Online Supplement

Central EP3 receptors mediate salt sensitive hypertension and immune activation

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Short Title: EP3 receptor in hypertension

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DETAILED MATERIALS AND METHODS

Materials: Most of the antibodies for flow cytometry were purchased from Biolegend (San Diego, CA) and included: 7-AAD for live/dead staining; BV510-conjugated anti-CD45 (30-F11); APC-conjugated anti-CD4 (GK1.5);APC/Cy7-conjugated anti-CD8 (53-6.7); PE/Cy7-conjugated anti-CD3 (145-2C11); FITC-conjugated CD44 (IM7);APC-conjugated anti-CD62L(MEL-14); PE/Cy7-conjugated anti-I-Ab (AF6-120.1);APC/Cy7-conjugated anti-CD11c (Bu15), APC-conjugated anti-CD64 (X54-5/7.1), Brilliant Violet 421-conjugated anti-CD86 (GL-1). PE-conjugated anti-Mer (FAB5912P) was purchased from R&D Systems (Minneapolis, MN). Isolevuglandin-protein adducts were analyzed by flow cytometry using an Alexa Fluor 488 labelled D11 ScFv antibody as previously described.[1] Primary antibodies for immunoblotting included rabbit anti-p47phox (Millipore, 07-500) and mouse anti-gp91phox (BDbioscience, 611414). All primers and probes for real time PCR (COX1: Mm04225243_g1, COX2:Mm03294838_g1, Ptger1:Mm00443098_g1, Ptger2:Mm00436051_m1, Ptger3: Mm01316856_m1, Ptger4: Mm00436053_m1) were from Applied Biosystems. GIPZ lentiviral mouse Ptger3 shRNA (V2LMM_65001, V2LMM_79034, V3LMM_463909) and non-silencing control shRNA were purchased from Dharmacon (Lafayette, CO).

Animals: Wild type C57BL/6 mice were obtained from Jackson Laboratories and were studied at 3 months of age. Generation of EP3 receptor knockout mice on a C57Bl/6 background has been previously described.[2] Only male mice were used, because blood pressure in female mice are influenced by hormonal cycle. Female mice are relatively protected from hypertension and associated T cell activation.[3, 4] All mice were housed in temperature-controlled room (22-24°C) with free access to water and food. For each experiment, mice randomly assigned to either experimental or control group, using a completely randomized design. The number and use of mice in each experiment are listed in Table S1.

Telemetry recording and Power Spectral Analysis: Blood pressure was monitored using radiotelemetry as previously described.[5] In brief, telemetry transmitters (Data Science International, Model PA-C10 or HD-X10) were implanted in mice with the catheter tip inserted into ascending aorta via the carotid artery on the left side. The mice were given at least 10 days for recovery after surgery. Blood pressure was recorded 10 minutes every hours at a sampling rate of 500 Hz by DSI PhysioTel Digital telemetry platform.

With waveform pressure data exported from the recording system, power spectral analyses on blood pressure and heart rate were performed using the HemoLab software suite version 20.7 as previously described.[5] In brief, interbeat interval (IBI), systolic, and diastolic blood pressure were derived from artifact free beat-to-beat blood pressure. The data were spline interpolated at a sampling rate of 25 Hz and converted from non-equidistant to equidistant time series. Power spectra were computed as areas under curves from equidistant data by fast Fourier transformation with 50% overlapping segments of 512 values. The cutoff frequency boundary for the low-frequency range was 0.2–0.6 Hz and a fixed high-frequency boundary of 2.5-5 Hz was maintained for all the

analyses. Telemetry data acquired during 11 pm – 2 am and 11 am – 2pm were used for nighttime and daytime analyses.

Measurement of renal superoxide: Superoxide levels were by quantifying the formation of 2-hydroxyethidium from Dihydroethidium (DHE). kidneys were rapidly removed after euthanasia, immersed in optimal cutting temperature (OCT) media and frozen in dry ice. Thirty micrometer sections were prepared and then incubated with 20 μM DHE in PBS for 30 minutes at 37°C in a humidified chamber protected from light as described previously.[6] Sections were imaged using confocal microscopy with an excitation wavelength of 405 nm and an emission wavelength of 530-560 nm. After initial microscopic adjustment, all images were taken using the same settings, including magnification, laser intensity, confocal pinhole size, emission filter, digital gain, etc.

Urinary norepinephrine: Spot urine samples were collected before animals were sacrificed, and immediately frozen in liquid nitrogen and stored at -80ºC freezer. EGTA-glutathione solution was added into be samples (1 μL stocking solution containing 9% EGTA and 6% glutathione per 50 μL urine), and then norepinephrine was measured by high-performance liquid chromatography as previously described.[7]

Immunohistochemistry: Five-micron sections were obtained from formalin fixed, paraffin embedded kidneys and Masson Trichrome blue staining was performed by the Translational Pathology Shared Resource Vanderbilt University Medical Center as previously described.[1, 7] The slides were then scanned using Leica SCN400 Slide Scanner at the Digital Histology Shared Resource at Vanderbilt. Blue staining collagen was quantified using ImageJ and expressed as the percentage of blue pixels to total pixels for each image. Values for each mouse represent the average from at least five random images that contained glomeruli and vasculature from the renal cortex of that animal.

Preparation Samples for Flow Cytometry: Immediately after animals were euthanized, the chest was opened, and an incision was made on the right atrium. Saline was perfused at physiological pressure through left ventricle via a 21G needle until the perfusate outflow from the right atrium was cleared of blood. The kidneys were then collected, homogenized and digested as previously described to prepare single cell suspensions.[8]

Real-time PCR: After euthanasia, brains were quickly removed from the animals and immediately frozen on dry ice. Tissue samples from organum vasculosum laminae terminalis (OVLT), subfornical organ (SFO), and paraventricular nucleus (PVN) were punched in a cryostat according to coordinates taken from Paxinos and Franklin's mouse brain atlas.[9] RNA were extracted from tissue lysates using Qiagen RNeasy Mini Kit and then reverse-transcribed to cDNA. The expression of COX-1, COX-2, EP receptors and beta actin were quantified by real time PCR.

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Table S1: Animals used in each experiments.

	Wild type (n=106)																EP3$^{-/-}$ (n=42)								
Experimental Procedure	**NS**					**L-NAME/HS**				**L-NAME only**	**control shRNA**	**EP3 shRNA**	**naïve**				**NS**				**L-NAME/HS**				**naïve**
	28					34				6	7	7	24				18				20				4
Fig.1: telemetry						9																5			
Fig.1: HR/BP PSA						6†																5			
Fig.1: Urinary NE			5				5												5					5	
Fig.2: kidney DHE	4					4											4				4				
Fig.2: kidney trichrome		6					8											4				4			
Fig.3: kidney FC	9*					12											8				10				
Fig.3: kidney cytokine FC			8				8												5				5		
Fig.4: spleen DC FC				5					5											5				5	
Fig.5: Brain real time PCR					5					9	6#														
Fig.5: telemetry											7	7													
Fig.5: HR/BP PSA											7	7													
Fig.5: kidney FC											7	7													
Fig.6: Effects of PGE_2 on isoLG and CD86													7												
Fig.6: Effects of EP1/3 antagonists on isoLG														6											
Fig.6: Effects of EP1/3 agonists on isoLG															5										
Fig.6: Effects of PGE_2 on NADPH oxidase assembly																6									4

Each number represent the number of mice used in each specific experiment. Numbers in the same column indicate same mice that were used in multiple measurements. Abbreviations: HR, heart rate; BP, blood pressure; PSA, power spectral analysis; FC, flow cytometry; NS, normal salt; and HS, high salt.

* one sample was excluded due to cell clotting during preparation.

† telemetry recording of 6 in 9 mice reached enough quality for power spectral analysis.

cortex samples were retrieved from 3 samples during revision.

Supplemental Figures

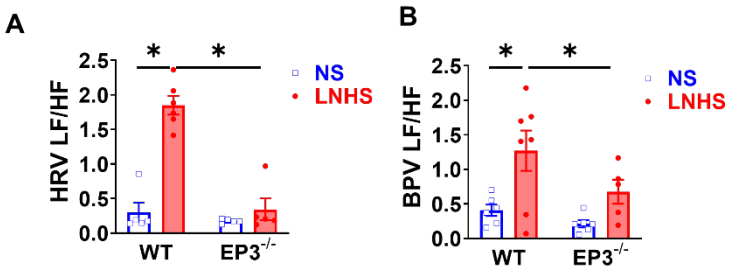

Figure S1: Effects of EP3 deficiency on L-NAME/high salt induced autonomic function at daytime. Data were analyzed with 2-way ANOVA and Bonferroni post-hoc multiple comparisons. n=5 to 9 in each group. *P<0.05, **P<0.01.

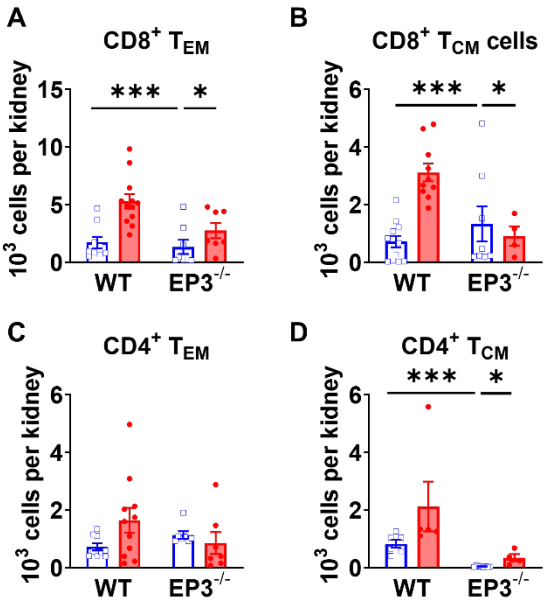

Figure S2: Effects of EP3 deficiency on renal memory T-cell infiltration. $CD8^+$ and $CD4^+$ effector memory T (T_{EM}) cells and central memory T (T_{CM}) cells are shown. Data were analyzed using 2-way ANOVA followed by Bonferroni post-hoc test, n=5 to 12 in each group. *P<0.05, ***P<0.001.

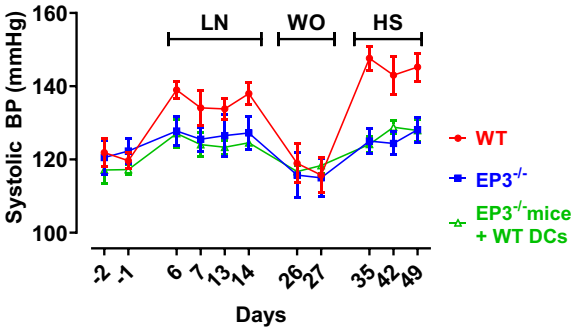

Figure S3: Effects of wild type DCs on blood pressure of $EP3^{-/-}$ mice subjected to L-NAME/high salt protocol. DCs were obtained from wild type mice, and 1×10^6 cells were adoptively transferred to $EP3^{-/-}$ recipient mice. Ten days after DC adoptive transfer recipient mice were subjected to L-NAME/high salt protocol. Systolic pressures were measured using telemetry and compared with previous data by 2-way ANOVA with repeated measurements.

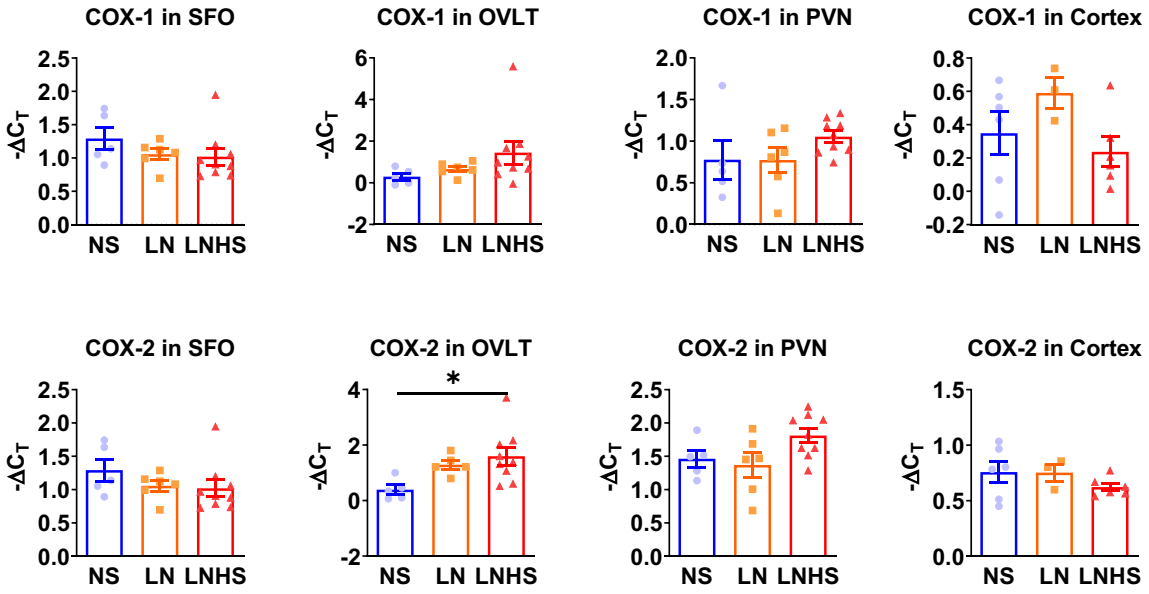

Figure S4: Expression of cyclooxygenases in the central nervous system of wild type mice at different stage of L-NAME/high salt induced hypertension. Data were analyzed with one-way ANOVA and post hoc multiple comparisons. *P<0.05, **P<0.01.

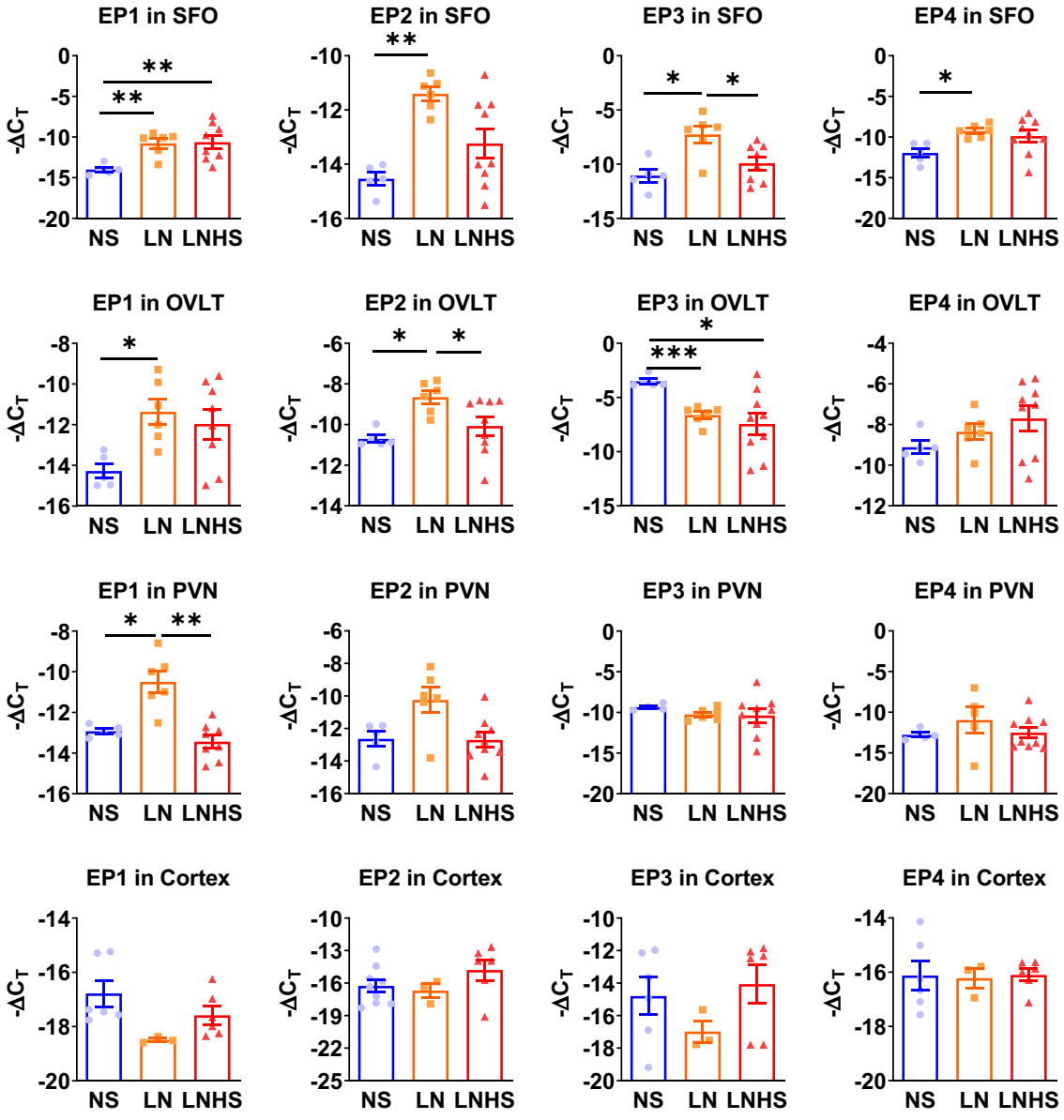

Figure S5: Expression of EP receptors in the central nervous system of wild type mice at different stages of L-NAME/high salt induced hypertension. Data were analyzed with one-way ANOVA and post hoc multiple comparisons. *P<0.05, **P<0.01, ***P<0.001.

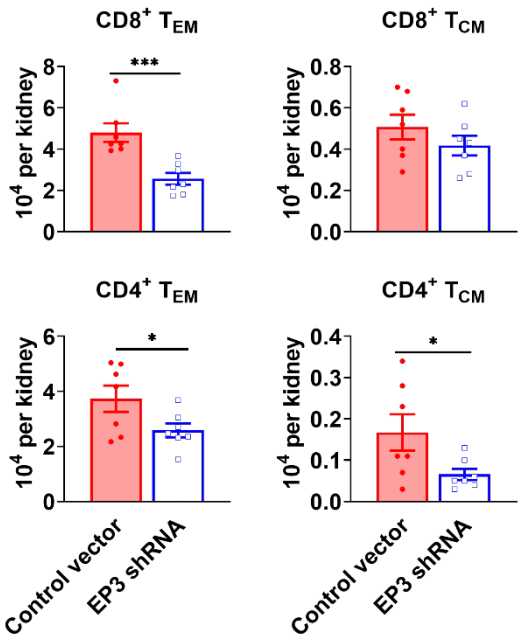

Figure S6: Effects of central EP3 receptors on renal memory T-cell infiltration. $CD8^+$ and $CD4^+$ effector memory T (T_{EM}) cells and central memory T (T_{CM}) cells are shown. Data were analyzed using student t test, n=7 in each group. *P<0.05,***P<0.001.

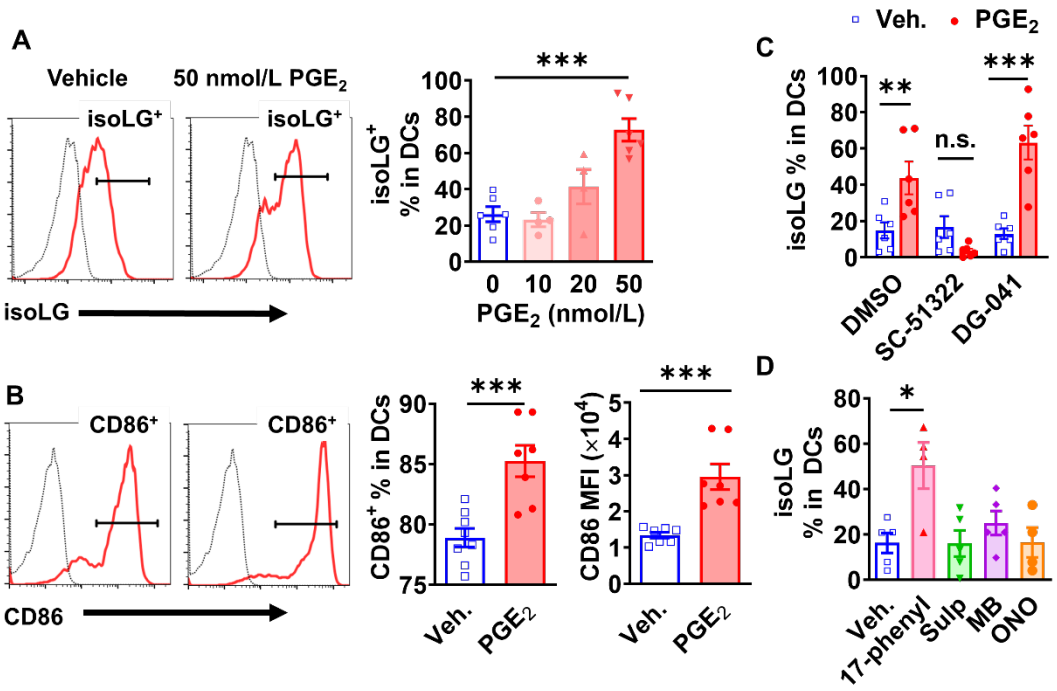

Figure S7: Effects of PGE_2 and its EP1/EP3 receptors on the formation isoLG protein adducts and NADPH oxidase activation in DCs. Splenic DCs were isolated by magnetic separation and cultured for 24 hours. (A) Formation of isoLG adducts were measured by flow cytometry in response to increased dose of PGE_2, n=4 to 6 in each group. (B) Effect of 50 nmol/L PGE_2 on the expression of co-stimulatory molecule CD86, n=8 in each group. Fluorescence minus one controls (FMO) were shown in black dashed lines. (C) Effects of EP1 or EP3 blockades on isoLG adduct formation. EP1 receptor antagonist SC-51322 (20 μmol/L) and EP3 receptor antagonist DG-041 (30 nmol/L) were added 30 minutes before PGE_2 treatment, n=6 in each group. (D) Effect of EP1 or EP3 agonists on isoLG adduct formation. DCs were treated with EP1 and EP3 receptor agonist 17-phenyl trinor PGE_2 (17-phenyl, 100 nM), EP3 receptor agonists sulprostone (Sulp, 10 nmol/L), MB-28767 (MB, 10 nmol/L), and ONO-AE-248 (ONO, 100 nmol/L) for 24 hours, n= 5 in each group. N= 4 to 6 in each group. Data were analyzed using one-way ANOVA in (A) and (D), with Student t tests in (B) and with two-way ANOVA in (C) and (E), *P<0.05, ***P<0.001.