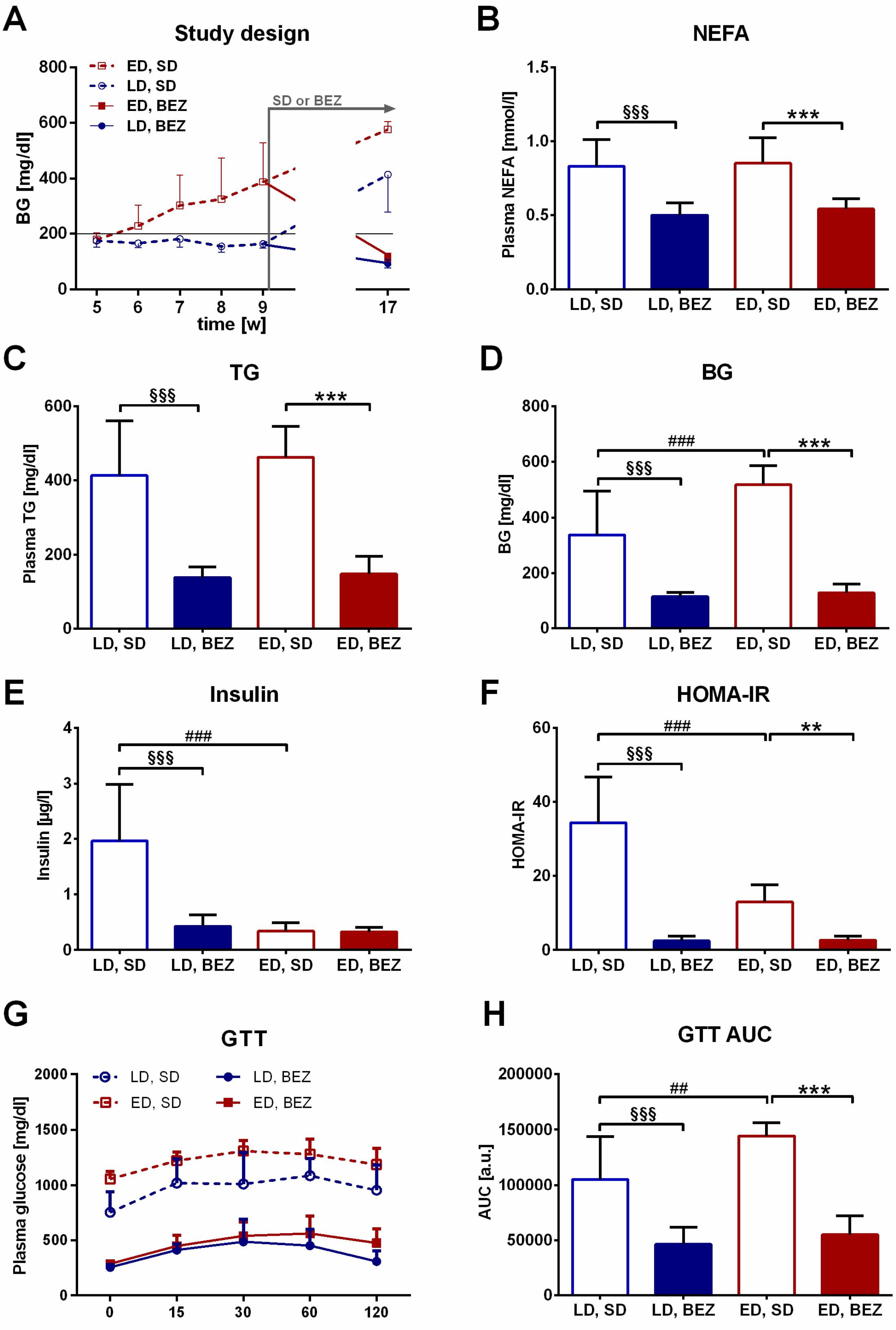
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ED, SD
LD, BEZ
ED, BEZ
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120
time [min]
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LD, SD
LD, BEZ
ED, SD
ED, BEZ

Figure 1

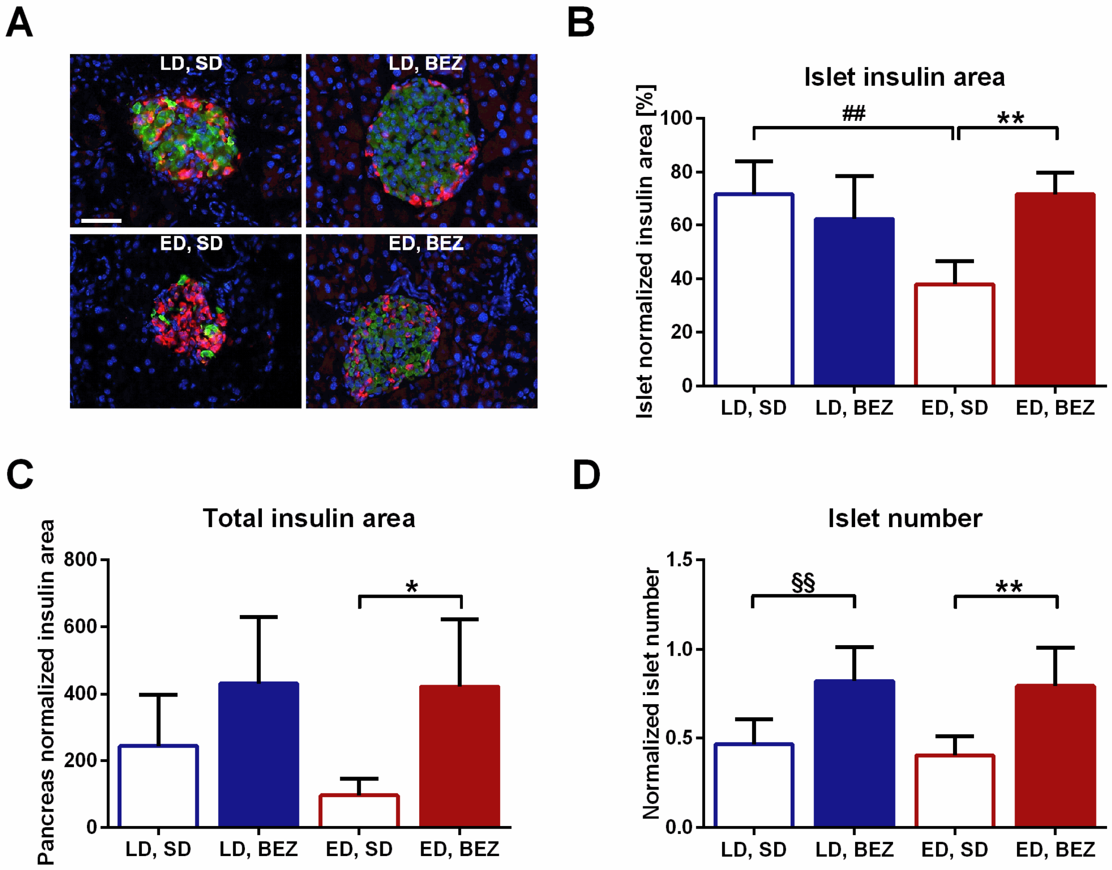

Figure 2

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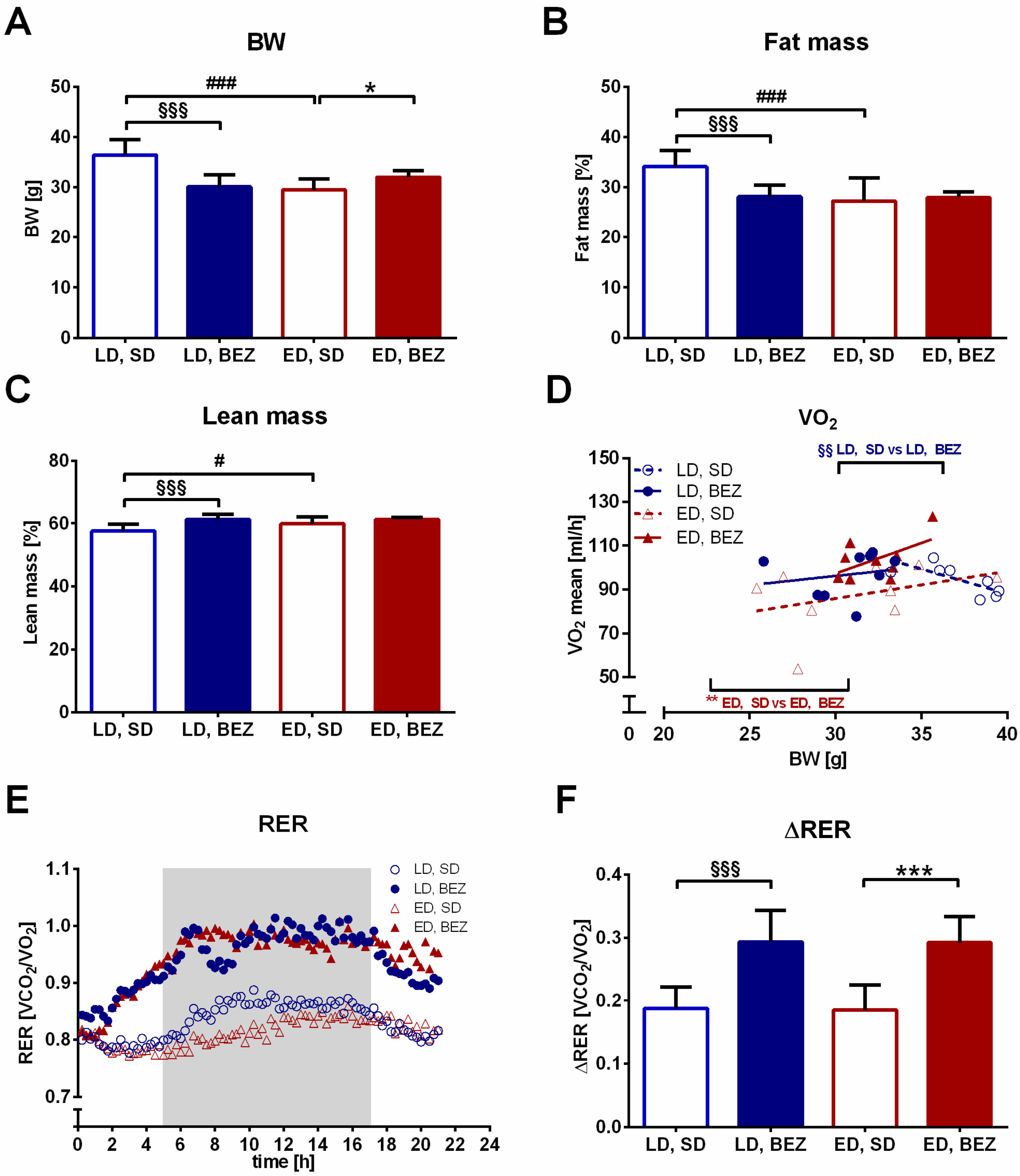
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BW [g]
LD, SD
LD, BEZ
ED, SD
ED, BEZ
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** ED, SD vs ED, BEZ
E
RER
RER [VCO$_2$/VO$_2$]
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0.8
0.7
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2
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6
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10
12
14
16
18
20
22
24
time [h]
LD, SD
LD, BEZ
ED, SD
ED, BEZ
F
ΔRER
ΔRER [VCO$_2$/VO$_2$]
0.4
0.3
0.2
0.1
0.0
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***
LD, SD
LD, BEZ
ED, SD
ED, BEZ

Figure 3

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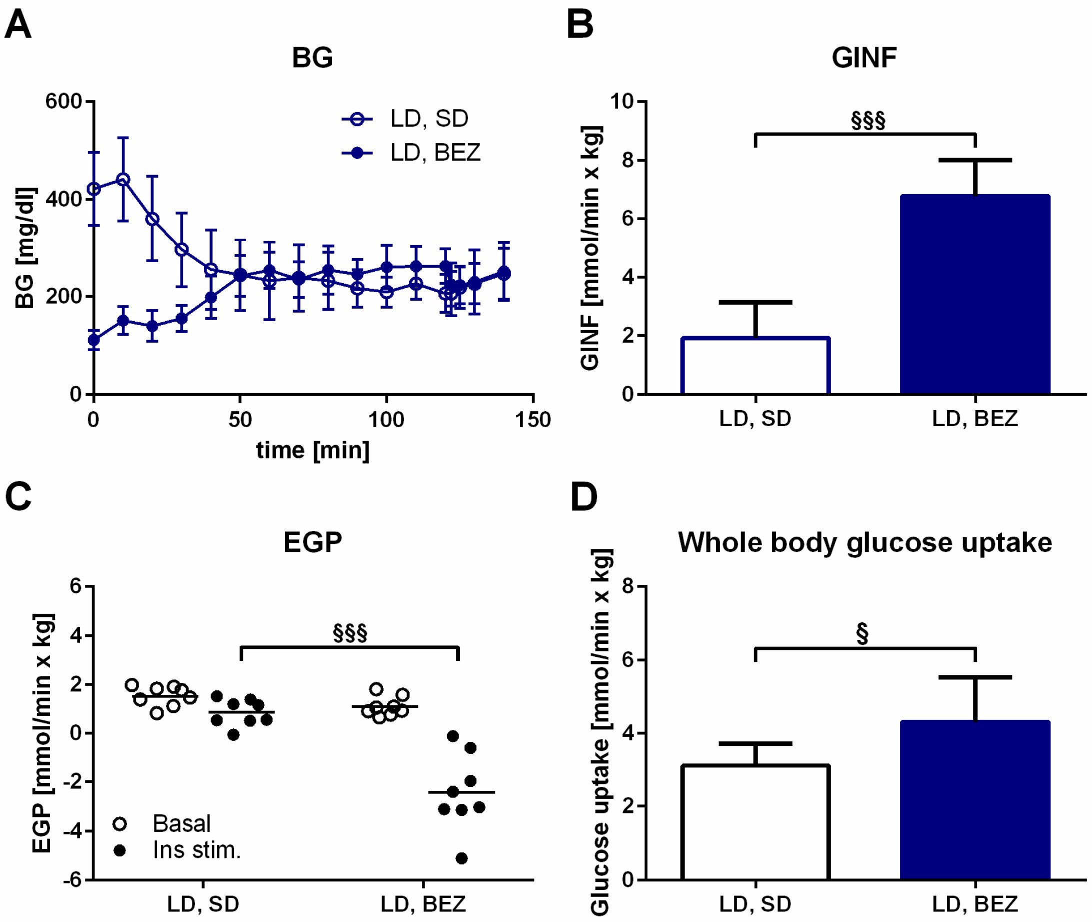

Figure 4

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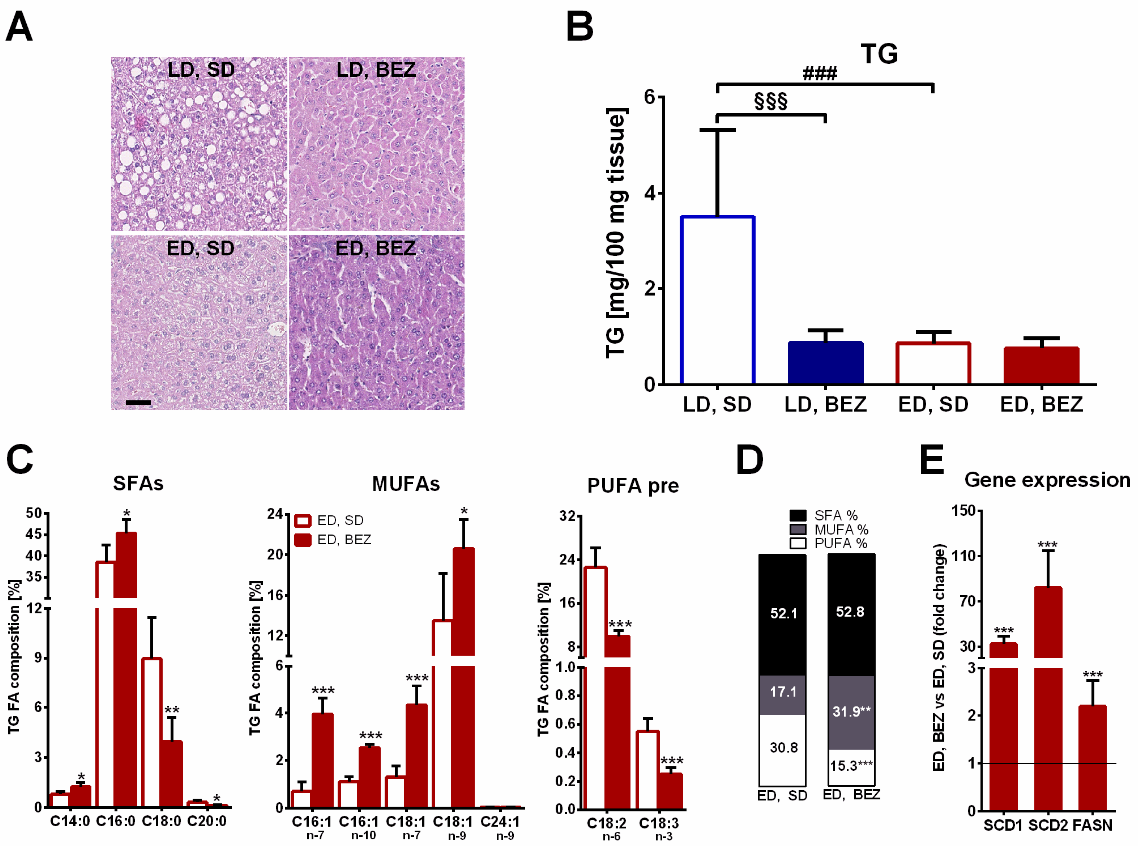

Figure 5

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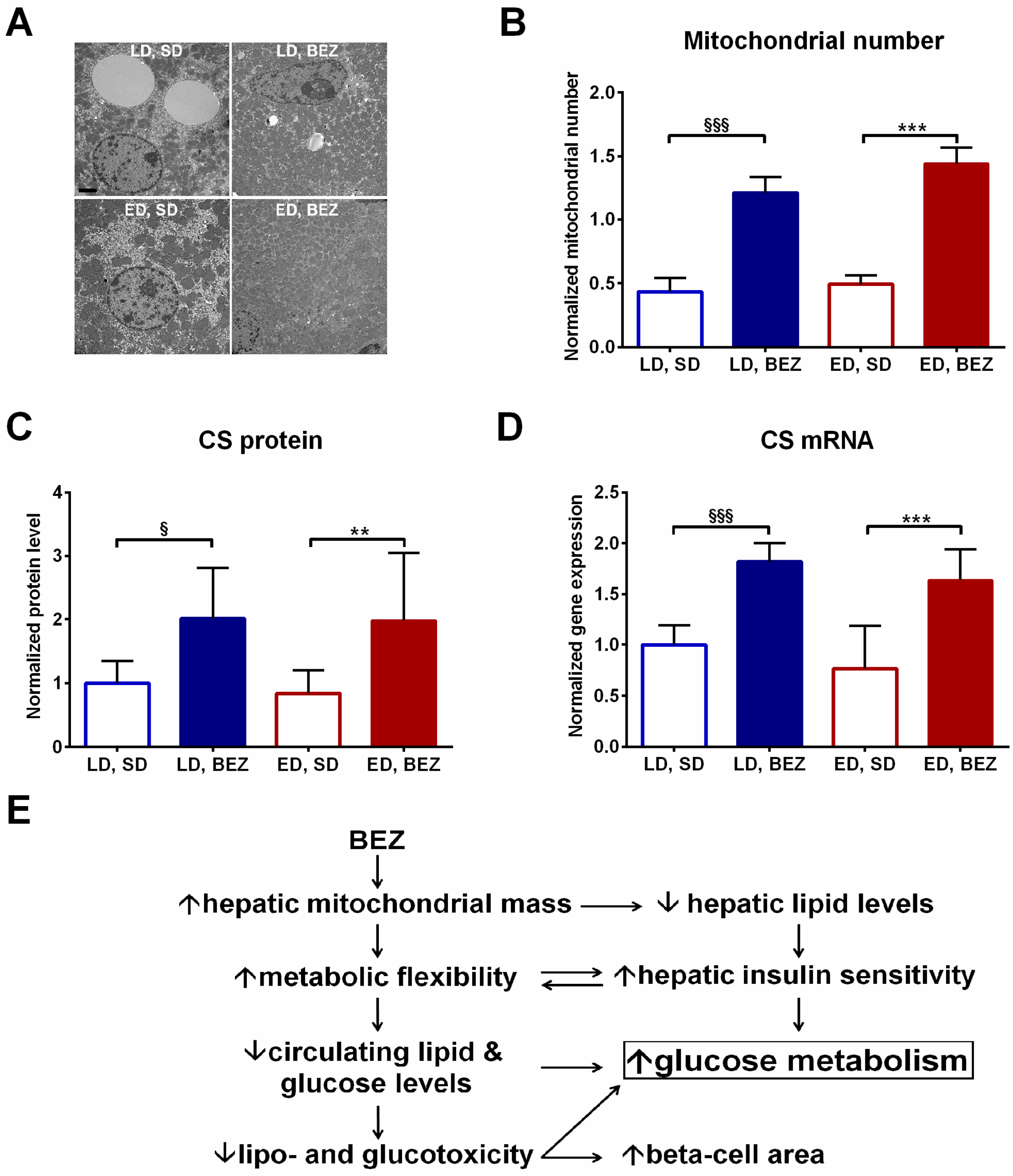

Figure 6

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Highlights

- Bezafibrate treatment reduced steatosis and ameliorated hepatic insulin resistance.
- Bezafibrate treatment enhanced hepatic mitochondrial mass and metabolic flexibility.
- Bezafibrate treatment elevated beta-cell mass.
- Bezafibrate treatment protected mice from developing diabetes.
- Bezafibrate treatment normalized hyperglycemia in the manifest diabetic state.

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