ONLINE-ONLY SUPPLEMENT

AN ORALLY ACTIVE EPOXYEICOSATRIENOIC ACID ANALOG ATTENUATES KIDNEY INJURY IN HYPERTENSIVE DAHL SALT SENSITIVE RAT

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Extended Materials and Methods

Vascular reactivity study

Second order mesenteric artery segments were mounted between two glass cannulas in a pressure myograph system (Danish Myo Technology model 111P, DMT, Aarhus, Denmark). The vessel was oxygenated in 95% O_2/5% CO_2 Krebs physiological salt solution at pH 7.4 and 37°C. Under no-flow conditions, the pressure within the vessel was increased in 10-mmHg increments from 20 to 65 mmHg. The vessel was then equilibrated at 65 mmHg for 30 min in the presence of the nitric oxide synthase (NOS) inhibitor, N_ω-nitro-L-arginine-methyl ester (L-NAME, 100μM) and the cyclooxygenase inhibitor indomethacin (10μM). Pressure was maintained at 65 mmHg for the duration of the experiment. Lumen diameter measurements were acquired using the MyoView 1.2P user interface (DMT). Control lumen diameter was calculated as the mean diameter during the last minute of the 30-min equilibration. Vessels were constricted with the thromboxane mimetic U-46619 and diameter of the constricted vessel was calculated as the mean during the last minute of the 15 minute period. EET antagonist 14,15-EEZE (10μM) or vehicle was added to the bath solution for 10 minutes prior to addition of EET-B. Graded concentrations of EET-B (10^{-9} to 10^{-5}M) were added to the bath solution every 5 minutes, and lumen diameter to each EET-B concentration was measured. The nitric oxide donor, sodium nitroprusside (100μM) was added to the bath at the end of the experimental period to ensure the vascular integrity.

Biochemical analysis

Urinary biochemical analysis was done using commercially available colorimetric, ELISA and EIA assay kits; creatinine, 8-isoprostane and nitrate/nitrite from Cayman Chemical (Ann Arbor, MI, USA), albumin and nephrin from Exocell (Philadelphia, PA, USA), and monocyte chemoattractant protein-1 (MCP-1) from BD Biosciences (San Jose, CA, USA). The levels of blood urea nitrogen (BUN) (BioAssay Systems, Hayward, CA, USA) and plasma creatinine (Cayman Chemical) were measured spectrophotometrically using commercial kits. Kidney tissue malondialdehyde (MDA) was measured in the kidney using a commercially available kit (Cayman Chemical). To determine the kidney tissue MDA level, the rat kidney was homogenized with buffer containing 1.5% potassium chloride to obtain a 1:10 (w/v) whole kidney homogenate. MDA was measured spectrophotometrically after reaction with thiobarbituric acid.

Kidney histology

For histological analysis, kidneys were excised, longitudinally sectioned, immersion-fixed in 10% neutral buffered formalin and paraffin embedded. The kidney sections were embedded and cut into 4 μm slices for use in histology protocols. Formalin-fixed paraffin-embedded kidney slices were deparaffinized, re-hydrated and stained with Masson's Trichromeor Periodic Acid-Schiff (PAS) reagents. Glomerulosclerosis and mesangial matrix expansion were blindly scored from kidney sections stained by PAS reagent at a magnification of 400x to determine the glomerular damage using the following numeric scale: 0= no damage; +1= very mild; +2= mild; +3= moderate and +4= severe). Two observers in a blinded fashion conducted histological analysis of glomerular injury. Tubules containing proteinaceous casts were determined in the PAS stained slides at magnification of 200x using Nikon NIS Elements Software. The percentage area positive for cast was calculated from the mean of 8 cortical and 5 medullary fields (200x) for each animal. Renal fibrosis was assessed in Masson's Trichrome stained slides and the percentage of the total area positive for interstitial collagen was calculated as an index of fibrosis using Nikon NIS Elements Software. The percentage area positive for interstitial collagen was calculated from the mean of 8 cortical and 5 medullary fields (200x) for each animal. Two observers who were blind to the treatment protocol and the animal models carried out the renal cast and fibrosis area calculations. Cardiac fibrosis was assessed from interstitial collagen positive areas in Picro-sirius Red stained tissue slides using Nikon NIS Elements Software.

Immunohistochemical analysis

The kidney sections were embedded and cut into 4 μm slices for use in immunohistochemistry protocols. Formalin-fixed paraffin-embedded kidney slices were deparaffinized, re-hydrated and subjected to immunohistochemistry protocols. Kidney sections were immunostained with anti-CD68 (1:100; Serotec, Raleigh, NC, USA) and anti-CD43 to determine macrophage/monocyte infiltration and T-lymphocytes, respectively. Biotinylated rat anti-mouse secondary antibody (1:200) was used for development with avidin-biotinylated HRP complex (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA, USA) followed by counterstaining with hematoxylin and mounted for image capturing. Stained sections were visualized by light microscopy at 400x magnification and digital images of the stained kidney were taken for analysis. Macrophage/monocyte infiltration was determined by point counting of CD68-positive cells by an experienced reviewer blinded to the experimental groups. The number of positive cells per picture was divided by the metric area of the image, established by micrometer slide image to obtain the number of positive cells per mm^2. Infiltration of T-lymphocytes in the kidney was determined as the percentage area positive for CD43 (1:200; Santa Cruz Biotechnology, Santa Cruz Biotechnology, CA, USA) immunostaining was assessed at a magnification of 200x using Nikon NIS Elements Software.

Additional kidney sections were also immunostained with antibodies against ER stress markers, glucose regulatory protein 78 (GRP78, 1:100; Santa Cruz Biotechnology, USA) and C/EBP homologous protein (CHOP, 1:200, Santa Cruz Biotechnology) in order to determine the relative expression level and localization of these ER stress markers in the kidney. Biotinylated horse anti-goat or anti-rabbit secondary antibodies (1:200, Santa Cruz Biotechnology) were used for development with avidin-biotinylated HRP complex (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA, USA). Finally the slides were counterstained with hematoxylin and mounted for image capturing.

Real-Time PCR Analyses

Total RNA was isolated from kidney homogenate using TRIzol LS reagents (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The isolated RNA was treated with RNase-free DNase (Invitrogen Life Technologies, Carlsbad, CA, USA) to remove traces of genomic DNA contamination. The mRNA samples were quantified by spectrophotometry at 260 nm and

1 µg of total RNA was reverse-transcribed to cDNA using iScript™ Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The target gene expression was quantified by iScript One-Step RT-PCR Kit with SYBR green using MyiQ™ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Each amplified sample in all wells was analyzed for homogeneity using dissociation curve analysis using iQ5 Optical System Software, Version 2.1(Bio-Rad Laboratories, Hercules, CA, USA). After denaturation at 95°C for 2 min, 40 cycles were performed at 95°C for 10 s and at 60°C for 30s. Each sample was run in triplicate, and the comparative threshold cycle (C_t) method was used to quantify fold increase ($2^{-\Delta\Delta Ct}$) in the expression of the target genes compared to controls. In analyzing the relative expression of the target genes, the C_t values were normalized to a housekeeping gene (pgk1). Statistical analyses were carried out for at least 5-7 experimental samples in each experimental group.

Endothelial Cell Culture Studies

Human umbilical vein endothelial cells (HUVECs) grown in 6-well plates were pretreated with vehicle (n=4), EET-B (5µM, n=4), and a combination (n=4) of EET-B (5µM) and EET antagonist 14,15-EEZE (5µM) for 16h. All samples were exposed to TNF-α (10ng/ml) for 6h at 37°C and the culture medium was assayed for the measurement of monocyte chemoattractant protein-1 (MCP-1) (Thermo Fisher Scientific, Rockford, IL, USA).

Na^+ Transport Studies

Immortalized mouse cortical collecting duct ($mpkCCD_{c14}$) principal cells were grown in defined medium on permeable supports (Costar Transwells, 0.4-µm pore, 24-mm diameter) as described previously.[1,2] A Millicel Electrical Resistance System (Millipore, Billerica, MA) was used to measure voltage and resistance across the $mpkCCD_{c14}$ cell monolayers grown on permeable supports as described previously.[2,3] Equivalent transepithelial Na^+ currents were calculated as the quotient of transepithelial voltage to transepithelial resistance under short-circuit conditions.

Determination of plasma levels of endogenous EETs and DHETs

Plasma levels of endogenous EETs and DHETs were measured by LC–ESI–MS (Agilent 6460 Triple Quad LC/MS). Samples for chromatographic analysis were prepared from 200µl of plasma using solid phase extraction with Varian Bond Elut® C18 column (Agilent Technologies, Santa Clara, CA, USA). In the LC–ESI–MS analysis process, the samples (5µl) were separated on a reverse phase C18 column (Kromasil, 250 x2 mm) using water/acetonitrile containing 0.005% acetic acid as a mobile phase at the flow rate of 0.20 mL/min. The mobile phase started at 90% methanol for 2 min, linearly increased to 100% methanol in 10 min, and held for 10 min. Drying gas flow was 12 l/min, drying gas temperature was 350°C, nebulizer pressure was 35 psig, vaporizer temperature was 325°C, capillary voltage was 3000 V, and fragmentor voltage was 120 V.

References

1. Pavlov TS, Levchenko V, Karpushev AV, Vandewalle A, Staruschenko A. Peroxisome proliferator-activated receptor gamma antagonists decrease Na^+ transport via the epithelial Na^+ channel. *Mol Pharmacol.* 2009;76:1333-1340.
2. Staruschenko A, Patel P, Tong Q, Medina JL, Stockand JD. Ras activates the epithelial Na (+) channel through phosphoinositide 3-OH kinase signaling. *J Biol Chem.* 2004;279:37771-37778.
3. Staruschenko A, Pochynyuk O, Vandewalle A, Bugaj V, Stockand JD. Acute regulation of the epithelial Na^+ channel by phosphatidylinositide 3-OH kinase signaling in native collecting duct principal cells. *J Am Soc Nephrol.* 2007;18:1652-1661.

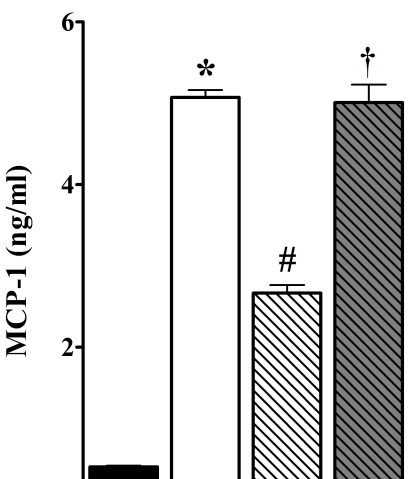

Figure S1: Human umbilical vein endothelial cells (HUVEC) were pretreated with vehicle, EET-B (5μM) or a combination of EET-B (5μM) and EET antagonist 14,15-EEZE (5μM) for 16h followed by 6h incubation with TNF-α (10ng/ml). TNF-α increased monocyte chemoattractant protein-1 (MCP-1) levels (*$p<0.05$ vs. vehicle), and EET-B attenuated TNF-α mediated increase in MCP-1 levels (#$p<0.05$ vs. TNF-α). 14,15-EEZE abolished the anti-inflammatory effect of EET-B (†$p<0.05$ vs EET-B). Data expressed as mean ± SEM, n=4.

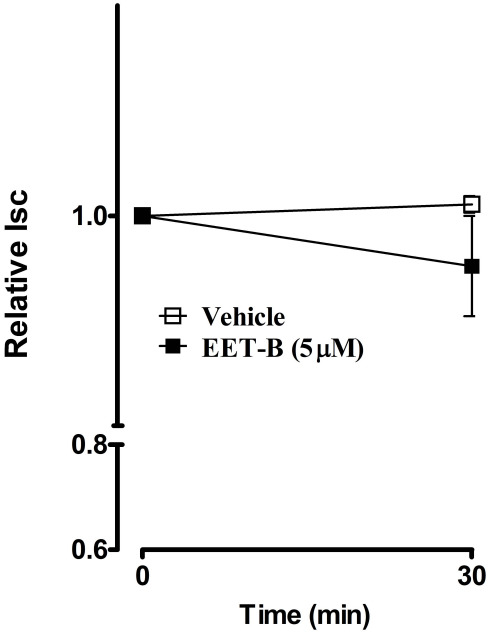

Figure S2: Effect of EET-B on basal Na^+ ion transport in mpkCCDc14 cells. The figure is demonstrating the equivalent short circuit current (Isc) in mpkCCDc14 principal cells in response to EET-B administration. EET-B did not alter the transepithelial Na^+ current in mpkCCDc14 cells. Data expressed as mean ± SEM, n=4.

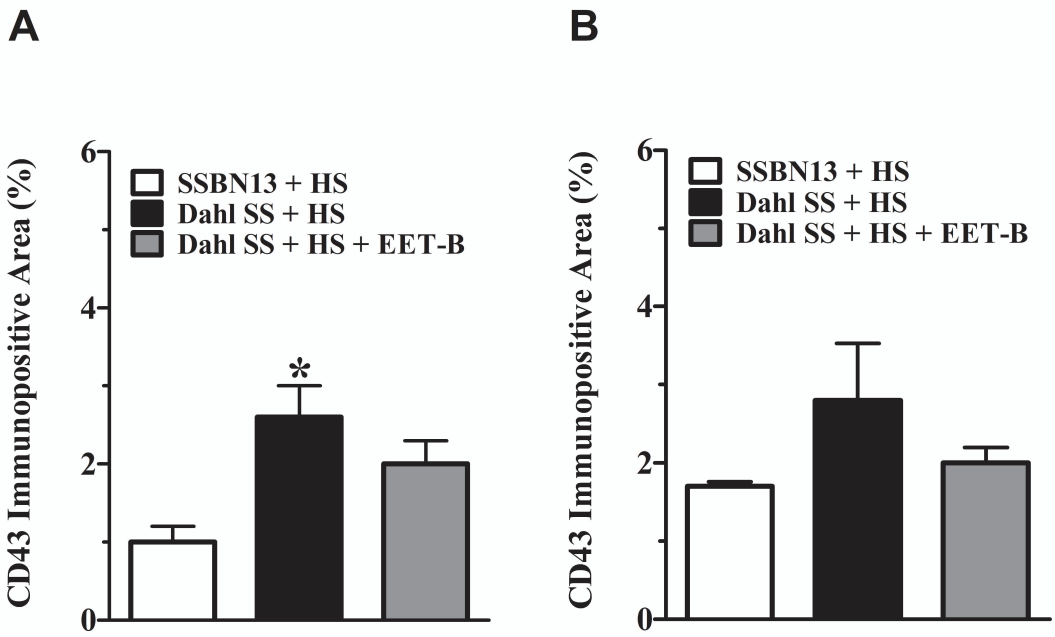

Figure S3: Percentage (%) of CD43 immunopositive kidney areas showing the presence of T-lymphocytes in the kidney cortex (A) and medulla (B) of rats from different experimental groups. Data expressed as mean ± SEM, n=4.*$p<0.05$ vs. SSBN13.

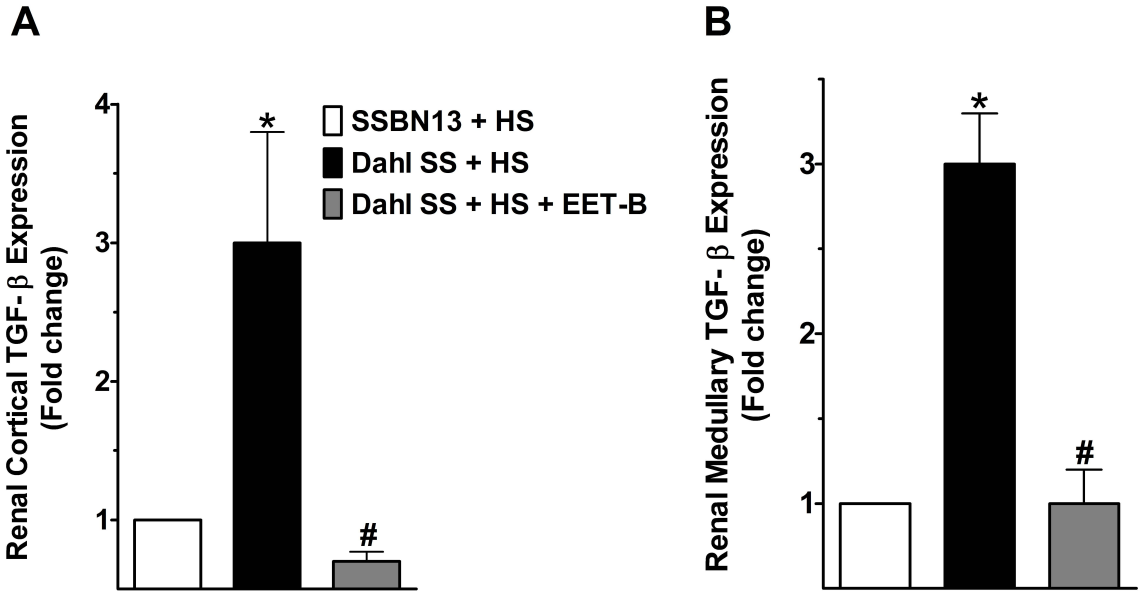

Figure S4: Renal mRNA expression of tumor growth factor- β (TGF-β) in the kidney cortex (A) and medulla (B) of rats from different experimental groups. *$p<0.05$ vs. SSBN13 rats treated with vehicle; #$p<0.05$ vs. vehicle treated Dahl SS rats. Data expressed as mean ± SEM, n=6-7.

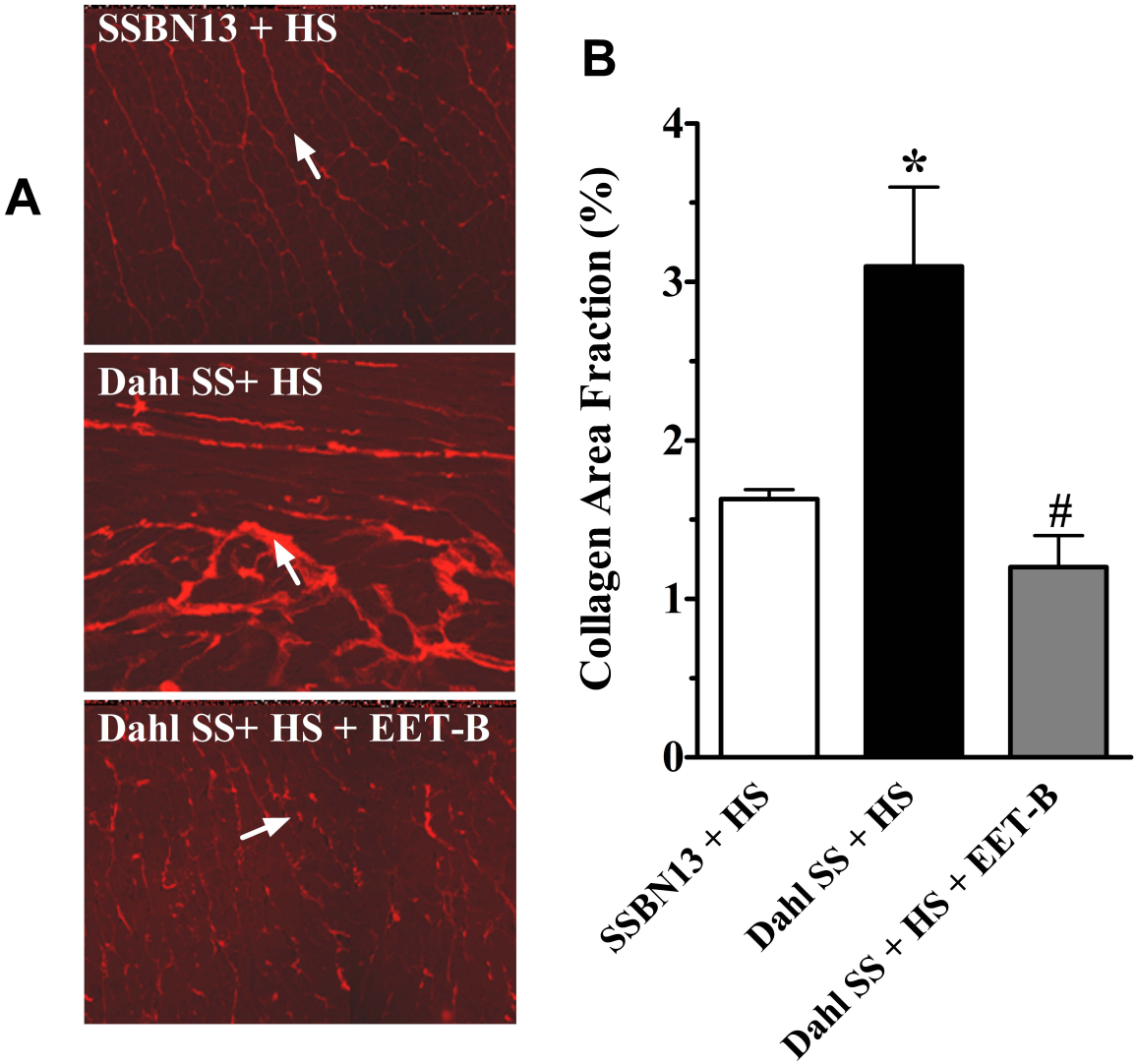

Figure S5: Representative photomicrographs of Picro-sirius Red staining of heart tissues depicting collagen positive areas (white arrows) (A) along with the calculated collagen positive area (%) in the heart sections of rats from different experimental groups (B). *$p<0.05$ vs. SSBN13 rats treated with vehicle; #$p<0.05$ vs. vehicle treated Dahl SS rats. Data expressed as mean ± SEM, n=4.

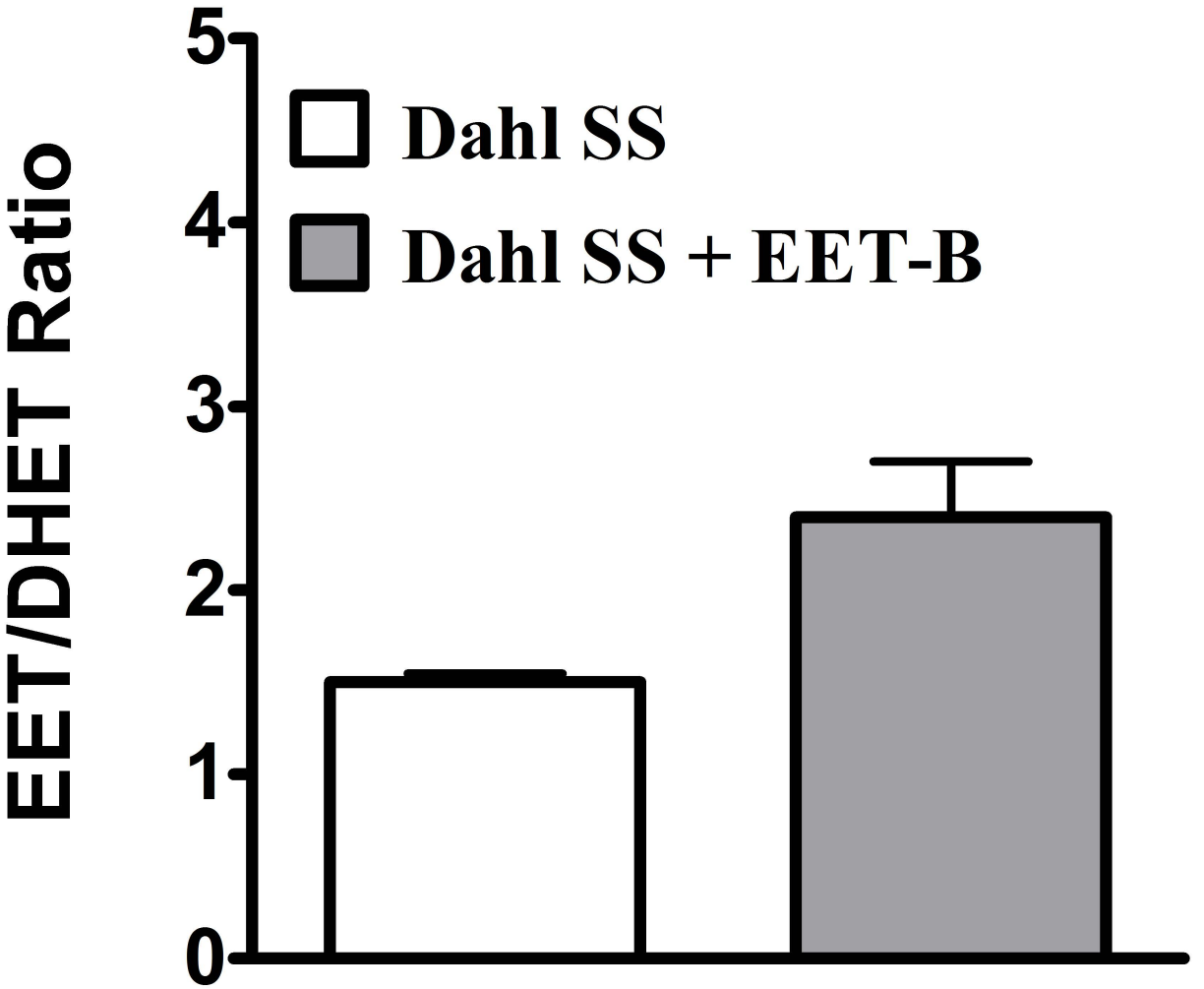

Figure S6: Plasma EET to DHET ratio in Dahl SS rats treated with vehicle or EET-B. Plasma EETs and DHETs were measured using LC-ESI-MS and all four regioisomers of EET were detected. EET levels averaged 19±2 and 5.2±0.4 ng/ml in vehicle or EET-B treated Dahl SS rats; respectively. DHET levels averaged 13±2 and 2.1±0.1ng/ml in vehicle or EET-B treated Dahl SS rats; respectively. Data expressed as mean ± SEM, n=4.