Online Data Supplement

Disruption of endothelial peroxisome proliferator-activated receptor gamma (PPARγ) reduces vascular nitric oxide production

Jennifer M. Kleinhenz, Dean J. Kleinhenz, Shaojin You, Jeffrey D. Ritzenthaler, Jason M. Hansen, David R. Archer, Roy L. Sutliff, and C. Michael Hart

Materials and Methods

Characterization of ePPARγ-/- mice. Polymerase chain reaction (PCR) genotyping of tail snips was employed to identify wild-type, floxed and null PPARγ, and Cre alleles. Tail snips were obtained from mice following 2% isoflurane inhalational anesthesia, and tail snip DNA was isolated and amplified on a thermal cycler using primers and protocols provided by the Jackson Laboratory. For PCR amplification of the null allele, a previously reported forward primer before the upstream loxP site (1) was used in conjunction with a reverse primer below the downstream loxP site (primer oIMR1935, Jackson Laboratories). PCR amplification conditions for null PPARγ were: 5 min at 94°C, followed by 26 cycles (30 sec at 94°C, 1 min at 62°C -0.4°C/cycle, 1 min at 72°C), 7 min at 72°C. To verify the integrity of each DNA sample, an unrelated gene (GAPDH) was also PCR amplified using forward primer: 5' – TGTAAAGGTCATTAAGAGGATTGGGTGTCC – 3'. Reverse primer: 5' ATCAGGGCGGAGTGGAGAGATCTGGTTTCT – 3'. PCR amplification conditions for GAPDH were: 5 min at 94°C, followed by 28 cycles (30 sec at 94°C, 1 min at 61°C, 30 sec at 72°C), 7 min at 72°C. Resulting PCR products were analyzed by agarose gel electrophoresis.

Blood pressure monitoring and angiotensin II treatment. Data Sciences International (St. Paul, MN) PA-C10 blood pressure probes, sterilized in 2% glutaraldehyde and soaked in sterile saline were used for blood pressure monitoring. Mice were anesthetized with inhalational isoflurane. The anterior neck was shaved and disinfected with 70% alcohol and Betadine solution. Atropine (0.25 mg/kg) was injected

subcutaneously to minimize airway secretions, and the mouse was covered with a sterile surgical drape. A ventral midline incision was made from the lower mandible to the sternum. The left common carotid artery was isolated and sutures were passed under the carotid and used for both ligation and retraction. The anterior suture was tied off just caudal to the carotid bifurcation, and the posterior suture was placed as far caudal as possible. A 25-gauge needle with a bend at the beveled tip was used to hold open the carotid while the transducer was advanced into the thoracic aorta. The loose ends of the two sutures were tied securely around the catheter anchoring it in the carotid. Through the same incision a subcutaneous pouch was formed using blunt dissection, and the transmitter battery was slipped under the skin and down into the pocket along the right flank close to the hindlimb. The neck incision was closed with suture. 1cc of warmed lactate ringers' buffer and buprenorphine (0.05 mg/kg) was administered post operatively. Buprenorphine (0.05mg/kg, twice daily) was administered for 3 days postoperatively to alleviate discomfort related to the procedure. The mice were singly caged and allowed to fully recover for 7 days prior to the initiation of data collection.

In selected ePPARγ-/- and littermate controls, osmotic pumps (Alzet, Cupertino, CA) were implanted 2 weeks after the introduction of the telemetry probe, and after stable values for basal blood pressure and heart rate were recorded. The mouse was anesthetized with isoflurane anesthesia, and an incision was made on the back of the neck. The Alzet minipump was implanted subcutaneously on the side of the back

opposite the transmitter for subcutaneous infusion of angiotensin II (Ang II, 0.7 mg/kg/day, Sigma). The incision was closed with suture.

Measurement of aortic NO production with electron spin resonance (ESR) spectroscopy. Immediately following sacrifice, aortas were rapidly dissected, placed in cold Krebs-Hepes buffer (pH 7.4), and any extraneous periadventitial fat was removed. Each aorta (~15 mm) was cut into 2 mm sections and placed into a single well of a 12-well plate. The NO spin trap, iron diethyldithio-carbamic acid ($Fe(DETC)_2$), was added to each well, along with calcium ionophore (A23187, EMD Chemicals, San Diego, CA) to stimulate NO production as previously reported (2). Following incubation at 37°C for 60 min, aortic segments were collected and snap frozen in liquid nitrogen in a 1 cc syringe. Electron spin resonance (ESR) spectroscopy was performed with a MEX EPR Spectrophotometer (Bruker, Karlruhe, Germany) with the following settings: microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 5 G; field center, 3290 G; sweep width, 90 G; microwave frequency, 9.39 GHz; conversion time, 328 ms; time constant, 5.24 s; number of scans, 4; sweep time, 168 s.

Electrophoretic mobility shift assays (EMSA) for NF-κB nuclear binding. Nuclear aortic proteins were isolated and pooled from two mice using the Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocols and stored at -80°C. Double-stranded NF-κB consensus oligonucleotide (5'- AGTTGAGGGGACTTTCCCAGGC) was radiolabeled with ^{32}P gamma ATP using T4 polynucleotide kinase enzyme. Aortic nuclear protein (2 μg) was incubated with

radiolabeled NF-κB (50,000 cpm/ng) for 2 h at room temperature. DNA-protein complexes were separated on 6% native polyacrylamide gels (20:1 acrylamide/bis ratio) in low ionic strength buffer (22.25 mM Tris borate, 22.25 mM boric acid, 500 mM EDTA) for 2-3 h at 4°C and 10 V/cm. Gels were fixed in a 10% glacial acetic acid/10% methanol solution for 10 min, vacuum dried and exposed to X-ray film. DNA-protein complexes were quantified by densitometric analysis using the Image J program (developed at the National Institutes of Health, http://rsb.info.nih.gov/ij/).

Glucose tolerance testing. Blood glucose levels were measured by applying tail blood to the OneTouch Profile portable blood glucose monitor (OneTouch, Johnson & Johnson, New Brunswick, NJ). For the glucose tolerance test, mice were fasted for 5 hours, then injected with glucose (2 g/kg, intraperitoneally). Blood glucose was then measured at intervals (pre-injection and 20, 40, 60, 120, 180, and 240 min post-injection) to determine glucose response. Glucose tolerance test results were analyzed by ANOVA with repeated measures.

Immunohistochemistry. Paraffin-embedded kidney sections were rehydrated, stained in hematoxylin for 1 minute, washed in water, stained in eosin for 1 minute, then dehydrated before mounting.

Results

Table S1: Phenotypic screening in littermate control and ePPARγ-/- mice.

	Littermate Control (n)	ePPARγ$^{-/-}$ (n)
Body Weight (g)	28.7 ± 1.0 (24)	27.2 ± 0.8* (24)
Organ Weights (mg/g body weight)		
Heart	5.35 ± 0.17 (7)	5.33 ± 0.14 (9)
Liver	44.78 ± 1.74 (5)	45.46 ± 1.05 (9)
Kidneys	6.66 ± 0.10 (14)	6.30 ± 0.14 (18)
Spleen	3.31 ± 0.30 (7)	4.26 ± 0.19* (9)
Testicles	3.80 ± 0.11 (14)	3.96 ± 0.08 (18)
Lungs	5.60 ± 0.34 (7)	6.05 ± 0.32 (9)
Blood Analysis		
Hematocrit (%)	47.17 ± 0.56 (9)	45.27 ± 0.48* (11)
Leukocyte Count (#/μl blood)	7,831 ± 812 (8)	9,833 ± 822 (12)
Fasting serum glucose (mg /dL)	141.1 ± 8.2 (10)	147.5 ± 5.9 (10)

After sacrifice, selected organs and blood were harvested from 6-12-week old ePPARγ$^{-/-}$ and age-matched littermate controls. Total body and organ weights were recorded, and hematocrit, total leukocyte count, and glucose levels were determined. Values are mean ± SEM; * $p<0.05$ versus littermate controls.

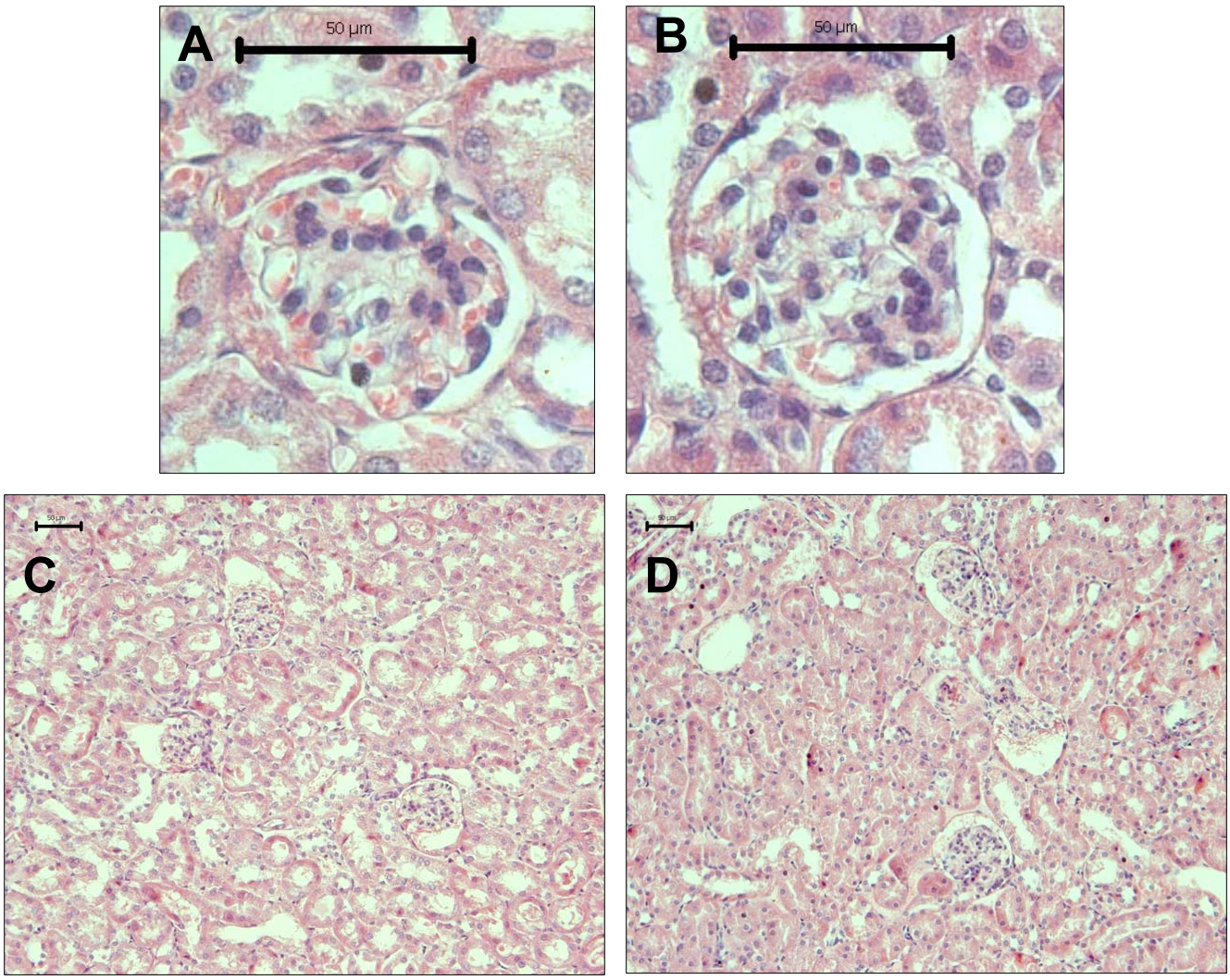

Figure S1: Highly vascularized tissues display no overt anatomical differences in ePPARγ-/- mice. Hematoxylin and Eosin-stained kidney sections from both ePPARγ-/- (B and D) and littermate control (A and C) mice were analyzed by light microscopy. No overt differences were seen in the morphology of the glomeruli, proximal tubules, or distal tubules. 40x and 20x images are displayed. Scale bar = 50 µm in all images.

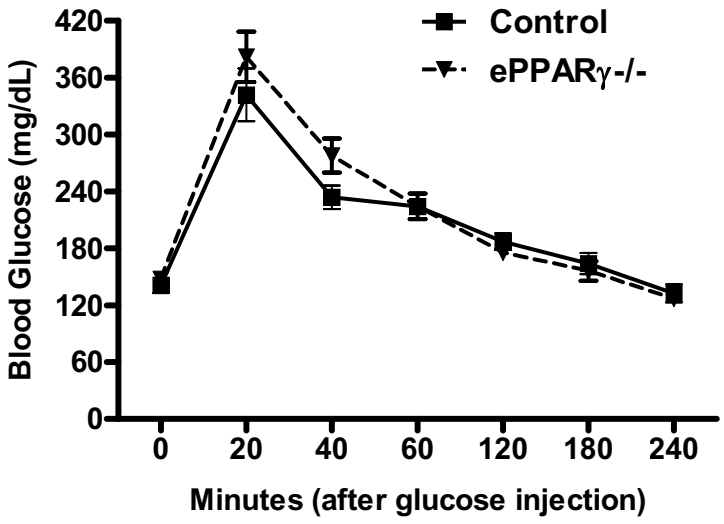

Figure S2: ePPARγ-/- mice show no difference in glucose tolerance relative to Controls. ePPARγ-/- and littermate control mice were administered 2g of glucose / kg body weight by IP injection, and blood glucose levels were monitored for 240 min. Each point represents the mean blood glucose (mg/dL) ± SEM for 10 individuals.

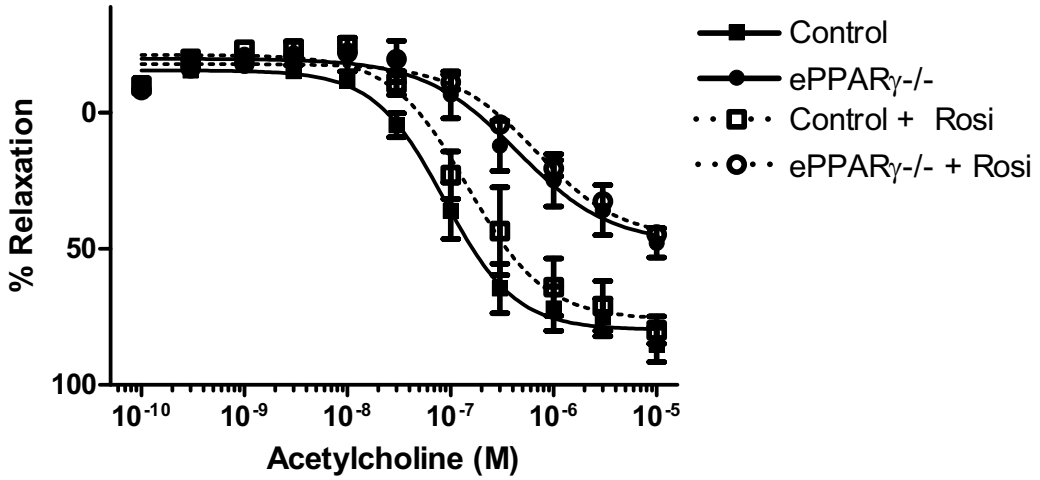

Figure S3: Lack of effect of acute PPARγ activation on relaxation responses of aortic rings from Control and ePPARγ-/- mice. Freshly dissected aortic rings from ePPARγ-/- and littermate control (Control) mice were contracted with L-phenylephrine, then treated with increasing concentrations of acetylcholine. The PPARγ agonist, rosiglitazone (10 μM) was added to the muscle bath of selected rings 1 hour prior to addition of acetylcholine. Each symbol represents mean percent relaxation from 80% maximal contraction ± SEM from 3-4 animals.

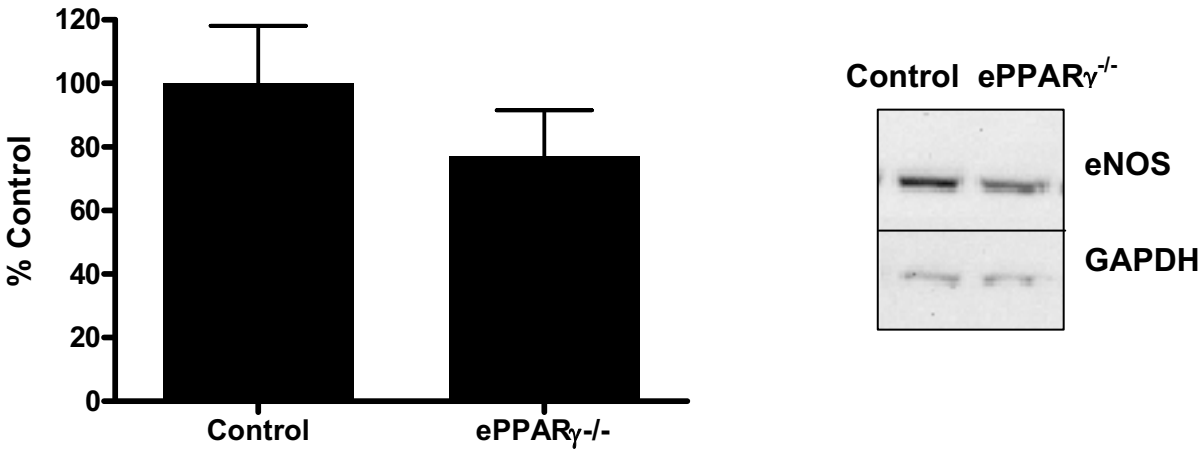

Figure S4. Aortic eNOS expression shows no statistical difference between ePPARγ-/- and Control mice. Aortic proteins from ePPARγ-/- and littermate control mice were analyzed by western blot, and quantified (A) using Quantity One 4.6 software (Bio-Rad Labs, Hercules, CA). Each bar represents % Control ± SEM from 8 individuals, where eNOS band density was normalized to GAPDH band density. Representative blots are shown (B).

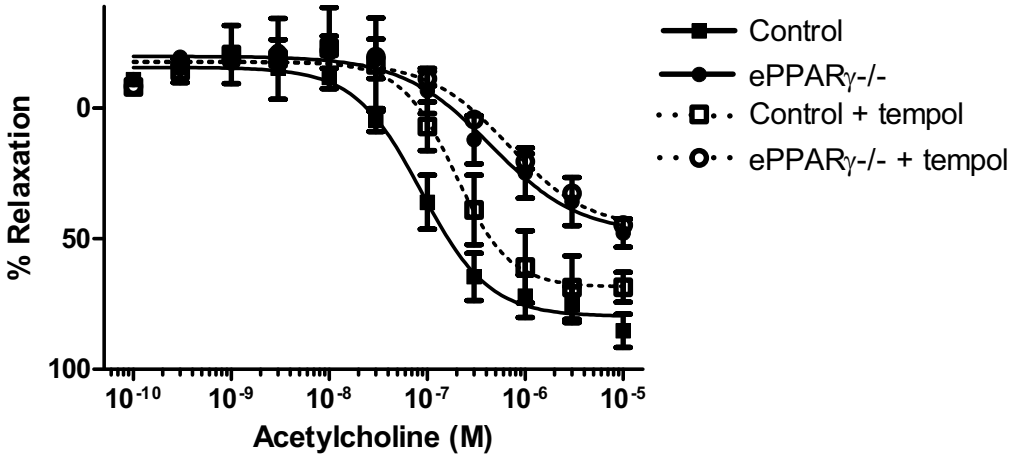

Figure S5: Lack of effect of acute tempol administration on relaxation responses of aortic rings from Control and ePPARγ-/- mice. Freshly dissected aortic rings from ePPARγ-/- and littermate control (Control) mice were contracted with L-phenylephrine, then treated with increasing concentrations of acetylcholine. The SOD mimetic, tempol (1 mM) was added to the muscle bath of selected rings 30 minutes prior to addition of acetylcholine. Each symbol represents mean percent relaxation from 80% maximal contraction ± SEM from 3 animals.

References

1. **He W, Barak Y, Hevener A, Olson P, Liao D, Le J, Nelson M, Ong E, Olefsky JM, and Evans RM.** Adipose-specific peroxisome proliferator-activated receptor gamma knockout causes insulin resistance in fat and liver but not in muscle. *Proc Natl Acad Sci U S A* 100: 15712-15717, 2003.

2. **Kleinhenz DJ, Sutliff RL, Polikandriotis JA, Walp ER, Dikalov SI, Guidot DM, and Hart CM.** Chronic ethanol ingestion increases aortic endothelial nitric oxide synthase expression and nitric oxide production in the rat. *Alcohol Clin Exp Res* 32: 148-154, 2008.