Supplemental Material

E-Prostanoid Receptor 3 Mediates Impaired Vasodilation in a Mouse Model of Salt-Sensitive Hypertension

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Supplemental Methods

Experiments described in Figures 1-3, Figure S2 and Table 1-3 were completed at the University of Iowa and all mice were maintained on a standard laboratory rodent chow diet containing 0.8% sodium chloride or 0.3% sodium (the NIH-31 Modified Open Formula Mouse/Rat Sterilizable Diet, Teklad Catalog # 7013) at baseline. During the 4-week study, some animals received a high salt diet (HSD) containing 4% sodium chloride equivalent to 1.57% sodium (Teklad Catalog # TD. 03095), while others remained on standard chow diet (Supplemental Figure 1A).

Experiments described in Figure 4, Figures S1B-D and S3-4 were performed at the Medical College of Wisconsin. All mice were weaned to a low salt diet containing 0.3% sodium chloride equivalent to 0.1% sodium (Teklad Catalog # 2920X). Both NT controls and S-P467L mice consumed the standard chow diet at baseline until they were provided with the 4% HSD (Teklad Catalog # TD. 03095) for 5 weeks.

Metabolic cage studies: During week 4 of the protocol depicted in Figure S1A, mice were placed in metabolic cages for the collection of urine samples. Mice were acclimated to the cages in the first 24 hours and urine samples collected in the second 24 hours were used for analysis of 8-isoprostane by ELISA.

Dihydroethidium HPLC Analysis: At the end of 4 weeks, kidneys were removed and cut into cortex and medulla. Renal cortical and medullary samples were incubated in pre-oxygenated Kreb's buffer loaded with 50 μM dihydroethidium (DHE) at 37 degrees Celsius for 30 min. DHE reacts with superoxide in renal tissues to yield specific product, 2-hydroxyethidium, which is detectable by HPLC.[1] At the end of 30 min incubation, the samples were snap-frozen and stored in -80 freezers until being analyzed with HPLC.

Vascular function: Wire myography studies was performed as previously described.[2-4] Briefly, the left and right common carotid arteries were dissected free of adventitial fat and each cut into two segments. Likewise, the first-order renal artery branches (segmental arteries) were also dissected from both kidneys. Carotid and renal arterial rings were then equilibrated for 45 min under a resting tension of 0.25 g, and concentration-dependent response to acetylcholine (ACh, 1 nmol/L-30 μmol/L) and sodium nitroprusside (SNP, 0.1 nmol/L-30 μmol/L) were performed after an initial submaximal precontraction (40%-60%) with a thromboxane A2 receptor agonist U46619 (60 nmol/L). In some vessel segments, the vasodilation responses to ACh and SNP were performed following the 30 min pre-incubation with various pharmacological agents (Y27632 1 μmol/L, Tempol 1 mmol/L, Calphostin C 50 nmol/L, DG041 100 nmol/L, Indomethacin 10 μmol/L, SC560 1 μmol/L, and Celecoxib 1 μmol/L) or the vehicle Dimethylsulfoxide (DMSO).

Liquid chromatography tandem mass spectrometry: Aortic tissues were harvested and placed immediately in 2 ml of cold methanol. The Internal standard d4 PGE2 (50 pg) was added and samples were kept at −80°C. Prostanoids were extracted using solid phase Strata-X Polymeric Reversed Phase 60 mg cartridges (Phenomenex, Torrance, CA). In brief, each sample was centrifuged for 15 min at 4°C. The supernatant was then collected, diluted with 5 vol of water, and acidified to pH 5.0 with HCl. Cartridges were primed with 6 ml of methanol followed by 6 ml of water. Samples were loaded, washed with 2 ml 20% methanol and eluted with 1.5 ml of ethyl acetate. The collected ethyl acetate fraction was dried under nitrogen and re-suspended in 50 μl of methanol and stored at −80°C until analysis. Protein pellets were saved and quantified by

Bradford protein assay. Identification and quantification of PGE2 and other cyclooxygenase-derived metabolites was performed on a Shimadzu Triple Quadrupole Mass Spectrometer LCMS-8050 equipped with a Nexera UHPLC using multiple reaction monitoring mode. Multiple reaction monitoring transition for PGE2 was m/z: 351.0 →171.3. MS conditions were Ionization mode: Negative heated electrospray (HESI), Applied voltage: −4.5 ~ −3 kV; Nebulizer gas: 3.0 L/min N2; Drying gas: 5.0 L/min N2; Heating gas: 12.0 L/min Air; Interface temp: 400°C; DL temp: 100°C; Heat block temp: 500°C. UHPLC conditions: Analytical column: Zorbax Eclipse Plus C18 RRHD (50mm L. X 2.1 mm I.D., 1.8 µm); Mobile phase A: 95% water 5% acetonitrile 0.05% acetic acid; Mobile phase B: Acetonitrile 0.05%; Time program 40% B. (0 min) →75%B. (3 min) →85% B. (7.5 min); Flow rate: 0.4 mL/min.; Injection volume: 5 µL; Column oven temp: 40°C. PGE2 standard was used to obtain a standard curve (0.5-500 pg) used to calculate the final concentrations of PGE2 and other prostanoids including PGD2, PGF2α and 8-Iso PGF2α. All solvents were HPLC grade or higher.

Quantitative real-time RT-PCR: RNA was extracted from aortic tissue and real-time RT-PCR (qPCR) was performed as previously described.[2] Briefly, using oligo (dT) primers, RNaseOUT (Invitrogen), and Superscript III (Invitrogen), cDNA was synthesized from 400-700 ng of total RNA extracted using RNeasy spin columns (RNeasy mini kit, QIAGEN). Each qPCR reaction was performed in duplicates. cDNA (10 ng) was subject to Taqman Gene Expression Assay using the Taqman Fast Advanced Master Mix (Applied Biosystems) and the targeted Taqman probes. The following probes were used on Applied Biosystems StepOne Plus System to evaluate gene expression level: mouse GAPDH (352932-0905028), COX-1 (Mm00477214_m1), COX-2 (Mm00478374_m1), PTGES-1 (Mm00452105_m1), PTGES-2 (Mm00460181_m1), PTGES-3 (Mm00727367_s1), HPGD (Mm00515121_m1), EP1 (Mm00443097_m1), EP2 (Mm00436051_m1), EP3 (Mm01316856-m1), and EP4 (Mm00436053_m1).

Measurement of blood pressure and heart rate: Blood pressure and heart rate were measured with radiotelemetry at a sampling rate of 500 Hz. Mice at 12-16 weeks of age were anesthetized with ketamine/xylazine and a radiotelemetry BP catheter (TA11PA-C10, DSI) was implanted into the left common carotid artery. The mice were given a 10-day recovery period before baseline blood pressure was recorded for 10 minutes every hour for 5 consecutive days (Figure 4A). Both the NT and S-P467L mice received 4% HSD for the following 5 weeks when blood pressure, heart rate and activity continued to be recorded. Daily blood pressure, HR and activity values for the baseline phase and HSD weeks 1-4 were plotted in Figure 4B and Supplemental Figures S3-S4. One outlier was eliminated from the NT group as determined by a Grubb's test.

DG041 Administration and Data Analysis: The BP-lowering effect of DG041 was examined in HSD week 5. Data from each animal were averaged hourly, and corresponding times across HSD days 29 and 30 were averaged for each animal to create a single consolidated 24-hour tracing as pre-DG041 blood pressure (Figure 4D-E and Supplemental Figure S5, solid blue and solid red). DG041 was solubilized and formulated as described and injected subcutaneously under isoflurane anesthesia at 10AM once daily during HSD days 31-34.[5] As shown in Supplemental Figure S5, 24-hour tracings for HSD days 31, 32, 33, and 34 were plotted against the pre-DG041 blood pressure. Due to the pharmacokinetic nature of DG041, plasma concentration of DG041 peaks approximately 2 hours following subcutaneous administration with a half-life of 4-6 hours.[5] Consistent with this, the BP-lowering effect of DG041 was transient and did not become statistically significant until after the third and fourth injections (HSD day 33

and 34, Supplemental Figure S5C-D). To quantify the BP-lowering effects of DG041, corresponding hourly blood pressure in HSD days 33 and 34 were averaged for each animal to create a consolidated post-DG041 tracing (Figure 5, open symbols). Tracing 2-6 hours in the efficacy window following DG041 administration (12PM-4PM) in Figure 5 was further averaged and compared to corresponding times prior to DG041 treatment (Figure 5C).

Chemicals: ACh, SNP, KCl, endothelin-1 (E7764) and Indomethacin (I7378) were obtained from Sigma Aldrich. U46619 (CAS 56985-40-1), Calphostin C (Cat. # 15383), SC560 (Cat. # 70340), Celecoxib (Cat. # 10008672) were obtained from Cayman Chemical. Tempol (ALX-430-081-G001) was obtained from Enzo Life Sciences. Y27632 (Cat. # 1254) and DG041 (Cat. # 6240) were obtained from TOCRIS. DMSO was used as a solvent and as the vehicle control in pharmacological studies.

Supplemental References

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A

ACh Response	Chow Diet				High Salt Diet			
	NT Vehicle	NT Tempol	S-P467L Vehicle	S-P467L Tempol	NT Vehicle	NT Tempol	S-P467L Vehicle	S-P467L Tempol
EC_{50} (logM)	-7.3±0.1	-7.7±0.1	-7.5±0.4	-7.5±0.5	-7.5±0.1	-7.5±0.1	-7.0±0.2	-7.1±0.2
E_{max} (%)	86±3	92±4	45±7†	44±7	85±2	80±4	33±3*	34±2

B

ACh Response	Chow Diet				High Salt Diet			
	NT Vehicle	NT PKCi	S-P467L Vehicle	S-P467L PKCi	NT Vehicle	NT PKCi	S-P467L Vehicle	S-P467L PKCi
EC_{50} (logM)	-7.4±0.1	-7.8±0.1	-7.4±0.3	-7.5±0.2	-7.4±0.1	-7.7±0.1	-6.4±0.3*	-7.0±0.2
E_{max} (%)	86±3	91±2	49±5†	63±5	82±3	86±2	34±4*	42±3

C

ACh Response	Chow Diet				High Salt Diet			
	NT Vehicle	NT Y27632	S-P467L Vehicle	S-P467L Y27632	NT Vehicle	NT Y27632	S-P467L Vehicle	S-P467L Y27632
EC_{50} (logM)	-7.5±0.1	-7.8±0.1	-7.0±0.1	-7.2±0.2	-7.3±0.1	-7.6±0.1	-7.0±0.2	-7.1±0.1
E_{max} (%)	84±2	96±3	46±2†	60±4$^{\#}$	81±2	91±2	40±3*	63±2$^{\#}$

Table S1. Vasodilation: Role of Superoxide, Protein Kinase C, and Rho kinase.
Dose-dependent vasodilation in response to acetylcholine (ACh) in Figure 1 was analyzed by nonlinear regression to generate EC_{50} and E_{max}. Effects of A) superoxide dismutase mimetic Tempol (1 mmol/L, n=4-8), B) a protein kinase C inhibitor (PKCi) Calphostin C (50 nmol/L, n=4-6), and C) a Rho kinase inhibitor Y27632 (1 µmol/L, n=4-8) are reflected by the EC_{50} and E_{max} of ACh dose responses. Data are presented as mean ± SEM. Two-way ANOVA was performed for statistical analysis. $^{\dagger}p<0.05$, S-P467L Chow Diet vs NT Chow Diet; *$p<0.05$, S-P467L HSD vs NT HSD; $^{\#}p<0.05$, Y27632-treated VS vehicle-treated.

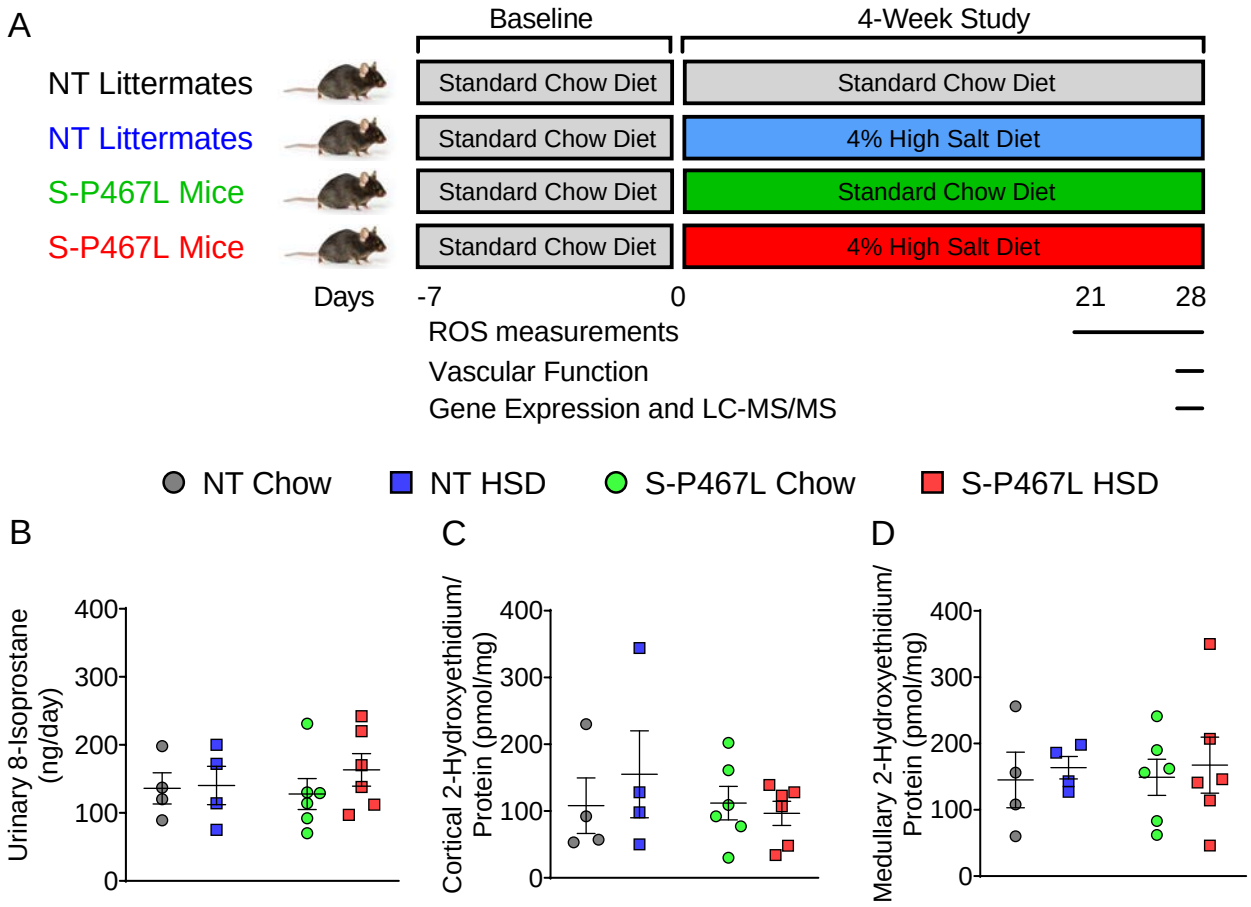
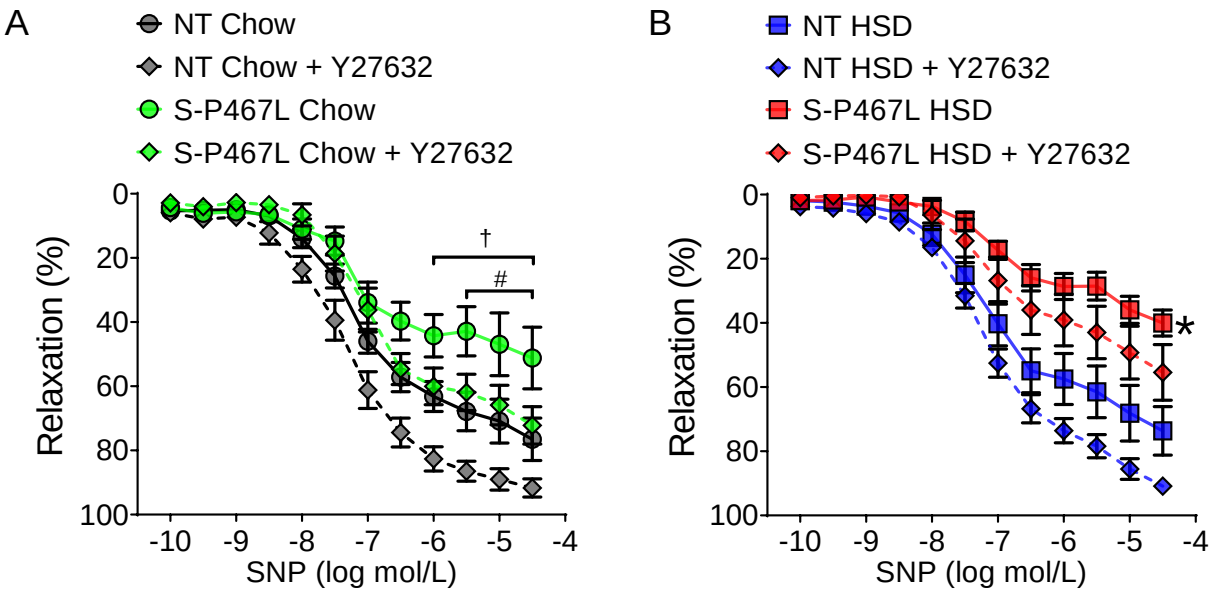


Figure S1. Study Design and Oxidative Stress

A) Schematic illustrating the study design. Non-transgenic (NT) littermates and S-P467L transgenic mice were maintained on a standard chow diet (0.8% salt) at baseline since weaning. Approximately half of the mice received a 4% high salt diet for 4 weeks while the other half remained on the normal salt diet. Experiments were performed in week 4 or at the end of 4 weeks. B) Mice were placed in metabolic cages to collect 24-hour urine during week 4. Urinary 8-isoprostane, a marker of oxidative stress, was measured by ELISA (n=4-6). C-D) Renal cortex and medulla were separated at the end of 4 weeks and immediately incubated with 50 µM dihydroethidium in pre-oxygenated Krebs buffer at 37 °C for 30 minutes. The levels of 2-hydroxyethidium, an oxidized product, was measured by HPLC (n=4-6).



C

SNP Response	Chow Diet				High Salt Diet			
	NT Vehicle	NT Y27632	S-P467L Vehicle	S-P467L Y27632	NT Vehicle	NT Y27632	S-P467L Vehicle	S-P467L Y27632
EC_{50} (logM)	-7.3±0.1	-7.4±0.1	-7.3±0.2	-7.1±0.1	-7.2±0.1	-7.2±0.1	-7.0±0.1	-7.1±0.2
E_{max} (%)	70±2	87±2$^{\#}$	47±3†	67±2$^{\#}$	66±3	83±2$^{\#}$	35±2*	48±3$^{\#}$

Figure S2. Role of Rho Kinase in Sodium Nitroprusside-Induced Vasodilation.
Isometric tension was measured on carotid artery rings from NT littermates on Chow Diet (NT Chow), NT littermates on high salt diet (NT HSD), S-P467L mice on chow diet (S-P467L Chow), and S-P467L mice on high salt diet (S-P467L HSD). A-B) Dose-dependent vasodilation (following precontraction with thromboxane A2 receptor agonist U46619) in response to sodium nitroprusside (SNP). Effects of Rho kinase inhibition (30 min preincubation with Y-27632, 1 µmol/L) were shown in curves with diamonds. Curves with filled circles (chow) and squares (HSD) represent vehicle-treated vessel segments (n=6-7). Data are plotted as mean ± SEM. Two-way ANOVA Repeated Measurements was first performed to determine whether two curves there different (main-group effect, denoted by statistical symbols on the right of the curve). When the main-group effect was not significantly different, Tukey's multiple comparison procedures were performed for comparisons at different concentrations of SNP (statistical symbols above the curves). C) These dose response curves were analyzed by nonlinear regression to generate EC_{50} and E_{max} values. Two-way ANOVA was performed for statistical analysis. $^{\dagger}p<0.05$, S-P467L Chow Diet vs NT Chow Diet; *p<0.05, S-P467L HSD vs NT HSD; $^{\#}p<0.05$, Y27632-treated VS vehicle-treated.

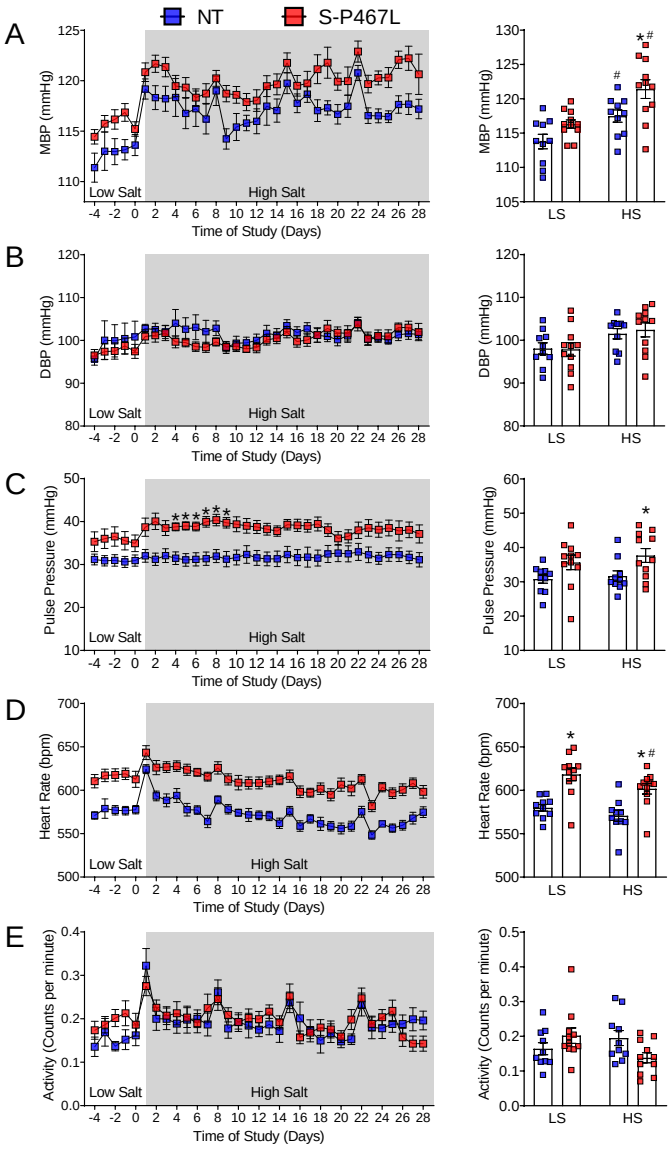


Figure S3 Mean, Diastolic, Pulse Pressure, Heart Rate, and Activity.
Mice were maintained on a 0.3% low salt diet since weaning and at 12 weeks of age were fed a 4% HSD for 4 weeks. Blood pressure, activity and heart rate was continuously recorded with radiotelemetry at baseline and during these 4 weeks. Daily mean blood pressure (MBP, A), diastolic blood pressure (DBP, B), pulse pressure (C), heart rate (D) and activity (E) in responses to 4-week HSD (left panels). BP and HR time course in the left panels were analyzed by two-way ANOVA repeated measurements. *p<0.05, S-P467L vs NT. In the right panels, average BP, heart rate and activity at baseline and during HSD days 26-28 were plotted. Two-way ANOVA results for MBP, $P_{genotype}$: 0.0032, P_{diet}: 0.0001, $P_{interaction}$: 0.50; DBP: $P_{genotype}$: 0.81, P_{diet}: 0.0098, $P_{interaction}$: 0.71; PP: $P_{genotype}$: 0.0045, P_{diet}: 0.43, $P_{interaction}$: 0.75; HR: $P_{genotype}$: 0.0002, P_{diet}: 0.005, $P_{interaction}$: 0.38; activity: $P_{genotype}$: 0.70, P_{diet}: 0.15, $P_{interaction}$: 0.0004. Sidak's multiple comparisons, *p<0.05, S-P467L LSD vs NT LSD or S-P467L HSD vs NT HSD; $^{\#}$S-P467L HSD vs S-P467L LSD or NT HSD vs NT LSD. One outlier was eliminated from the NT group as determined by a Grubb's test.

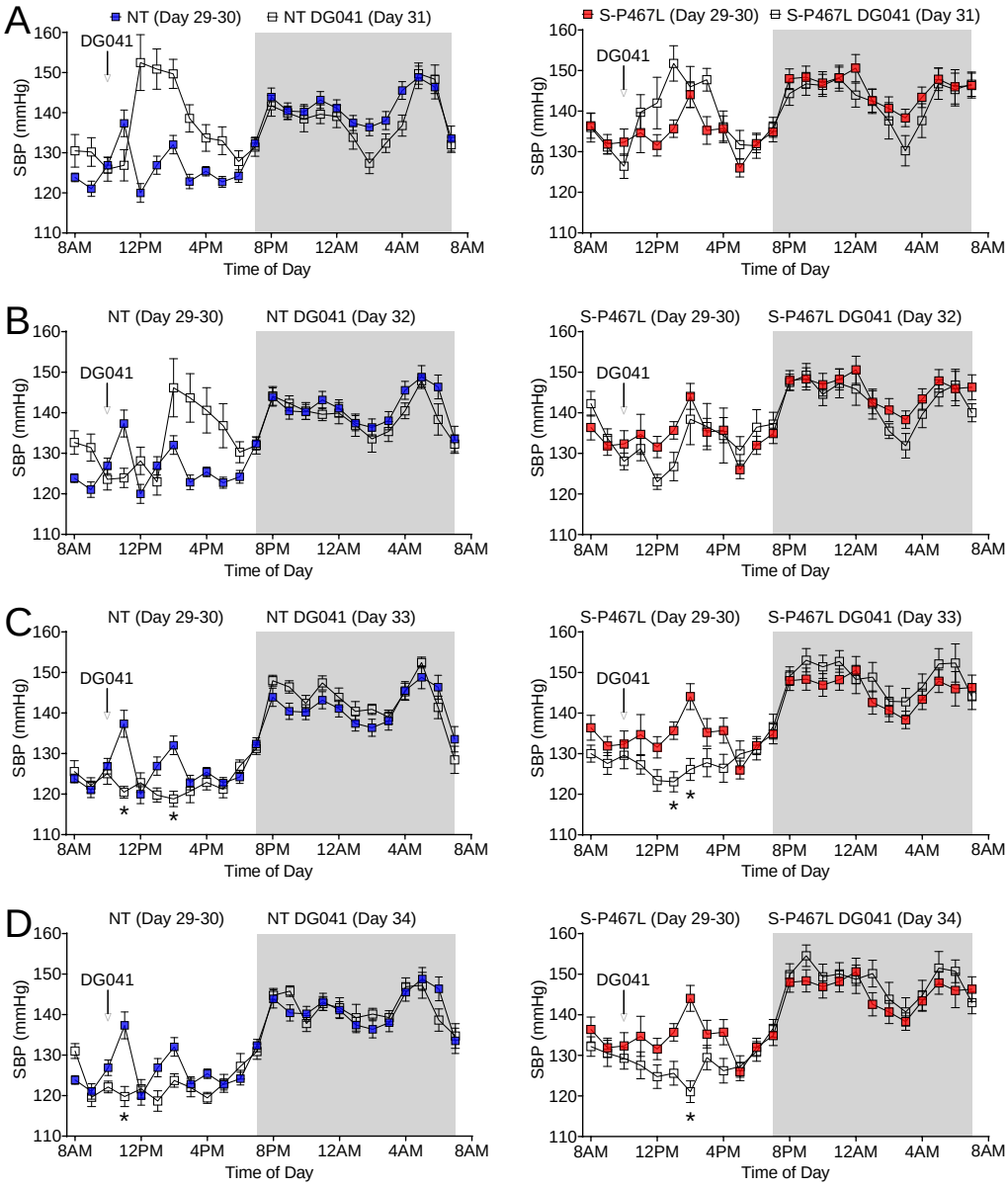


Figure S4. BP-Lowering Effects of EP3 Antagonism.
Mice were maintained on a 0.3% low salt diet since weaning and at 12 weeks of age were fed a 4% HSD for 5 weeks. Blood pressure was continuously recorded with radiotelemetry at baseline and during these 5 weeks. Salt-sensitive hypertension was established after HSD for 4 weeks (see Figure 4A-C). Hourly SBP for first two days in week 5 (HSD days 29-30, prior to DG041 treatment) was consolidated and plotted in 24-hour format (solid blue and solid red). During HSD days 31-34, mice received a subcutaneous injection of DG041 (20 mg/kg/day) at 10AM each day for 4 consecutive days. A-D) Hourly SBP in each day of DG041 administration (open squares) was plotted against the same pre-treatment SBP. In response to the third and fourth doses of DG041 (plasma half-life = 4-6 hours), transient dipping of BP was observed in S-P467L mice but not in NT controls. *$p<0.05$, two-way ANOVA repeated measurements. One outlier was eliminated from the NT group as determined by a Grubb's test.