ONLINE DATA SUPPLEMENT

NF-κB -induced oxidative stress contributes to mitochondrial and cardiac dysfunction in type II diabetes

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SUPPLEMENTAL MATERIALS AND METHODS

Chemicals and drugs

The spin probes 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine (CMH), and 1-hydroxy-3-carboxypyrrolidine (CPH); the metal chelators defferoxamine (DF) and diethyldithiocarbamate (DETC); Krebs-HEPES buffer (KHB); and oxygen label NOX-13.1-OS were obtained from Noxygen Science Transfer and Diagnostics (Elzach, Germany). Pyrrolidine dithiocarbamate (PDTC) was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade and were purchased from Sigma-Aldrich unless otherwise specified.

Experimental protocol

At 12 weeks of age, diabetic *db/db* mice were markedly obese and hyperglycemic relative to heterozygous controls. Appropriate groups (n=8 each) of mice were treated for 20 weeks, from age 12 weeks to age 32 weeks, with 100 mg/kg/day PDTC in drinking water. The dose of PDTC was calculated weekly according to animal weight and water consumption. No harmful effects of the full dose were seen; however, the taste of the water likely changed, as mice did not drink as much if the full dose was given acutely. Thus, the dose was gradually increased, which allowed the mice to acclimate, thereby not altering the amount of water consumed by each mouse. The average water intakes of each animal were approximately 7.66 ± 0.272, 12.4 ± 0.36.8 ±0.2, 11.5 ± 0.23 and 7.74 ± 0.23 ml/mouse/ for hz control, obese *db/db,* obese *db/db* + PDTC and heterozygous+ PDTC, respectively (Supplementary Figure 1).

Echocardiography

Echocardiography was performed in mice anesthetized with 1.5% isoflurane/oxygen with a Toshiba Aplio SSH770 system (Toshiba Medical Systems, CA) fitted with a PLT 1202 linear transducer (12 or 14 MHz), which generates two-dimensional images at frame rates ranging from 300 to 500 frames per second. Left ventricular end-diastolic dimension (LVD), LV end-systolic dimension (LVS) were measured using two-dimensional short-axis imaging. LV percent fractional shortening (%FS) was calculated as: %FS = (LVD – LVS)/LVD x100%.

Measurement of free radicals

Two different spin probes were used for EPR studies. CMH was used to measure total reactive oxygen species (ROS) and total superoxide ($O_2^{\bullet-}$) levels, and CPH was used to measure peroxynitrite ($OONO^-$) levels. All EPR measurements were performed using an EMX ESR eScan BenchTop spectrometer and super-high quality factor (Q) microwave cavity (Bruker Company, Germany).

LV total ROS,* $O_2^{\bullet-}$ and *$OONO^-$ production

Small portions of LV issue pieces were incubated with CMH (200uM) at 37°C for 30 minutes for ROS production; PEG-SOD (50 U/ml) for 30 minutes, then CMH (200uM) for another 30 minutes for $O_2^{\bullet-}$ measurement; or CPH (500 μM) for 30 min for $OONO^-$ measurement. Aliquots of incubated probe media were then taken in 50-μl disposable glass capillary tubes (Noxygen Science Transfer and Diagnostics) for determination of total heart ROS , $O_2^{\bullet-}$ and $OONO^-$ production, under the following EPR settings: center field g = 2.002; field sweep 50G; microwave power 20 mW; modulation amplitude 1.90 G; conversion time 10.24 ms; time constant 81.92 ms.

Quantification of NF-κB p65 activity

The NF-κB/p65 Active ELISA (Active Motif, USA) kit was used to measure the binding activity of free NF-κB p65 in nuclear extracts. The kit used contained a 96-well plate to which the consensus-binding site oligo for NF-κB p65 was immobilized. Nuclear extracts were added to each well. A primary antibody specific for an epitope on the bound and active form of the NF-κB was then added. The plate was then incubated with secondary antibody, developed, and read colorimetrically. Free p65 was captured by anti-p65 antibody-coated plates and the amount of bound p65 was detected by adding a second anti-p65 antibody followed by peroxidase-conjugated anti-rabbit IgG, and then using colorimetric detection in an ELISA plate reader at 450 nm.

Mitochondrial complex III enzymatic activity

Aliquots of mitochondria were mixed with oxygenated KHB (20 mmHg - pO2) containing 1 mM EGTA. Then, the oxygen spin label NOX-13.1-OS (5 μM), CMH (200μM), and the complex III substrate, pyruvate (5 mM), were added to the mitochondrial suspension. After adequate mixing, samples were taken in capillary tubes [1] and the enzymatic and respiratory activities of each complex ($O_2^{\bullet-}$ / H_2O_2 and oxygen consumption) were quantified by EPR.

***Immunofluorescence*:**

Anesthetized mice were perfused transcardially with heparinized saline followed by 10% neutral buffered formalin. Hearts were then collected and stored in 4% paraformaldehyde until analyzed. The hearts were embedded in paraffin and 10 μm thick sections were cut on Superfrost plus slides (Fischer Scientific) and incubated overnight at 56˚C. The slides were then deparaffinized in xylene, and rehydrated in descending grades of ethanol. Antigen retrieval was performed by incubating slides in Reveal decloaker, pH 6.0, for 30 min at 120°C in a decloaking

chamber (Biocare Medical, Concord, CA) at 17 to 19 lb/in^2 and cooled to 90°C. Following equilibration to RT, slides were incubated in 0.2% fish skin gelatin (FSG) (Sigma-Aldrich, St. Louis, MO) in PBS for 10 min. This solution was also used to wash slides between all incubations. Tissues were blocked in a humidity chamber for a minimum of 30 min with blocking solution consisting of 2% donkey serum (Sigma), 1% bovine serum albumin (BSA) (Sigma), 0.05% FSG, 0.1% Triton X-100 (Sigma), and 0.05% Tween 20 (Bio-Rad) in PBS. Slides were then incubated overnight at 4˚C with a 1/100 dilution of primary anti-mouse 3-nitrotyrosine (Cayman Chemicals), a footprint for peroxynitrite formation, followed by Alexa Fluor 594-labeled secondary immunoglobulin (IgG) (Molecular Probes) for 30 min at RT. The slides were washed and mounted with ProLong Gold anti-fade reagent (Molecular Probes) for fluorescent microscopy.

Mitochondrial swelling assay

LV mitochondria were isolated by differential centrifugation of heart homogenates as described previously [2,3]. Mitochondria were then resuspended in swelling buffer (for swelling experiments). Mitochondria were then resuspended in swelling buffer (for swelling experiments). Intact mitochondria (250 ug/ml) isolated from heart tissues were suspended in a buffer containing (in mM) 120 KCl, 10 Tris–HCl, 5 KH_2PO_4, and 20 MOPS. Mitochondrial swelling was assessed spectrophotometrically as a decrease in absorbance at 520 nm (A_{520}) [2].

Statistical analyses

All data illustrated are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software**.** Data were analyzed by ANOVA, followed by Bonferroni's multiple comparison tests. In all cases, $p<0.05$ was considered statistically significant.

REFERENCES

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SUPPLEMENTAL FIGURES

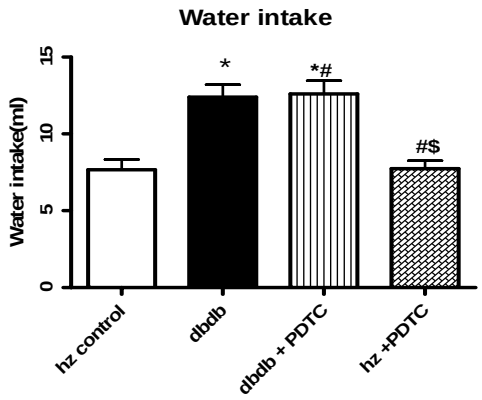

Figure 1. Daily water intakes for each group of mice. Values are represented as mean ± SEM.