Online Supplement

Title: Sympathetic regulation of the NCC in Dahl Salt-Sensitive Hypertension

Short Title: Sympathetic regulation of the NCC in DSS rats

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Materials and Methods

Animals

Groups of male Dahl Salt-Sensitive (DSS) or Dahl Salt-Resistant (DSR) rats (9-12 weeks of age, Envigo, Indianapolis, IN, USA) were randomly assigned to a 21-day or 42-day normal salt (NS, 0.6% NaCl, Envigo Teklad, Teklad Global Diet #2918, 18% protein, 5% crude fat, 5% fiber, total potassium (K^+) content 0.6%, total NaCl content 0.6% [174 mEq Na^+/kg]) or high salt (HS, 4% NaCl, Envigo Teklad Diets, TD.03095, 19% protein, 5% crude fat, 3% fiber, total K^+ content 0.8%, total NaCl content 4% [678 mEq Na^+/kg]) diet and tap water *ad libitum*. All animals were randomly assigned to experimental treatment groups. All animal protocols were approved by the Boston University School of Medicine Institutional Animal Care and Use Committee and performed using National Institutes of Health's "Guide for the Care and Use of Laboratory Animals."

Surgical Procedures

Subcutaneous osmotic minipump implantation

Rats were anesthetized using sodium brevital (20mg/kg IP). An osmotic minipump (2ML4, Alzet) was surgically implanted subcutaneously in the subscapular region. [1, 2]

Femoral vein, artery, and bladder cannulation

On day 21 or 42 of NS or HS intake, rats were anesthetized (sodium brevital, 20mg/kg IP supplemented with 10mg/kg IV as required) and the left femoral vein, left femoral artery, and bladder were cannulated with PE-50 tubing to deliver intravenous infusions, measure arterial blood pressure, and collect urine, respectively.[1, 2] Rats received an intravenous infusion of isotonic saline (20μL/min) during a 2-hour recovery period to allow the animal to regain full consciousness and stable cardiovascular and renal excretory function.[1, 2] Mean arterial pressure (MAP) was recorded continuously via the femoral artery cannula using BIOPAC data acquisition software (MP150 and AcqKnowledge 3.8.2; BIOPAC Systems) in conjunction with an external pressure transducer (P23XL; Viggo Spectramed).[1, 2]

Experimental Treatment Groups

21-day studies

DSS rats were randomly assigned to receive an osmotic minipump delivering a continuous subcutaneous infusion (2.5 μL/hr) of 50/50 isotonic saline/DMSO, terazosin hydrochloride (10mg/kg/day,[2] Sigma) dissolved in 50/50 saline/DSMO or propranolol hydrochloride (10mg/kg/day,[2] Sigma) dissolved in 50/50 isotonic saline/DMSO prior to random assignment to a 21-day HS diet (n=5/6 per group).

42-day studies

DSS rats were assigned to a 42-day HS diet. On day 21 of HS intake, rats were randomly implanted with an osmotic minipump to deliver a continuous subcutaneous infusion (2.5 μL/hr) of 50/50 isotonic saline/DMSO or terazosin hydrochloride (10mg/kg/day,[2] Sigma) in 50/50 isotonic saline/DMSO for the remaining 21 days of the 42-day experimental diet.

Acute Experimental Protocols

The following protocols were performed consecutively in a single experimental period in each animal on day 21 or 42 of NS or HS intake following femoral vein, artery, and bladder cannulation.

Cardiovascular function

After the 2-hour recovery period, baseline MAP was recorded continuously in conscious rats via the femoral artery cannula during the 1-hour isotonic saline infusion period of the *Renal sodium transporter activity* protocol (below). MAP values represent the average MAP during the entire 1-hour isotonic saline infusion period.

Renal sodium transporter activity

Rats received an intravenous infusion for a 1-hour control period (isotonic saline, 20μL/min[1-3]), a 1-hour Epithelial Sodium Channel (ENaC) blockade period (amiloride, ENaC antagonist; 2mg/kg bolus followed by 2mg/kg/hour at 20μL/min[1-3]), and a 1-hour Sodium Chloride Cotransporter (NCC) blockade period during which ENaC blockade was maintained (hydrochlorothiazide (HCTZ), NCC antagonist; 2mg/kg bolus followed by 2mg/kg/hour HCTZ + 2mg/kg/hour amiloride at 20μL/min[1-3]). The use of amiloride allows for the isolation of the NCC's contribution to urinary sodium excretion. MAP readings were obtained during the 1-hour control period. Throughout the protocol, urine was collected via the bladder cannula in 10-minute increments to assess urinary sodium concentration. Estimated NCC activity was assessed as the peak natriuretic response (ΔUNaV; μeq/min) to HCTZ, calculated by subtracting the average UNaV from the last two 10-min periods of ENaC blockade from the maximum UNaV during NCC blockade (occurred within the first two 10-min periods of NCC blockade in all animals). Estimated ENaC activity was assessed as the peak natriuretic response (ΔUNaV; μeq/min) to amiloride, calculated by subtracting the average UNaV from the last two 10-min periods of control from the maximum UNaV during ENaC blockade (occurred within the first two 10-min periods of ENaC blockade in all animals).

Assessment of vascular sympathetic tone

Following completion of the renal sodium transporter activity protocol, the peak change in MAP in response to an intravenous bolus of hexamethonium (30 mg/kg)[1] was assessed. Baseline MAP was determined as the average MAP recorded over a 10-min control period prior to hexamethonium injection. After baseline MAP measurement, animals received an intravenous bolus of hexamethonium, and blood pressure was monitored for an additional 30-min period. The peak depressor response, assessed over a 60-s period, occurred within 5 min post injection. [1] Following protocol completion, rats were decapitated while conscious, and both kidneys were collected and immediately frozen at −80°C for measurement of the NCC and associated regulatory kinases.[1]

Kidney Protein Extract Preparation

Kidneys harvested from rats post decapitation and following completion of the acute experiments were immediately stored at -80ºC. Approximately 200 mg of kidney cortex tissue was homogenized on ice using a potter elvehjem tissue grinder (Kimble, Cat. No.

885510-0021) in a homogenizing buffer containing Halt Protease inhibitors cocktail (Thermoscientific, Cat. No. 78429) and PhosSTOP phosphatase inhibitor (Roche, Cat. No. 04906845001).[2] The homogenate was then centrifuged at 6,000 RPM for 15 minutes at 4ºC. The supernatant was collected and a BCA assay was used to determine protein content. Prepared protein extracts were stored at -80ºC prior to use in immunoblotting studies.

Immunoblotting

Kidney cortex protein extracts were loaded at 20-40 µg of total protein per lane. Nitrocellulose membranes (GE, cat. No. 10600096) were blocked in 5% blocking grade blocker (BIO-RAD, cat. No. 170-6404) for 1 hour and probed overnight at 4ºC with primary antibodies in 0.1% PBS-Tween. Membranes were washed with 0.1% PBS-Tween and incubated for 2-hours with secondary antibodies in 0.1% PBS-Tween at room temperature. Membranes were visualized using chemiluminescence (SuperSignal West Pico Plus, cat. No. 34580; Thermo Scientific). Semi-quantitative analysis was performed using Image J version 1.52 (NIH) and protein expression was normalized to total protein using Coomassie staining (Bio-Safe, cat. No. 1610786, BIO-RAD). Antibodies and dilutions used are provided in Supplemental Table 3.

ELISA

Whole blood samples were obtained following conscious decapitation and used to determine plasma norepinephrine (NE) via ELISA per manufacturer's instructions (Immuno-Biological Labs America, Minneapolis, MN; cat. No. IB89552). Renal NE content was determined via ELISA per manufacturer's instructions (Immuno-Biological Labs America, Minneapolis, MN; cat. No. IB89537).[1, 2]

Analytical Techniques

Urine volume was determined gravimetrically, assuming 1g = 1mL. Urine sodium and potassium concentration was assessed using flame photometry (model 943; Instrumentation Laboratories).[1, 2] Plasma hematocrit (Hct) was determined using a micro-hematocrit centrifuge (Adams Readacrit, Clay Adams, NJ).[3] Hct was used to calculate estimated plasma volume (EPV) and estimated blood volume (EBV) using the following equations; EPV = (0.065 x body weight (kg)) x (100 – Hct), EBV = (EPV x 100)/(100-Hct). Fractional excretion of sodium was determined using standard techniques as previously described by our laboratory.

Statistical Analysis

Data are shown as mean ± SD. Comparisons were made between NS and HS dietary salt intake within group or HS and HS + adrenoceptor antagonist groups using a two-tailed Student's t-test, and One-way ANOVA was used to assess differences between groups, and a Tukey's post-hoc test was used to evaluate variation among groups. Statistical analysis was carried out using GraphPad Prism version 7 (GraphPad). Statistical significance is defined as $P < 0.05$.

References:

1. Walsh KR, Kuwabara JT, Shim JW, Wainford RD. Norepinephrine-evoked salt-sensitive hypertension requires impaired renal sodium chloride cotransporter activity in sprague-dawley rats. *Am J Physiol Regul Integr Comp Physiol*. 2016;310:R115-124
2. Frame AA, Puleo F, Kim K, Walsh KR, Faudoa E, Hoover RS, Wainford RD. Sympathetic regulation of the ncc in norepinephrine-evoked salt-sensitive hypertension in sprague-dawley rats. *Am J Physiol Renal Physiol*. 2019; 317: F1623-F1636
3. Ashek A, Menzies RI, Mullins LJ, Bellamy CO, Harmar AJ, Kenyon CJ, Flatman PW, Mullins JJ, Bailey MA. Activation of thiazide-sensitive co-transport by angiotensin II in the cyp1a1-Ren2 hypertensive rat. *PLoS One*. 2012;7:e36311

Treatment Group	EPV (ml)	EBV (ml)
DSR 0.6% NaCl	10.5±0.4	20.6±0.5
DSR 4% NaCl	10.3±0.3	20.9±0.4
DSS 0.6% NaCl	10.4±0.3	21.2±0.5
DSS 4% NaCl	11.6 ±0.2*	22.1±0.5
DSS 4% NaCl + vehicle	11.7±0.3	22.2±0.6
DSS 4% NaCl + terazosin	10.7±0.2τ	21.8±0.5
DSS 4% NaCl + propranolol	11.4±0.3	22.0±0.5

Table S1 Estimated Plasma and Blood Volume Estimated Plasma Volume (EPV) and Estimated Blood Volume (EBV) in milliliters listed for naive DSR and DSS rats and DSS rats receiving a subcutaneous infusion of vehicle, terazosin (10 mg/kg/day) or propranolol (10 mg/kg/day) maintained on either a normal (0.6% NaCl) or a high-salt (4% NaCl) diet for 21 days. Data are expressed as mean ± SD (N=5 or 6/group) *P<0.05 versus DSS 0.4% NaCl group value, τP<0.05 versus DSS 4% NaCl.

Treatment Group	Baseline Na^+ excretion (μeq/min)	Baseline K^+ excretion (μeq/min)	Baseline urine output (μl/min)
DSR 0.6% NaCl	2.33±0.26	1.92±0.33	19.6±3.1
DSR 4% NaCl	2.54±0.28	2.04±0.26	23.8±4.8
DSS 0.6% NaCl	2.47±0.36	2.11±0.38	20.8±3.1
DSS 4% NaCl	2.34±0.33	2.18±0.41	19.1±4.4
DSS 4% NaCl + vehicle	2.55±0.27	2.09±0.37	23.6±3.4
DSS 4% NaCl + terazosin	2.46±0.31	2.14±0.29	22.2±2.6
DSS 4% NaCl + propranolol	2.52±0.37	2.22±0.33	21.6±2.3

Table S2 Baseline renal excretory parameters during acute renal sodium transporter assay Baseline sodium excretion (μeq/min), baseline potassium excretion (μeq/min), and baseline urine output (μl/min) during the control isotonic saline infusion period of the acute renal sodium transporter activity assay in conscious naive male DSR and DSS rats and DSS rats receiving a subcutaneous infusion of vehicle, terazosin (10 mg/kg/day) or propranolol (10 mg/kg/day) maintained on either a normal (0.6% NaCl) or a high-salt (4% NaCl) diet for 21 days. Data are expressed as mean ± SD (N=5 or 6/group).

Primary Antibody	Dilution	Source
NCC (Sodium Chloride Cotransporter)	1:1,000	Millipore, Billerica, MA; cat. No. AB3553
Phosphorylated NCC- Thr53	1:1,000	Phosphosolutions, Aurora, CO; cat. No. p1311-53
WNK1 (With no lysine kinase 1)	1:200	Santa Cruz, Dallas, TX, cat. No 28897
WNK4 (With no lysine kinase 4)	1:1,000	Novus Biologicals, Centennial, CO, cat. No. NB600-284SS
OxSR1 (Oxidative Stress Response 1)	1:2,000	Abcam, Cambridge, MA, cat. No. ab125468
SPAK (STE20/SPS1-related proline-alanine-rich protein kinase)	1:500	Abcam, Cambridge, MA, cat. No. ab79045
Anti-phospho SPAK (Ser373)/ phosphor-OSR1 (Ser325)	1:500	Millipore Sigma, Burlington, MA, cat. No. 07-2273
Anti-phospho OxSR1 (T185)/ SPAK (T233)	1:500	Abcam, Cambridge, MA cat. No. ab138655
Secondary Antibody	**Dilution**	**Source**
HRP (Horseradish peroxidase) donkey anti-rabbit IgG	1:2,000	Abcam, Cambridge, MA, Cat. No. ab16284

Table S3 Antibodies used for immunoblotting

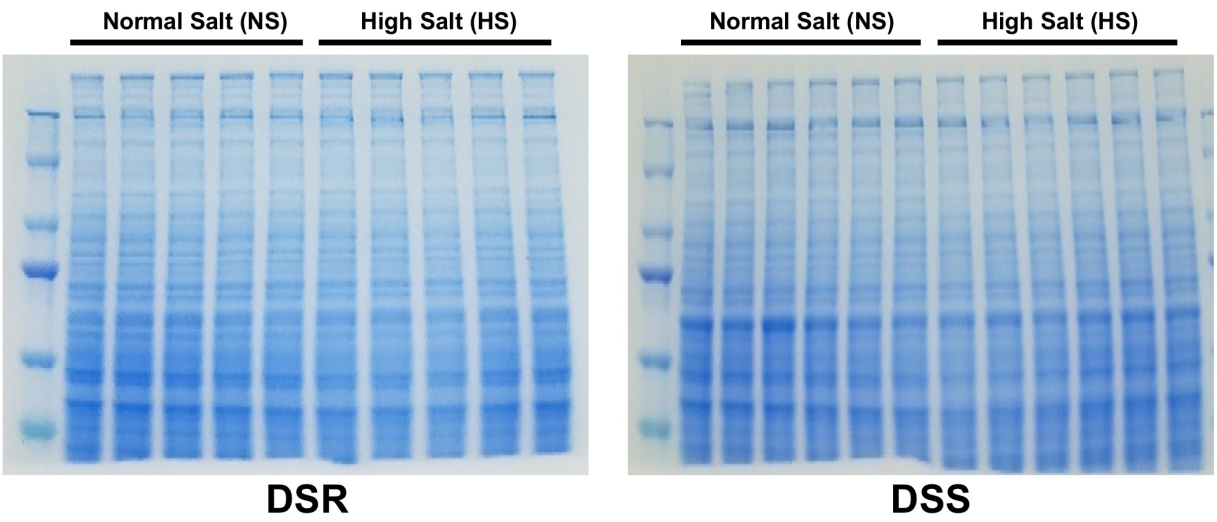

Figure S1 Coomassie blue stain of total protein from Dahl Salt Resistant (DSR) and Dahl Salt Sensitive (DSS) rat samples for which immunoblotting data is presented in Figures 1 and 2. Left panel represents total protein loaded after Coomassie stain in 3-month old male DSR rats fed a 21-day normal salt (NS; 0.6% NaCl) or high salt (HS; 4% NaCl) diet. Right panel represents total protein loaded after Coomassie stain in 3-month old male DSS rats fed a 21-day normal salt (NS; 0.6% NaCl) or high salt (HS; 4% NaCl) diet.

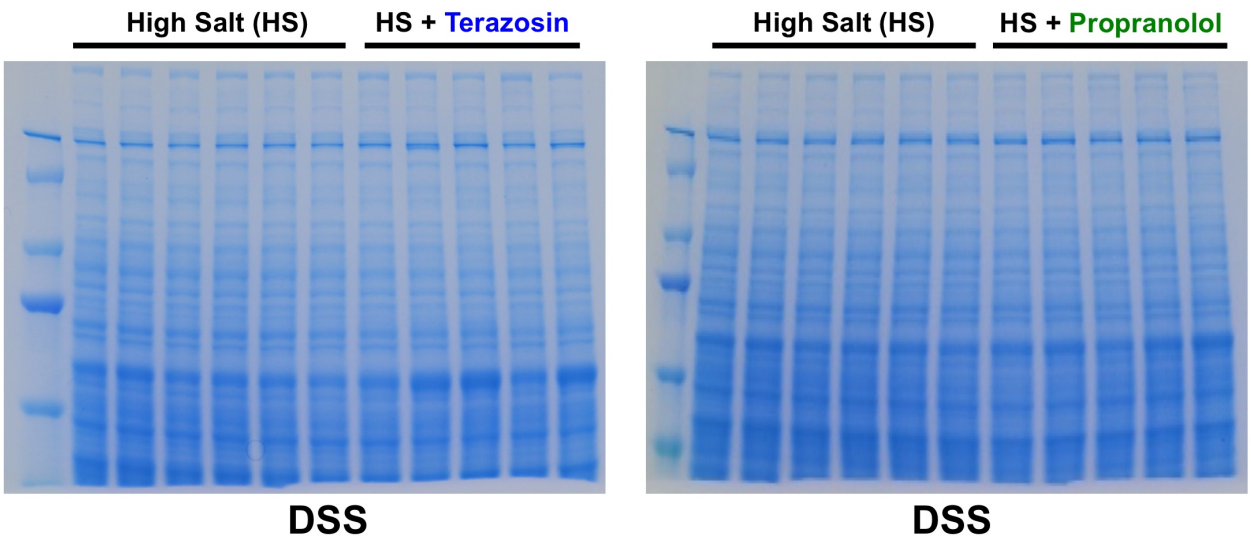

Figure S2 Coomassie blue stain of total protein from Dahl Salt Sensitive (DSS) rat samples for which immunoblotting data is presented in Figures 3 and 4. Left panel represents total protein loaded after Coomassie stain in 3-month old male DSS rats fed a 21-day high salt (HS; 4% NaCl) diet during vehicle or s.c. terazosin infusion. Right panel represents total protein loaded after Coomassie stain in 3-month old male DSS rats fed a 21-day high salt (HS; 4% NaCl) diet during vehicle or s.c. propranolol infusion.

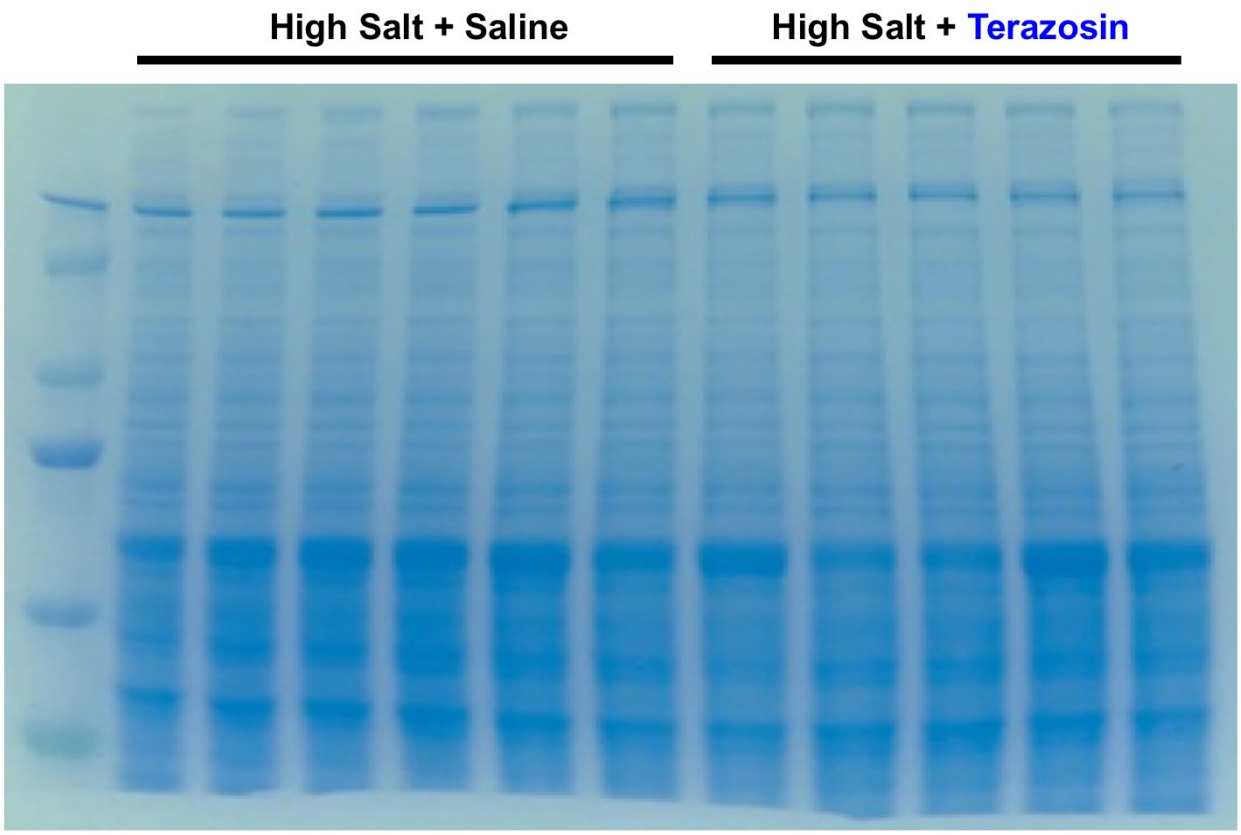

Figure S3 Coomassie blue stain of total protein from Dahl Salt Sensitive (DSS) rat samples for which immunoblotting data is presented in Figures 5 and 6. The panel represents total protein loaded after Coomassie stain in 3-month old male DSS rats fed a 42-day high salt (HS; 4% NaCl) diet during vehicle or s.c. terazosin infusion of the last 21-days of the high salt intake period.

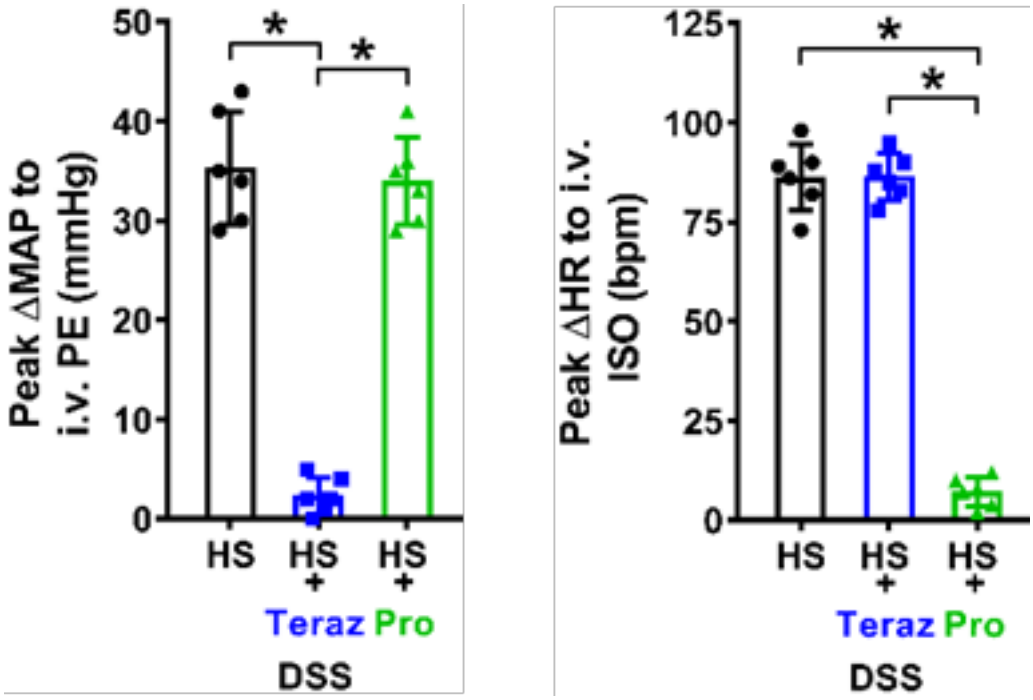

Figure S4 Confirmation of adrenoceptor blockade. (Left) peak pressor response (ΔMAP; mmHg) to i.v. phenylephrine (α-adrenoceptor agonist, PE; 4μg/kg bolus), (right) peak tachycardic response (ΔHR; beats per minute [bpm]) to i.v. isoproterenol (β-adrenoceptor agonist, ISO; 0.7μg/kg bolus) in conscious untreated 3-month old Dahl Salt Sensitive (DSS) rats or DSS rats that received a subcutaneous (s.c) infusion of terazosin (Teraz) or propranolol (Pro) during a 21-day high salt (HS; 4% NaCl) diet. Terazosin treated rats do not show a pressor response to PE confirming α_1-adrenoceptor blockade. Propranolol treated rats do not show a tachycardic response to ISO confirming β-adrenoceptor blockade. Data are expressed as mean ± SD (N=6/group).

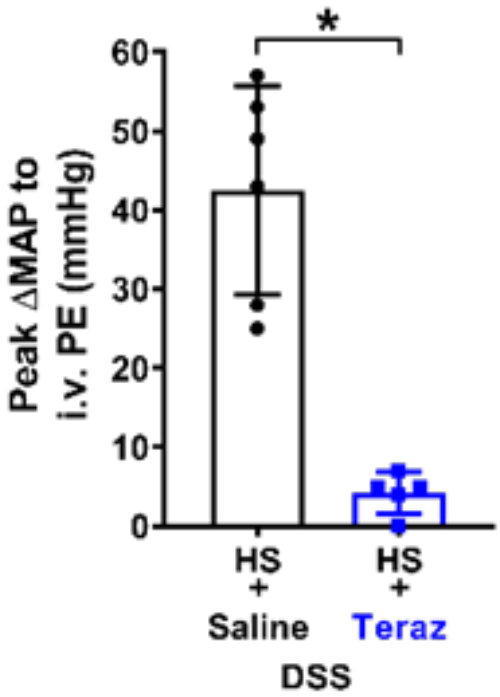

Figure S5 Confirmation of α_1-adrenoceptor blockade. Peak pressor response (ΔMAP; mmHg) to i.v. phenylephrine (α-adrenoceptor agonist, PE; 4μg/kg bolus in conscious 3-month old Dahl Salt Sensitive (DSS) rats that received a subcutaneous (s.c) infusion of saline/DMSO or terazosin/DMSO (Teraz) during days 21-42 of a 42-day high salt (HS; 4% NaCl) diet. Terazosin treated rats do not show a pressor response to PE confirming α_1-adrenoceptor blockade. Data are expressed as mean ± SD (N=5-6/group).