ONLINE SUPPLEMENTAL MATERIAL

Critical role for hepatocyte-specific eNOS in NAFLD and NASH

Rory P. Cunningham[1,3], Mary P. Moore[1,3], Ryan J. Daskek[1,5], Grace M. Meers[1, 3], Takamune Takahashi[7], Ryan D. Sheldon[8], Andrew A. Wheeler[4], Alberto Diaz-Arias[6], Jamal A. Ibdah[1,2], Elizabeth J. Parks[2,3], John P. Thyfault[9,10], R. Scott Rector[1,2,3]

[1]Research Service, Harry S Truman Memorial Veterans Medical Center, Columbia, Missouri 65212, USA; [2]Departments of Medicine-Division of Gastroenterology and Hepatology, [3]Nutrition and Exercise Physiology, [4]Surgery, University of Missouri, Columbia, MO 65211, USA; [5]Comparative Medicine Program, University of Missouri, Columbia, Missouri, USA; [6]Boyce & Bynum Pathology Professional Services, Columbia MO, 65201, USA; [7]Division of Nephrology and Hypertension, Vanderbilt University School of Medicine, Nashville, Tennessee 37232, USA; [8]Metabolic and Nutritional Programming, Center for Cancer and Cell Biology, Van Andel Institute, Grand Rapids, MI 49503, USA; [9]Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, KS 66160, USA; [10]Kansas City VA Medical Center, Kansas City, MO 64128, USA.

SUPPLEMENTAL MATERIAL FOR DATA PRESENTED IN SUPPLEMENT

Animal care and terminal procedures

For all animal experiments, room temperature was kept constant at 21-22 degrees Celsius with a 12:12 light/dark cycle. Food intake and body weight of the animals was recorded weekly, and body composition (4in1-1100 Analyzer; EchoMRI, Houston, TX) measured monthly. Food consumption was measured by taking the difference in grams of food given and grams of food remaining 7 days later and multiplying total grams consumed by energy content per gram of the diet (CD - 3.5kcal/gram, WD - 4.75kcal/gram) and dividing by 7 to give kilocalories per day. On the day of euthanasia, mice were fasted overnight for 12 hr (2000-0800), before being anesthetized with pentobarbital sodium (50 mg/kg). Blood was collected via cardiac puncture, and the animals were euthanized via removal of the heart. Livers were quickly excised from anesthetized mice and prepared for mitochondrial isolation, homogenization for palmitate oxidation, and fixed in 10% formalin or snap-frozen in liquid nitrogen for later processing as described in detail in the following sections.

Glucose, insulin, and pyruvate tolerance testing

A subset of CD and WD-fed $eNOS^{fl/fl}$ and $eNOS^{hep-/-}$ male mice (22-30 weeks of age) were used for glucose, insulin, and pyruvate tolerance tests, to determine the effects of hepatocellular eNOS deficiency on whole body glucose homeostasis. For all testing, mice were fasted in single cages with fresh bedding and *ad libitum* access to water. For glucose tolerance testing (GTT), mice were fasted overnight prior to baseline testing. At the end of the fast, the tail was nicked and blood sampled via a glucometer (Alpha Trak, Abbott Labs), to determine baseline blood glucose. Mice

were then administered a sterile solution of 50% dextrose (2g/kg of body weight) via intra peritoneal (IP) injection, as previously performed (1). Blood glucose measures were taken at 15, 30, 45, 60, and 120 min post glucose injection. For insulin tolerance testing (ITT), mice were fasted 3 hr prior to baseline testing, from the start of their light cycle. After baseline glucose measurement, mice were administered with insulin (Humulin R; 100 U/ml) made in a 3% BSA sterile saline (0.9% NaCl, Hospira; NDC 0409-4888-20) solution at a dose of 0.75U insulin/kg of body weight via IP injection. Blood glucose measurements were taken at the same intervals as GTT. Mice were fasted 5 hr prior to pyruvate tolerance testing (PTT) at the start of their light cycle. After baseline glucose measured, mice were administered a pyruvate solution (0.2g/mL of sterile saline) at a dose of 2g/kg of body weight via IP injection. Blood glucose was measured at the same intervals as GTT and ITT. All area under the curves were calculated using the trapezoidal method.

Primary antibodies

Primary antibodies used are as follows: eNOS (no. 610297; BD Biosciences, San Jose, CA), phosphorylated eNOS ser1117 (no. 612393; BD Biosciences, San Jose, CA), oxidative phosphorylation (OXPHOS) mitochondrial profile (ab110413; Abcam, Cambridge, MA), peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1α, no. WH0010891M3, Millipore-Sigma, Burlington, MA), mitochondrial transcription factor A (TFAM; Santa Cruz Biotechnology, Dallas, TX), Bnip3 (no. 3769; Cell Signaling Technology, Danvers, MA), 1A/1B light chain 3B (LC3; no. 4108S, Cell Signaling), p62 (no. 5114; Cell Signaling Technology), Unc-51 Like Autophagy Activating Kinase 1 (D8H5) (ULK1, no. 8054S; Cell Signaling Technology), pULK1 Ser555 (D1H4) (no. 5869S, Cell Signaling Technology), Parkin (no. 4211; Cell Signaling

Technology), autophagy related 12-5 ATG12-5 (D88H11), (ATG12-5, no. 6904S; Cell Signaling Technology), autophagy related 7 (ATG7, no. 8558S; Cell Signaling Technology), PTEN-induced kinase 1 (PINK1, no. 6946; Cell Signaling Technology), AMP-activated protein kinase (AMPK, no. 2532S; Cell Signaling Technology), pAMPK (Thr172) (no. 2351; Cell Signaling Technology), dynamin related protein 1 (D8H5) (DRP1, no. 5319S; Cell Signaling Technology), pDRP1 (S616) (no. 3455; Cell Signaling Technology), dynamin-like 120 kDa protein, mitochondrial (D6U5N) (OPA1, no. 80471S; Cell Signaling Technology), Protein kinase B (AKT, no. 9272, Cell Signaling Technology), phosphorylated AKT (ser473) (no. 9271; Cell Signaling Technology), phosphorylated AKT (thr308) (no. 13038; Cell Signaling Technology), glycogen synthase kinase-3β (GSK-3β, no. 9315; Cell Signaling Technology), insulin receptor substrate 1 (IRS1, no. 2382; Cell Signaling Technology), phosphorylated IRS1 (ser612) (no. 3193; Cell Signaling Technology), IRS2 (no. 3089; Cell Signaling technology), phosphorylated IRS2 (ser731) (no. 3690; Cell Signaling Technology). Primary antibodies were used at 1:1,000 dilution, and secondary antibody at 1:5,000 dilution. Blots were analyzed via densometric analysis (Image Laboratory Beta 3, Bio-Rad Laboratories, Hercules, CA). Total protein was assessed with Amido black (0.1%, Sigma) to control for differences in protein loading and transfer as previously described (2; 3). Blots in primary hepatocytes were normalized to β-actin.

Serum assays

Quantification of serum alanine aminotransferase (ALT) levels were performed by a commercial laboratory (Comparative Clinical Pathology Services, Columbia, MO) using an Olympus AU680 automated chemistry analyzer (Beckman Coulter, Brea, CA) according to the manufacturer's instructions. Analysis of serum glucose, triglycerides, free fatty acids (FFA) were carried out using an AU680 automated chemistry analyzer (Beckman Coulter, Brea, CA) according to the

manufacturer's instructions. Plasma insulin concentrations were determined using a commercially available ELISA (EZRMI-13K, Sigma).

Metabolomics

Metabolomic analysis was carried out via ion-paired liquid chromatography/mass spectrometry (LC/MS) metabolite profiling on frozen whole liver from $eNOS^{fl/fl}$ and $eNOS^{hep-/-}$ mice, as previously described with slight modifications (4). Briefly, metabolites were extracted by the addition of extraction solvent (80% methanol) kept at -20°C directly to frozen liver sample at a concentration of 40mg frozen liver/mL of extraction solvent. Extracts were vortexed for 15 seconds, sonicated in a water bath sonicator for 5 min, and then incubated on ice for 10 min. Protein was pelleted by centrifugation at 17,000g for 10 min at 4C. 800µL of supernatant (4 million cell equivalents) was transferred to a fresh Eppendorf tube and dried in a rotor speedvac. For LCMS analysis samples were resuspended in 50µL of LCMS grade water. For GCMS analysis samples were incubated at 70C for 15 min in 15uL of 10mg/mL methoxyamine HCL (Sigma# 226904) in pyridine (Millipore #PX2012-7), then 35uL of MTBSFA+1%TMCS (Sigma#M-108) was added and samples were further incubated at 70°C for an additional 90 minutes. Ion-paired LC/MS metabolite profiling was conducted on an Agilent 6470 QQQ mass spectrometer coupled to an Infinity 1290 UHPLC system with a Zorbax RRHD Extend-C18 analytical column (2.1x150mm, 1.8µm; Agilent# 759700-902) and Zorbax Fast Extend-C18 guard column (2.1mm 1.8µm; Agilent# 821725). 2uL of resuspended cell extracts (160,000 cell equivalents on column) were injected. Mobile phase A was 97% H2O, 3% methanol, 10mM tributylamine (Sigma# 90780), and 15mM acetic acid. Mobile phase B was 100% methanol with 10mM tributylamine, and 15mM acetic acid. The gradient is as follows: 0-2.5min 100%A, 2.5-7.5 min ramp to 80%A, 7.5-13min ramp to 55%A, 13-20 min ramp to 99%B, 20-24 min hold at 99%B. Flow rate was held at

0.25mL/min, and the column compartment heated to 35C. The mass spectrometer was operated in dynamic MRM mode pre-programmed per with two transitions within a 2-4-minute retention time window per analyte. The ESI source was operated in negative mode with the following conditions: gas 13 L/min at 150C, nebulizer pressure 45 psi, sheath gas 12L/min at 325C, nozzle voltage 500V, and capillary voltage 2000V. After each analytical run the column was re-equilibrated by backflush with 90% acetonitrile (0.8mL/min) for 8 min and 100% A for 8 min (0.4mL/min) to achieve run-to-run retention time consistency. GC/MS metabolite profiling was conducted on an Agilent 5977B MSD with a 7890 GC with a DuraGuard J&W DB-5ms GC Column (30m, 0.25mm, 0.25µm, plus DuraGuard, 10m; Agilent #122-55326). 1uL of derivatized cell extracts were injected (80,000 cell equivalents on column) into a split/splitless inlet heated to 250C and operated in splitless mode. Column flow was set to 1mL/min and the oven ramp was 60C for 1 min, then ramped to 320C at 10C/min, and held at 320C for 10 min. The EI source was operated at -70EV and 250C.The MS was set to scan from 50-800m/z with a step of 0.1m/z at a rate of 2hz. 2-3 fragment ions at a standard-verified retention time were used to identify compounds. Data analysis for both GC and LC profiling were conducted with Agilent MassHunter Quantitative software (v. 9.0) and peaks were visually inspected by a trained technician for accuracy. All metabolic profiling and analysis were completed at the Van Andel Institute (Grand Rapids, MI), by trained technicians.

SUPPLEMENTAL RESULTS

Supplemental Table 1: Gene primer sequences.

Primers	Forward Sequence 5'-3'	Reverse Sequence 5'-3'
arg-1	GATCACATCCCAAAATTCCC	CTTCATCTTTCTTCCCACAC
αsma	AAACAGGAATACGACGAAG	CAGGAATGATTTGGAAAGGA
bnip3	ACCACAAGATACCAACAGAG	AATCTTCCTCAGACAGAGTG
cat	CTCCATCAGGTTTCTTTCTTG	CAACAGGCAAGTTTTTGATG
cd11c	TCACACCTGCAGAGATTT	TACTCAGACGGCCATGGT
cd68	GTGTCTGATCTTGCTAGGACC	GTGCTTTCTGTGGCTGTAG
col1a1	ACGCCATCAAGGTCTACTGC	ACTCGAACGGGAATCCATCG
emr1	TTTCAAATGGATCAGAAGG	CAGAAGGAAGCATAACCAAG
Il-1β	GCTACCTGTGTCTTTCCCGT	CATCTCGGAGCCTGTAGTGC
mcp-1	ACTGAAGCCAGCTCTCTCTTCCTC	TTCCTTCTTGGGGTCAGCACAGAC
nfe212	CATTCCCGAATTACAGTGTC	GGAGATCGATGAGTAAAAATGG
nqo1	CCTTTCCAGAATAAGAAGACC	AATGCTGTAAACCAGTTGAG
pdgrf	GATTGACATCCTGCCTGACC	CATGGAACTCCACCAAATCC
ppib	TGGAGATGAATCTGTAGGAC	CAAATCCTTTCTCTCCTGTAG
tgfβ1	AAGTTGGCATGGTAGCCCTT	GCCCTGGATACCAACTATTGC
tnf-α	GTGACAAGCCTGTAGCCCAC	GCAGCCTTGTCCCTTGAAGA

Supplemental Table 2: Characteristic of human patients across NAS groupings. Data are presented as mean ± SD. BMI, body mass index; ALT, alanine aminotransferase; AST aspartate amino transferase. *significantly different from NAS 0 and NAS 1-3 ($P < 0.05$).

	NAS 0	**NAS 1-3**	**NAS 4-6**
Number of patients	7	45	38
Age (years)	39 ± 7.8	47.8 ± 11.4	45.8 ± 12.6
BMI (kg/m²)	47 ± 9.0	48.8 ± 7.0	48.6 ± 7.4
Sex (Male/Female)	0/7	10/35	7/31
ALT (IU/L)	24.3 ± 9.8	29.8 ± 16.8	54.7 ± 52.4*
AST (IU/L)	23.6 ± 5.8	26.9 ± 9.4	48.6 ± 51.2*
Glucose (mg/dL)	87.9 ± 12.2	105.8 ± 36.9	108.8 ± 33.9
Total cholesterol (mg/dL)	175 ± 29.6	162.5 ± 35.6	176.5 ± 42.6
Triglycerides (mg/dL)	112.9 ± 31.5	133.1 ± 63.1	138.4 ± 55.5

Supplemental Figure 1: Hepatocellular eNOS deficiency has no effect on animal characteristics and glucose tolerance. A) $eNOS^{fl/fl}$ and $eNOS^{hep-/-}$ mice were randomized to either a CD or WD and body weight was tracked for 16 weeks (*n* = 13-17/group). B) Final body fat % and C) liver weight, after 16 weeks on respective diets (*n* = 13-17/group). D). Serum glucose, TGs, and ALT and insulin concentrations (*n* = 13-17/group). E) GTT, ITT, and PTTs were performed on a subset of $eNOS^{fl/fl}$ and $eNOS^{hep-/-}$ mice on either a CD or WD for 10 weeks (*n* = 4-11/group). F) Total glucose AUC for GTT, ITT, and PTT (*n* = 4-11/group). Data are presented as mean ± SD.* main effect of diet ($P < 0.05$ vs CD). CD, control diet; WD, western diet; TGs, triglycerides; ALT, alanine aminotransferase; GTT, glucose tolerance test; ITT, insulin tolerance test; PTT, pyruvate tolerance test; AUC, area under the curve.

Supplemental Figure 2: Hepatocellular eNOS deficiency does not exacerbate WD-induced hepatic inflammation. mRNA expression of markers of hepatic inflammation in $eNOS^{fl/fl}$ and $eNOS^{hep-/-}$ mice fed a CD or WD for 16 weeks (*n* = 13-17/group). Data are presented as mean ± SD. *main effect of diet ($P < 0.05$ vs CD). CD, control diet; WD, western diet.

Supplemental Figure 3: **Effect of hepatocellular eNOS deficiency on hepatic mitochondrial function in female $eNOS^{fl/fl}$ and $eNOS^{hep-/-}$ mice**. A) Whole liver complete, incomplete, and total [1-^{14}C] palmitate oxidation to CO_2 (*n* = 7-8/group). B) Oxygen consumption in isolated liver mitochondria (*n* = 7-8/group). Data are presented as mean ± SD. *main effect of diet ($P < 0.05$ vs CD), #main effect of genotype ($P < 0.05$ vs $eNOS^{fl/fl}$).

Supplemental Figure 4: Effects of hepatocellular eNOS deficiency on markers of mitochondrial content, biogenesis, and fission/fusion. A) Whole liver β-HAD activity and B) whole liver citrate synthase activity (*n* = 13-17/group). Protein expression of C) electron transport chain complexes and D) markers of mitochondrial biogenesis and fission/fusion (*n* = 13-17/group), and E) their Western blot representative images. Data are presented as mean ± SD.*main effect of diet ($P < 0.05$ vs CD), #main effect of genotype ($P < 0.05$ vs $eNOS^{fl/fl}$). CD, control diet; WD, western diet.

Supplemental Figure 5: Hepatocellular eNOS deficiency impairs mitochondrial morphology, quality, and turnover. In $eNOS^{fl/fl}$ and $eNOS^{hep-/-}$ mice on either a CD or WD for 16 weeks - A) Protein expression of markers of mitophagy in whole liver homogenate (*n* = 13-17/group), and B) their representative Western blot images. Data are presented as mean ± SD.*main effect of diet ($P < 0.05$ vs CD), $ significant diet and genotype interaction ($P < 0.05$). CD, control diet; WD, western diet.

Supplemental Figure 6: Effect of hepatocellular eNOS over expression on hepatic mitochondrial function and content. A) Whole liver complete, incomplete, and total [1-^{14}C] palmitate oxidation to CO_2 (n = 10/group). B) Isolated liver mitochondria complete, incomplete, and total [1-^{14}C] palmitate oxidation to CO_2 (*n* = 10/group). C) Oxygen consumption in isolated liver mitochondria (n = 10/group). D-F) Protein expression of the electron transport chain (ETC) complexes, markers of mitochondrial turnover, and their representative Western blot images (n = 10/group). Data are presented as mean ± SD.*main effect of diet ($P < 0.05$ vs CD), #main effect

of overexpression ($P < 0.05$ vs AAV-GFP). $ significant diet and genotype interaction ($P < 0.05$). CD, control diet; WD, western diet.

Supplemental Figure 7: Metabolomic analysis of whole livers from eNOS$^{fl/fl}$ and eNOS$^{hep-/-}$ mice. Metabolomic analysis was carried out via ion-paired liquid chromatography/mass spectrometry (LC/MS) metabolite profiling on frozen whole liver from eNOS$^{fl/fl}$ and eNOS$^{hep-/-}$ mice. A) Heat map of metabolite abundance segregated by genotype and diet. B) Relative abundance of metabolites (n = 9-11/group). Data are presented as mean ± SD. *main effect of diet ($P < 0.05$ vs CD), #main effect of genotype ($P < 0.05$ vs eNOS$^{fl/fl}$). CD, control diet; WD, western diet.

Supplemental Figure 8: Hepatic insulin signaling is not compromised in eNOS$^{hep-/-}$ mice. For Acute *in vivo* insulin stimulation studies in eNOS$^{fl/fl}$ and eNOS$^{hep-/-}$ male mice (20-22 weeks of age) were performed via intraperitoneal injections of insulin. A-F) Whole liver protein expression of markers of hepatic insulin signaling and their representative Western blot images (n = 4-6/group). Data are presented as mean ± SD.*main effect of insulin ($P < 0.05$ vs saline).

Supplemental Figure 9: Genetic and viral manipulation of hepatocyte eNOS does not alter eNOS content in other tissues. A) Genetic ablation of eNOS does not alter eNOS protein content in other tissues in eNOS$^{hep-/-}$ mice (n = 4-6/group). B) In hepatocyte eNOS overexpression studies, liver-specific adeno-associated viral overexpression of eNOS does not alter eNOS protein content in other tissues (n = 4-6/group). Data are presented as mean ± SD.*main effect of diet ($P < 0.05$ vs saline). CD, control diet; WD, western diet.

REFERENCES

1. Winn NC, Acin-Perez R, Woodford ML, Hansen SA, Haney MM, Ayedun LA, Rector RS, Vieira-Potter VJ, Shirihai OS, Sacks HS, Kanaley JA, Padilla J: A Thermogenic-Like Brown Adipose Tissue Phenotype Is Dispensable for Enhanced Glucose Tolerance in Female Mice. Diabetes 2019;68:1717-1729
2. Rector RS, Thyfault JP, Morris RT, Laye MJ, Borengasser SJ, Booth FW, Ibdah JA: Daily exercise increases hepatic fatty acid oxidation and prevents steatosis in Otsuka Long-Evans Tokushima Fatty rats. Am J Physiol Gastrointest Liver Physiol 2008;294:G619-626
3. Moore MP, Cunningham RP, Kelty TJ, Boccardi LR, Nguyen NY, Booth FW, Rector RS: Ketogenic diet in combination with voluntary exercise impacts markers of hepatic metabolism and oxidative stress in male and female Wistar rats. Appl Physiol Nutr Metab 2020;45:35-44
4. Roy DG, Chen J, Mamane V, Ma EH, Muhire BM, Sheldon RD, Shorstova T, Koning R, Johnson RM, Esaulova E, Williams KS, Hayes S, Steadman M, Samborska B, Swain A, Daigneault A, Chubukov V, Roddy TP, Foulkes W, Pospisilik JA, Bourgeois-Daigneault MC, Artyomov MN, Witcher M, Krawczyk CM, Larochelle C, Jones RG: Methionine Metabolism Shapes T Helper Cell Responses through Regulation of Epigenetic Reprogramming. Cell Metabolism 2020;31:250-266.e259