SUPPLEMENTAL MATERIAL

Resting afferent renal nerve discharge and renal inflammation: Elucidating the role of afferent and efferent renal nerves in DOCA-salt hypertension

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SUPPLEMENTAL METHODS AND MATERIALS

Animals

Male Sprague Dawley rats (Weight: 275-300g; Age: 10-12 weeks) were purchased from Charles River Laboratories (Wilmington, MA) and housed in pairs in a temperature and light controlled room. Rats were allowed access to standard rat chow and distilled water *ad libitum* during this pre-experimental period.

Experiment 1: Direct recording of resting afferent renal nerve discharge in DOCA-salt rats

General Procedures

Rats were anesthetized with 5% isoflurane, and maintained at 2-3% during surgical preparation. The femoral vein and artery were cannulated with PE50 tubing for 0.9% saline perfusion (50µl/min) to establish steady state urine production and for arterial pressure measurement respectively. The renal pelvis was cannulated with a 32G triple-lumen catheter (Part Number: 0040EO; ReCathCo, Allison Park, PA) for intrapelvic perfusion and withdrawal of infusates and monitoring of pelvic pressure. A renal nerve bundle near the renal hilus was isolated and gently placed on an electrode consisting of two single stainless steel wires (0.003"; Sigmund Cohn Corp., Mount Vernon, NY). A ground wire was placed nearby in the abdominal muscle. Excess fluid was removed from the site by vacuum, and the nerve and electrode were encased in silicone (Kwik-Sil, WPI, Sarasota, FL) once a recording was obtained. Finally, the central end of the nerve was cut to eliminate efferent nerve traffic in the signal leaving only afferent nerve discharge and background noise. Following the surgical preparation, animals were maintained at 1.5% isoflurane for 30-minutes prior to initiating the protocol.

To determine the background noise contribution to the signal, the distal end of the nerve was cut upon completion of the experiment. This integrated voltage was subtracted from the final integrated ARNA signal (∫ARNA) during subsequent analysis. The nerve signal was amplified (Gain: 20,000X) and filtered by band-pass (0.05‑3kHz) using a single-channel amplifier (Nihon Kohden, Model: Meg2100). All data was recorded at 2kHz and analyzed using LabChart 8.0 software (ADInstruments, USA).

Establishment of Maximal Afferent Renal Nerve Activity for Normalization Analysis

A separate pilot study was conducted in young male Sprague Dawley rats (n=6) to establish a stimulus that would induce maximal activation of afferent renal nerve activity (ARNA) in each animal to be used for normalizing multiunit recording of ARNA between animals (see Figure S1). We compared the ARNA response to non-selective depolarization by intrapelvic administration of increasing concentrations of potassium chloride (KCl; 40, 80, 60, and 320 mM) to increasing concentrations of the TRPV1 agonist capsaicin (10, 50, and 100 µM). Each stimulation was followed by flushing the renal pelvis with isotonic saline. Resting afferent activity was allowed to return to baseline between each stimulation.

Measurement of Resting ARNA and Responsiveness to Stimuli in DOCA-Salt Rats

This experiment was designed to test the hypothesis that DOCA-salt treatment alters resting ARNA. Twenty male Sprague Dawley rats (Weight: 275-300g; Age: 10-12 weeks) underwent the 21-day DOCA-salt treatment as previously described [1, 2]. All rats were nephrectomized, and allowed three weeks to recover. Half of the rats were switched to 0.9% saline, and five days later, administered 100mg DOCA in a subcutaneous silicone implant (DOCA; n=10). Controls remained on distilled water and received a silicone vehicle implant (Vehicle; n=10). After 21 days of DOCA-salt (DOCA; n=10) or Vehicle (n=10) treatment, animals were anesthetized with 5% isoflurane, and maintained at 2-3% during surgical preparation.

Resting ARNA was recorded over a 10-minute period. Next, we measured the ARNA responses to randomized mechano- and chemo-sensitive stimuli. These included a 20mmHg increase in pelvic pressure with isotonic saline, intrapelvic perfusion of hypertonic saline (600mM NaCl), and intrapelvic administration of 20μg/mL bradykinin. A minimum of a five-minute recovery period was allowed between each stimulus to allow activity to return to resting level. Finally, a 50μM capsaicin solution was perfused into the renal pelvis to elicit a maximum level of afferent renal nerve discharge.

Nerve Activity Quantification and Analysis

The mean value of the 10-minute baseline ∫ARNA was used to quantify resting ARNA. Further, to control for intra-experimental variability of the electrode placement and nerve units, resting afferent nerve activity was also normalized to the peak ∫ARNA response to intrapelvic 50μM capsaicin ($\int ARNA_{peak}$). Resting ∫ARNA was expressed as a percent of $\int ARNA_{peak}$ using the following calculation:

$$\%A_{max} = 100 * \frac{\int ARNA_{baseline}}{\int ARNA_{peak}}$$

Experiment 2: Effect of total and selective afferent renal denervation on DOCA-salt hypertension and renal inflammation

Experimental Groups and Surgical Procedures

Thirty-eight rats underwent a unilateral nephrectomy 14 days prior to treatment as previously described [1]. Rats were then randomly assigned to one of four experimental groups: (1) 100 mg DOCA-salt + total renal denervation (DOCA T-RDNx; n=12), (2) 100 mg DOCA-salt + selective afferent ablation (DOCA A-RDNx; n=11), (3) 100 mg DOCA-salt + sham denervation (DOCA Sham; n=10), or (4) 0 mg DOCA + sham denervation (Control; n=5).

For all surgical procedures, rats were anesthetized with 2-3% isoflurane (Phoenix Pharmaceutical, St. Joseph, MO). Atropine sulfate (0.2 mg/kg, i.p.; West-Ward Pharmaceuticals, Eatontown, NJ), ketoprofen (5 mg/kg, s.c.; Fort Dodge Animal Health, Overland Park, KS) and gentamicin sulfate (2.5 mg/kg, i.m.; Hospira, Lake Forest, IL) were administered prior to surgery.

Total RDNx was achieved via a midline abdominal incision by surgical sectioning of the renal nerves followed by a perivascular application of 10% phenol solution (in 100% ethanol) as previously reported [1, 2]. Selective afferent renal nerve ablation (A-RDNx) was performed by periaxonal application of a concentrated capsaicin solution (33mM capsaicin in 5% ethanol; 5% Tween 80; 90% 150mM NaCl) to the renal artery and vein as recently described [2]. Sham procedures were performed with 0.9% saline. Following renal denervation or sham, rats were instrumented with a radiotelemeter for continuous measurement of arterial pressure and heart rate [1]. Rats were allowed a seven-day post-operation recovery period and then housed individually in metabolic cages for the duration of the experiment (Techniplast, USA). For the three-day post operation recovery, rats were given ketoprofen (2.5 mg/kg, s.c; s.i.d.), and the drinking water was supplemented with amoxicillin (1 mg/ml; Sandoz International, Holzkrichen, Germany).

Experimental Protocol

Following the seven-day recovery period, drinking water was replaced with a 0.9% saline solution for the three DOCA-salt groups (the Control group remained on normal drinking water) and baseline measurements of arterial pressure, heart rate and sodium intake were begun. At the end of the 5-day control period, rats were briefly anesthetized with isoflurane and received either a subcutaneous implant of 100mg DOCA in silicone or silicone vehicle (Sylgard 184 silicone elastomer base; Dow Corning, Midland, MI) [1]. Arterial pressure (AP) and heart rate (HR) were monitored continuously by radiotelemetry (Model PA-C10, Data Sciences International, USA).

AP was sampled at 500 Hz for 10 seconds every four minutes using commercially available software (Dataquest A.R.T., Data Sciences International, USA). HR was determined from the AP profile. Mean 24-hour (24h) averages of AP (MAP) and HR were calculated and plotted for each day of the study. Daily food and water intake were measured gravimetrically. Sodium intake was calculated by summing the intake of the 0.1% (g/g) NaCl chow diet and 0.9% (g/ml) saline intake. Cumulative sodium intake was calculated by area-under-the-curve analysis of 21 days of daily sodium intake data following DOCA or silicone vehicle implantation. Individual curves for each rat were analyzed and then group averages were analyzed for each treatment group. At the end of the three weeks of treatment, rats were anesthetized and euthanized by exsanguination. Kidneys were collected and dissected to isolate the renal pelvis from the renal cortical-medullary tissue (combined) sample. Tissues were flash-frozen in liquid nitrogen and stored for subsequent assessment of efficacy of T-RDNx and A-RDNx and measurement of renal inflammatory protein content.

Confirmation of T-RDNx and A-RDNx

Renal norepinephrine (NE) content was measured to assess whether efferent renal nerves were ablated effectively. Protein homogenates were assayed by high-performance liquid chromatography (HPLC) analysis with electrochemical detection [3].

Effectiveness of afferent nerve ablation was assessed through the enzyme linked immunosorbent assay (ELISA) detection of calcitonin gene-related peptide (CGRP) renal tissue content as recently described [2]. In isolated renal pelvic samples, CGRP tissue content was assayed according to the assay's manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI). Data is expressed in picograms of analyte per milligrams of total protein. Homogenate total protein concentration was measured by Bradford assay.

Renal Cytokine Analysis

The renal inflammatory profiles of DOCA-salt and control rats were determined by measuring several pro-inflammatory chemo- and cytokines. Pro-inflammatory analytes measured were as follows: GRO/KC, MCP-1, IL-1β, IL-2, IL-6, IL-17a, TNFα, and IFNγ. Data is expressed in picograms of analyte per milligrams of total protein. Homogenate total protein concentration was measured by Bradford assay.

Staining and Flow Cytometry

At day of necropsy, the kidney was halved and further dissected into approximately 2-3mm cubes with a sterile scalpel. The tissue contents were incubated in Collagenase Type I buffer (100 U/mL) (Worthington Biochemical Corporation) containing RPMI 1640/5%FCS/2mM $MgCl_2$/2mM $CaCl_2$ for 45 min at 37°C. Samples were homogenized gently with MACS C-tubes and poured through a 70µm filter. To isolate renal leukocytes, cell solutions further isolated by 44%/67% percoll gradient (800 x g at 20°C) (GE Heathcare, USA), and the leukocyte interface was transferred to a new tube and washed with FACS buffer to prepare for cell staining.

Cells were stained with either a T-effector or T-regulatory panel. T-Effector Panel: Anti-CD44 (Clone: IM7); Anti-CD3 (Clone: IF4); Anti-CD4 (Clone: OX-35); Anti-CD8 (Clone: OX-8); Anti-CD62L (Clone: OX-85); Anti-CD80 (Clone: 3H5); Anti-CD86 (Clone: 24F). T-regulatory panel: Anti-CD25 (Clone: OX-39); Anti-CD3; Anti-CD4; Anti-CD8; Anti-FoxP3 (Clone: FJK-16S); Anti-CD80; Anti-CD86. All antibodies were diluted 1:200 (5ug/ml). Intracellular staining with APC anti-FOXP3 was performed using the FoxP3 kit in accordance with the manufacturer's directions (eBioscience). Samples were measured with the flow cytometer (BD LSR II, BD Biosciences, USA) and data was analyzed by FlowJo Software 8.0 (Ashland, OR, USA).

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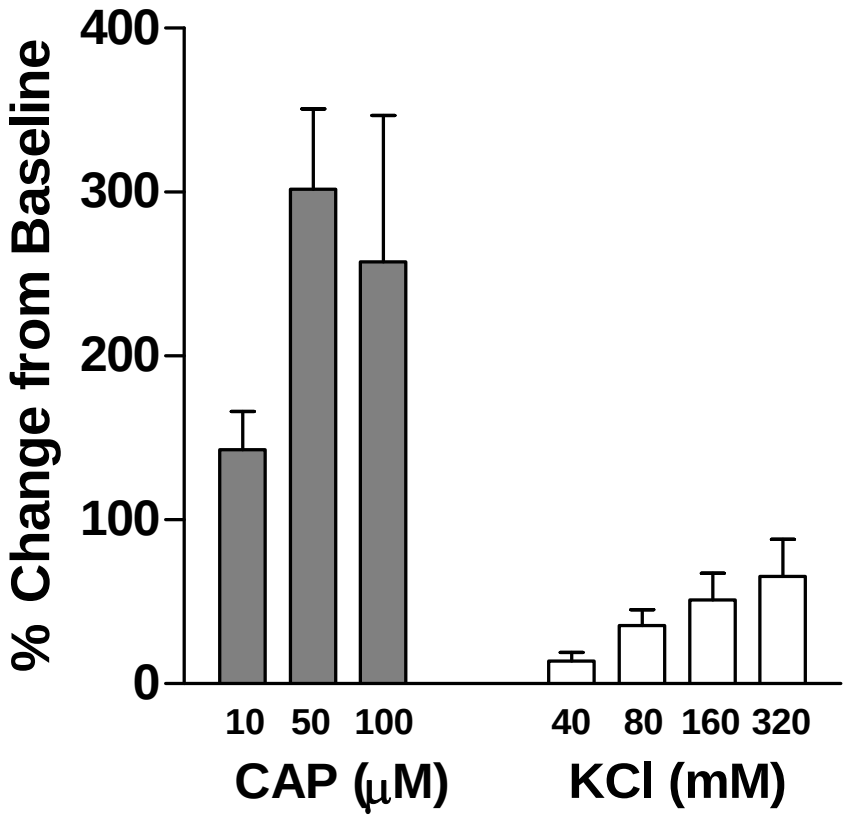
1. Jacob F, Clark LA, Guzman PA, Osborn JW. Role of renal nerves in development of hypertension in doca-salt model in rats: A telemetric approach. *Am J Physiol*. *Heart Circ Physiol*. 2005;289:H1519-1529.
2. Foss JD, Wainford RD, Engeland WC, Fink GD, Osborn JW. A novel method of selective ablation of afferent renal nerves by periaxonal application of capsaicin. *Am J Physiol*. *Regul Integr Comp Physiol*. 2015;308:R112-122.
3. Li M, Galligan J, Wang D, Fink G. The effects of celiac ganglionectomy on sympathetic innervation to the splanchnic organs in the rat. *Autonomic Neuroscience : Basic & Clinical*. 2010;154:66-73.

SUPPLEMENTAL DATA

Necropsy Data

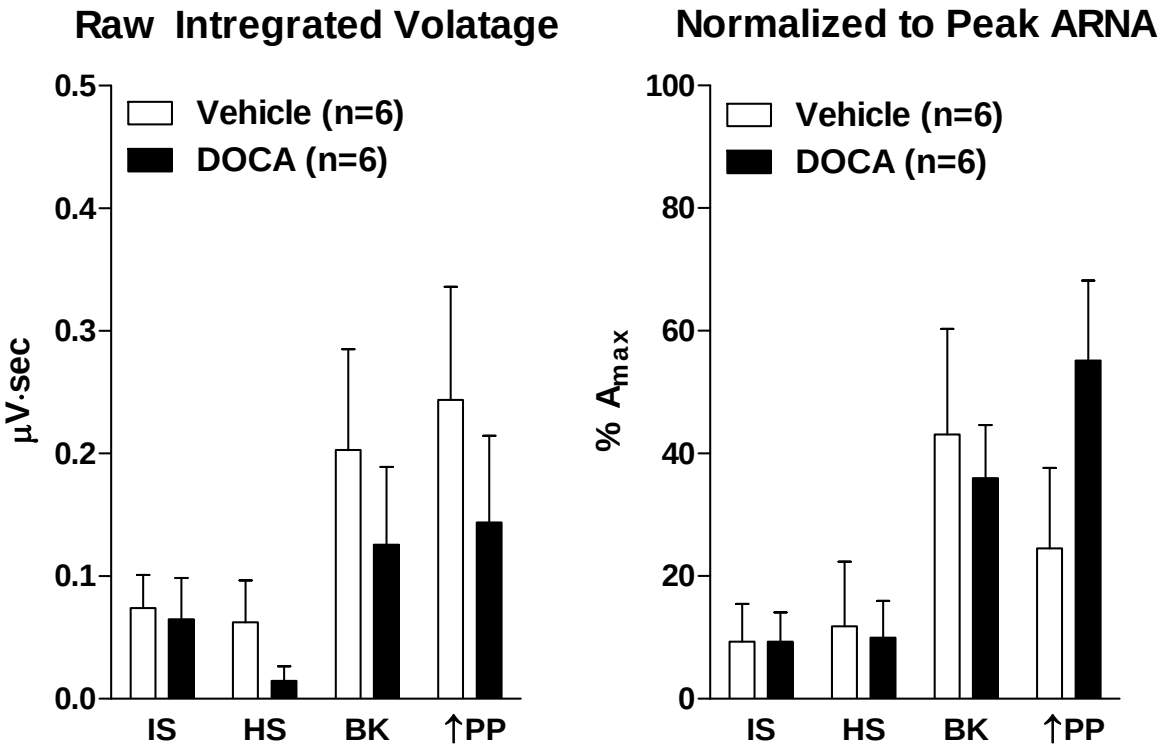
Body weight (BW) was similar in all four groups at the end of the protocol (Vehicle-Sham 478±21; DOCA-Sham 485±12; T-RDNx 496±24; A-RDNx 516±15g). Heart size was increased in DOCA-Sham rats compared to Vehicle-Sham, and T-RDNx attenuated (#p<.05) this hypertrophic effect (Vehicle-Sham 2.71±0.09; *DOCA-Sham 3.25±0.14; #T-RDNx 3.02±0.05; *A-RDNx 3.22±0.10g/kg BW). Similarly, renal mass was increased (*p<.05) in DOCA-Sham rats compared to Vehicle-Sham, and remained increased in the T-RDNx or A-RDNx rats (Vehicle 3.79±0.27; *DOCA-Sham 4.59±0.21; *T-RDNx 5.10 ±0.21; A-RDNx 5.59±0.19g/kg BW).

SUPPLEMENTAL FIGURES
Figure S1



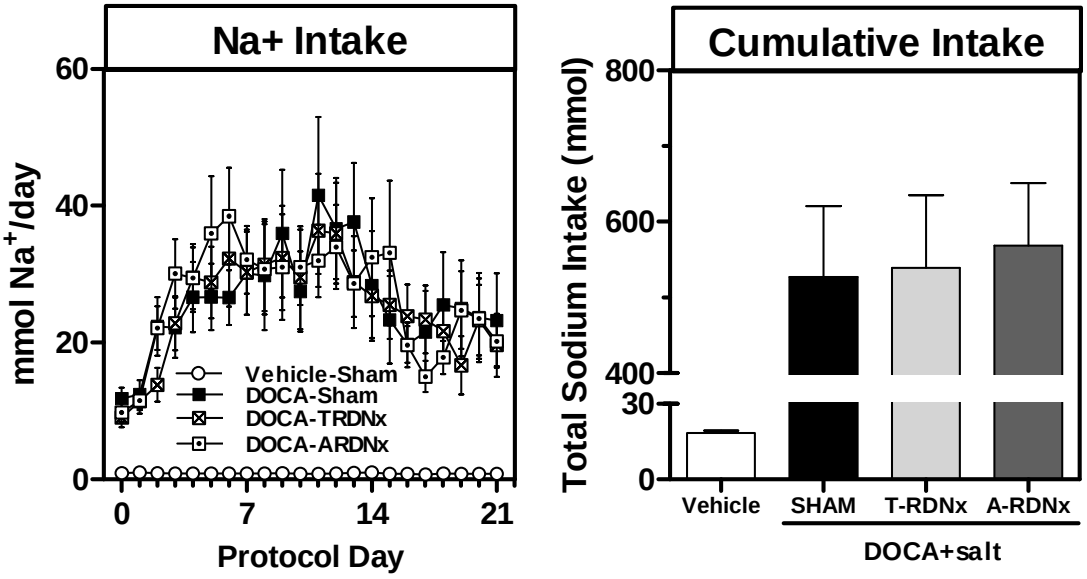
Capsaicin and Potassium Chloride Afferent Activation Response. Pilot experiments in six male Sprague Dawley rats were designed to determine the appropriate chemical and dose for the peak afferent activation normalization. We tested both capsaicin (TRPV-1 agonist) and KCl (non-specific nerve depolarization). The peak afferent nerve response was achieved with 50μM capsaicin. Response to intrapelvic KCl was lesser, even at supraphysiological (320mM) concentrations. All data presented as mean±SEM.

Figure S2



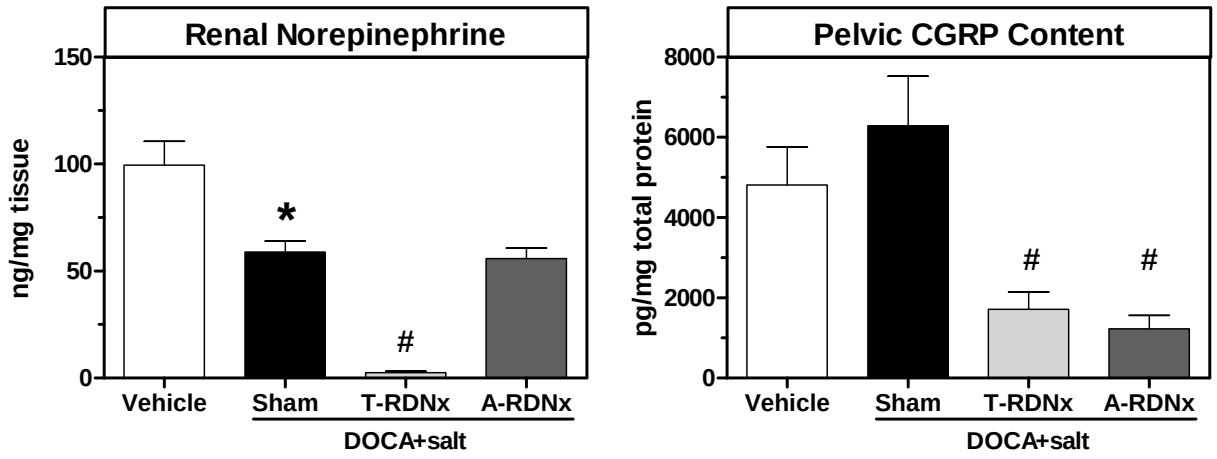
Responsiveness to Chemical and Mechanical Afferent Stimulation. Sensitivity of intrapelvic afferent renal nerves was assessed in DOCA and Vehicle rats using mechano- and chemo-sensitive stimuli. Represented in raw integrated voltage and as a percent of peak response to capsaicin, no difference in sensitivity was observed between DOCA and Vehicle rats across all stimuli. Abbreviations: IS=isotonic saline; HS=600mM hypertonic saline; BK=20ug/mL bradykinin; PP: 20mmHg increased pelvic pressure. All data presented as mean±SEM (n=6/group).

Figure S3



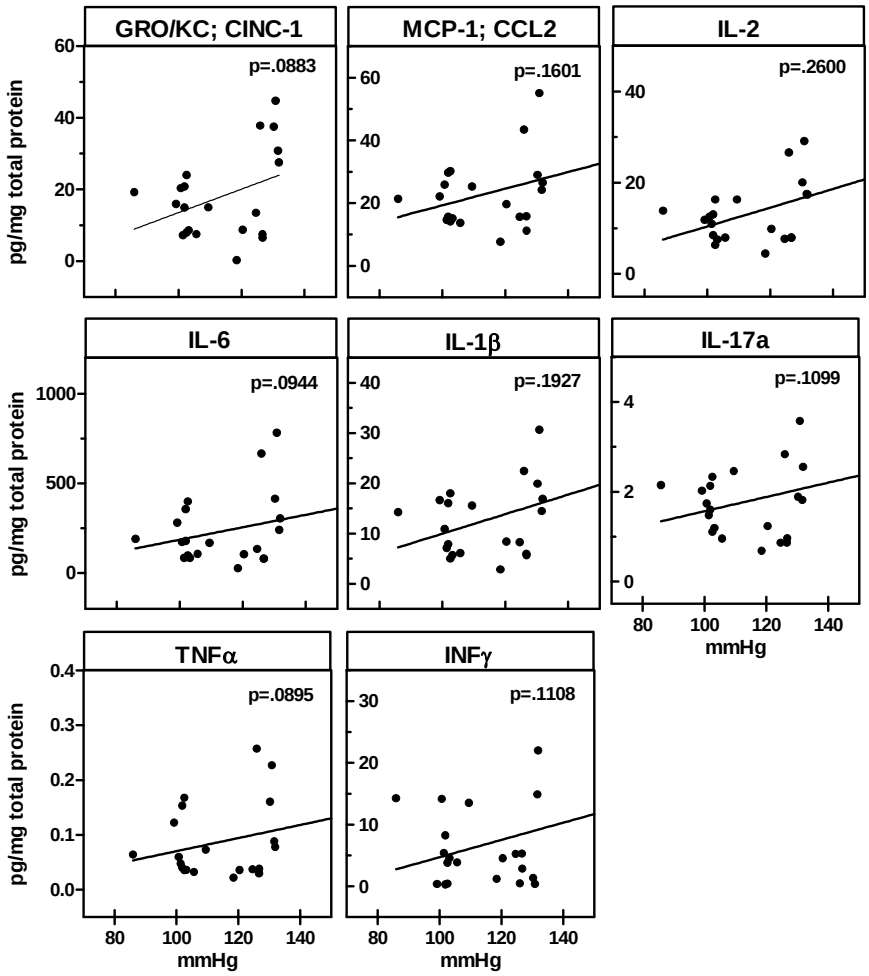
Daily and Cumulative Sodium Intake. Panel A: Daily sodium intake (mmol Na/day) was increased in all DOCA-salt groups (Sham, T-RDNx, and A-RDNx) compared to Vehicle-Sham rats. Panel B: Cumulative sodium intake (mmol) over the 21-day DOCA-salt treatment was no different between Sham, T-RDNx, and A-RDNx animals. All data presented as mean±SEM. *p<.05 vs. Vehicle-Sham; #p<.05 vs. DOCA-Sham.

Figure S4



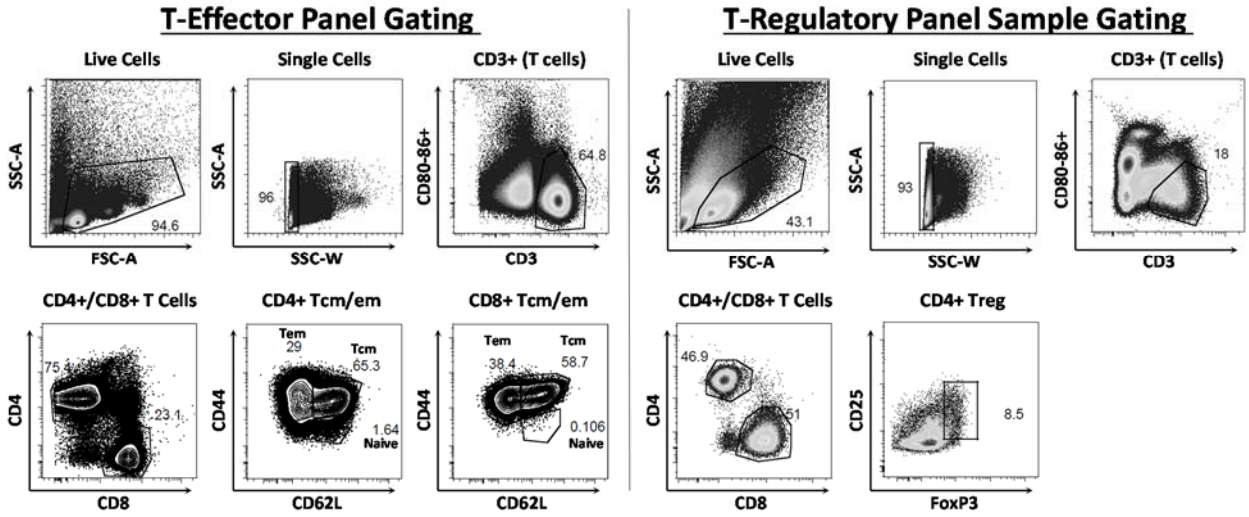
Confirmation of Denervation Efficacy. To confirm efferent and afferent nerve ablation efficacy in each subject, renal norepinephrine (NE) and calcitonin gene-related peptide (CGRP) were measured in each subject. T-RDNx substantially (>90%) decreased (#p<.05) renal NE content and A-RDNx had no observed effect (DOCA 59±5; #T-RDNx 3±1; A-RDNx 56±5 ng/mg tissue). Further, T-RDNx and A-RDNx both decreased (#p<.05) CGRP content vs. sham DOCA+salt rats (DOCA 6287±1238; #T-RDNx 1720±423; #A-RDNx 1230±336 pg/mg tissue). The average CGRP and NE values are statistically significantly different from the theoretical value of zero. All data presented as mean±SEM. *p<.05 vs. Vehicle-Sham; #p<.05 vs. DOCA-Sham. Vehicle n=5; DOCA n=10; T-RDNx n=12; A-RDNx n=11.

Figure S5



Correlation Between Mean Arterial Pressure and Renal Cytokine Content. The relationship between final mean arterial pressures (MAP) and renal cytokine content in DOCA-treated animals (DOCA, T-RDNx, and A-RDNx) was assessed to elucidate a potential effect of blood pressure on cytokine content. No significant ($p>.05$) correlation was observed between MAP and any individual cytokine. DOCA n=6; T-RDNx n=5; A-RDNx n=5.

Figure S6



Sample Gating for Flow Cytometry. The gating samples for the T cell effector (left) and regulatory (right) panels are depicted above.