Supplemental Data Section

Endothelin activation of reactive oxygen species mediates acute stress-induced pressor response in Dahl salt-sensitive pre-hypertensive rats

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Running head: Mechanism of acute behavioral stress induced pressor response

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Methods

Telemetry: Telemetry transmitters (Data Sciences, Inc.) were implanted according to the manufacturer's specifications. Rats were anesthetized with ketamine/xylazine (50 mg/kg / 10 mg/kg, i.p.). The abdominal aorta was then exposed by a midline incision, and briefly occluded. The transmitter catheter was inserted into a hole made by a 21-gauge needle just proximal to the iliac bifurcation, and secured in place with tissue glue (Vetbond). The transmitter body was attached to the abdominal wall along the incision line with 4-O proline suture as the incision was closed. The skin was closed with staples that were removed 7 days after the incision had healed. Rats were returned to individual housing for data collection and allowed 8-10 days to recover from surgery before being subjected to the stress protocol. Animals were housed in a room separate from that used for studying the stress response. The individual rat cages were placed on top of the telemetry receivers, and mean arterial pressure (MAP) and heart rate (HR) were continuously (i.e. 10-sec sampling periods at regularly scheduled 10-min intervals) recorded throughout the study using the Dataquest A.R.T. Acquisition program.

Air jet stress: DS rats were subjected to 2 sessions of acute air jet stress spaced one week apart without treatment. All animals were previously subjected to at least two 15-minute restraint sessions on days prior to an experiment to reduce the stress associated with the restraint itself. On the day they were subjected to air jet stress, rats were quietly brought to a sound-proofed room. Immediately after starting the telemetry recording software, the room was vacated. The animals were then allowed to acclimate to their surroundings for 15-30 minutes in their cages, that is, until which time they ceased exploring the new environment and their pressures stabilized. Animals were then placed in tubular Plexiglas restrainers with sufficient aeration, and MAP and HR were continuously monitored by telemetry for at least 15 minutes before initiating air jet stress. When necessary, animals were monitored for up to an additional 10 minutes to allow the animals to adapt to being restrained, such that 3-5 minutes of stable MAP and HR recordings were obtained prior to exposure to air jet stress. Air jet stress consisted of pulses (2 sec duration delivered every 10 sec for 3 min) of compressed air (15 lb/in^2) aimed at the forehead from a 1/8" opening at the front of the tube. After the 3-minute stress period, MAP and HR were monitored for 20 additional minutes while the animals were still in the restrainer, and post-air jet values obtained. At the end of this post-stress period, animals were returned to their cages and brought back to their holding room.

Determination of plasma concentrations of 8-isoprostane, ET-1, and catecholamines: Rats were anesthetized with ketamine/xylazine (50 mg/kg / 10 mg/kg, i.p.), and catheters (Braintree Scientific Inc., Braintree, MA) were inserted into the jugular vein. Catheters were routed subcutaneously, and exteriorized at the back of the neck; catheters were filled with heparin (1000 U/ml). For four days after the surgery, animals were placed into the restrainers for at least 15 min, consistent with the duration used for the acute stress protocol, and catheters were flushed to maintain patency. On days 5 and 6 post-surgery, blood (1 ml) was drawn from restrained animals to determine baseline (unstressed) plasma levels. Red blood cells were resuspended in 1 ml sterile saline (0.9% NaCl) containing 6.2% bovine serum albumin and 50 μl of 7.5% EDTA, and replaced in the animal; catheters were subsequently refilled with heparin. On day 7 post-surgery, animals were subjected to the acute stress

protocol, and blood (1 ml) was drawn over the 30-60 sec interval of air jet stress. As before, red blood cells were resuspended in a sterile saline solution, re-infused, and catheters refilled with heparin. Catheter patency was maintained for the next 4 days, after which time the animals were placed on a 3-day regimen of either tempol (1 mM in the drinking water), the selective ET_A receptor antagonist ABT-627 (5 mg/kg/day in the drinking water), or A-182062 (30 mg/kg/day in the food). Separate groups of animals were used for each of the treatments. Blood (1 ml) was drawn on each of the first 2 days of treatment with tempol, ABT-627, or A-182062, and on the third day of treatment, animals were again subjected to the acute stress protocol. Blood samples were centrifuged at 10000 x g for 10 min at 4 °C, and plasma was removed, aliquoted, and stored at –80 °C until analyses could be performed. The following plasma measurements were made: 8-isoprostane concentration by EIA (Cayman Chemical Company, Ann Arbor, MI), ET-1 by ELISA (QuantiGlo; R&D Systems, Minneapolis, MN), and epinephrine (Epi) and norepinephrine (NE) by RIA (BI-CAT-RIA, ALPCO Diagnostics, Windham, NH) according to manufacturer's specifications.

Whole animal pressor responses: Separate groups of DS animals were either left untreated (tap water alone) or given tempol (1 mM in the drinking water for 3 days) (*n*=5 for each). Animals were anesthetized with thiobutabarbital (Inactin; 65 mg/kg, i.p.), and the right femoral artery and vein were isolated and cannulated with PE-50 for monitoring MAP and drug infusion, respectively. After a 30-minute equilibration period, animals were given chlorisondamine (5 mg//kg, i.v) to eliminate endogenous sympathetic vasomotor tone and baroceptor-reflex mediated responses. Effective blockade was confirmed by the absence of reflex bradycardia following constrictor administration. Phenylephrine (0.5, 1, 2, 4, 8, 16, and 24 μg/kg), an α_1 adrenergic specific agonist, was administered in randomized order of doses. MAP was allowed to return to baseline between each dose. Responses to ET-1 and sarafotoxin 6c (S6c; both ET-1 and S6c at 0.1, 0.3, and 1 nmol/kg), a selective ET_B receptor agonist, were examined in separate sets of animals. ET-1 and S6c was administered in ascending concentration order at 20-minute intervals due to the prolonged nature of pressor responses. Peak and steady-state (one minute before introduction of next dose) responses were reported. ET responses were obtained only in the presence of chlorisondamine. All measurements were recorded using a Power Lab data acquisition system. Responses to PE in anesthetized animals are reported as the peak change in MAP from the baseline MAP; both peak and steady-state responses are reported for S6c. PE and S6c dose-response curves were analyzed two-way analysis of variance with repeated measures.

Statistical Analysis: Data are expressed as means ± SE. All baseline MAP and HR values are reported as 24-hour means. Total pressor response refers to the change in MAP during the 3 minutes of air jet stress, and was determined by the equation: $\Sigma((P - P_{pre\text{-}stress}) \times 0.067))$, where P refers to each MAP data point recorded during the delivery of air jet stress, $P_{pre\text{-}stress}$ is the average pressure during the 3 minutes just prior to the onset of the air pulses, and 0.067 is the 4 second data collection interval in minutes. Data are expressed as the area under the curve (AUC; mmHg x min). Statistical analyses of baseline MAP and HR and of the total pressor response were made by paired t-test. Baseline plasma values of 8-isoprostane, ET-1, and catecholamines represent the average values obtained for the two days before subjecting the animals to air jet stress. Statistical analyses of plasma determinations were made by two-way analysis of variance, followed by Newman-Keuls test for multiple comparisons. Differences are considered significant at $p<0.05$.

Results

Two week successive air jet stress protocol. Changes in arterial pressure in untreated animals during air jet stress are shown in Figure S1. Figure S2 shows that the pressor response is indistinguishable from week one to week two in the same group of DS rats: thus, any effect of the treatments on the stress response during the second week could not be attributed to habituation.

Pressor responses in anesthesized rats. ROS have been shown to partially mediate the constrictor response to various agonists, including ET-1.[1, 2] Peak and steady-state pressor responses to exogenous ET-1 (Fig. S3) or S6c (Fig S4) were unaffected by pretreatment with tempol. The rapid response to stress is mediated by the sympathetic nervous system, and thus in part by α adrenergic-mediated vasoconstriction. We therefore examined the whole animal pressor response to exogenous phenylephrine in anesthetized animals to determine whether the blunted pressor response to air jet stress in tempol-treated rats was due to reduced responsiveness of the vascular smooth muscle to α_1 adrenergic stimulation. Experiments were performed in both the absence and presence of autonomic ganglion blockade. Chlorisondamine produced comparable decreases in MAP in untreated and tempol-treated animals (Fig S5). Tempol had no effect on the phenylephrine-mediated pressor response in the absence and presence of chlorisondamine (Fig. S6A and B, respectively).

REFERENCES

1. Just A, Whitten CL, Arendshorst WJ. Reactive oxygen species participate in acute renal vasoconstrictor responses induced by ET_A and ET_B receptors. *Am J Physiol Renal Physiol.* 2008;294:F719-728.

2. Just A, Olson AJM, Whitten CL, Arendshorst WJ. Superoxide mediates acute renal vasoconstriction produced by angiotensin II and catecholamines by a mechanism independent of nitric oxide. *Am J Physiol Heart Circ Physiol.* 2007;292:H83-92.

FIGURE S1

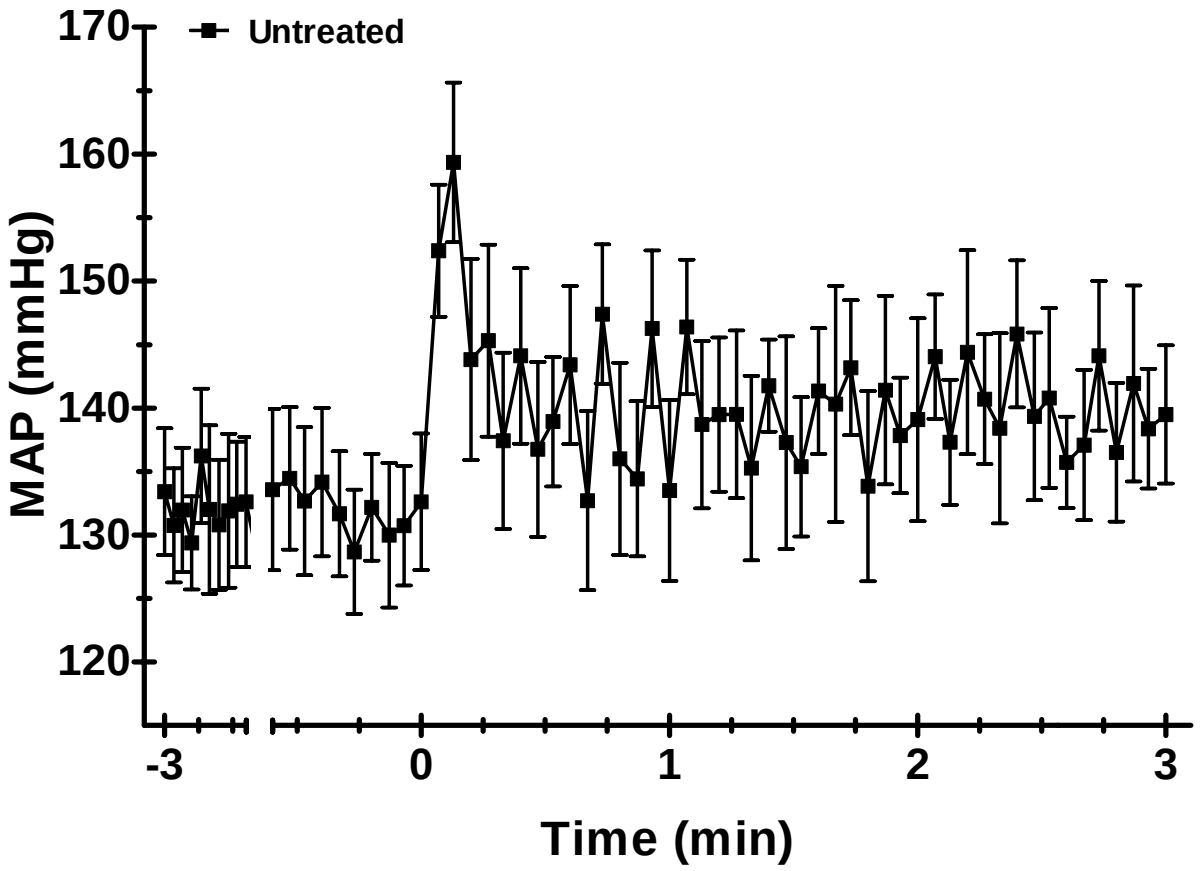

Figure S1: Effect of air jet stress on mean arterial pressure in pre-hypertensive Dahl salt-sensitive rats. Data represent average absolute mean pressure from 10 untreated animals.

FIGURE S2

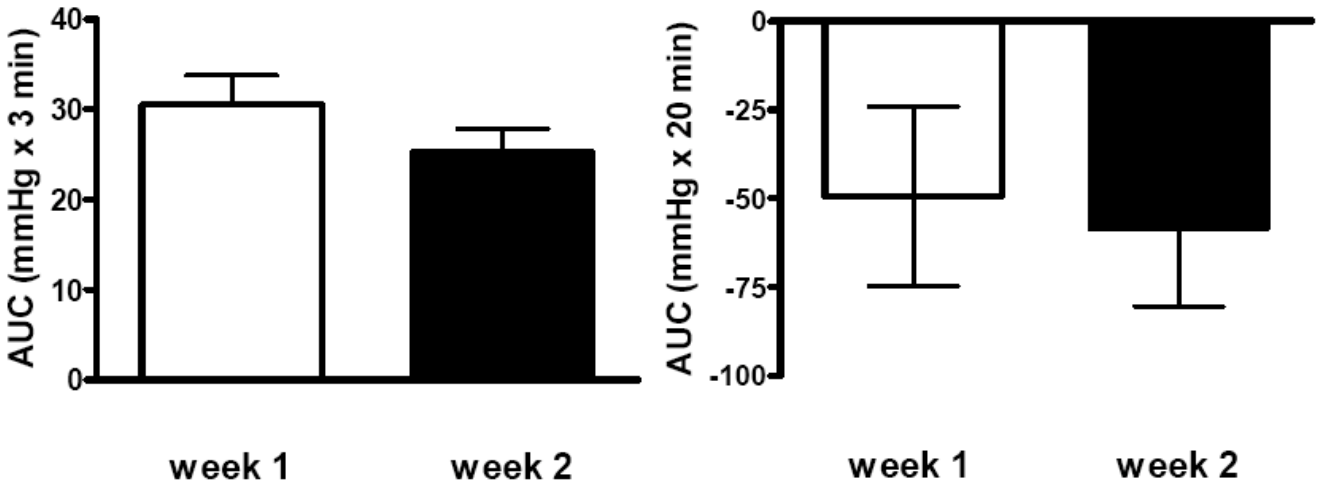

Figure S2: Summary of integrated pressor response (area under the curve; AUC) to acute air jet stress (left panel) and integrated mean arterial pressure during the 20-minute post-stress period (right panel) in pre-hypertensive Dahl salt-sensitive rats. Air jet stress was administered twice in untreated animals (*n*=8) at a weekly interval.

FIGURE S3

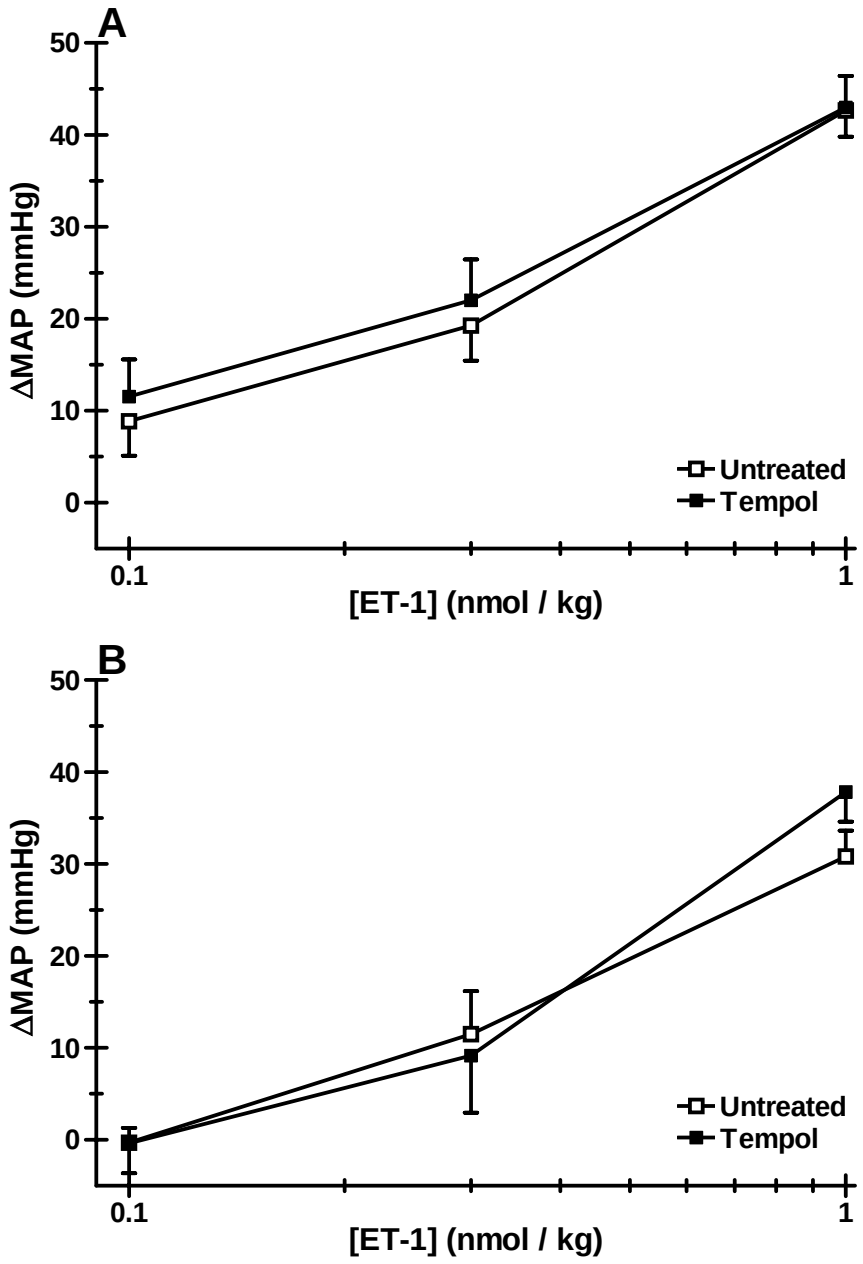

Figure S3: Effect of the free radical scavenger, tempol, on whole animal peak (A) and steady-state (B) pressor response to exogenous endothelin-1 (ET-1) (*n*=6) in anesthetized, pre-hypertensive Dahl salt-sensitive rats in the presence of autonomic ganglionic blockade using chlorisondamine (5 mg/kg). Animals were either untreated or given tempol (1 mM in the drinking water mg/kg/day) for 3 days.

FIGURE S4

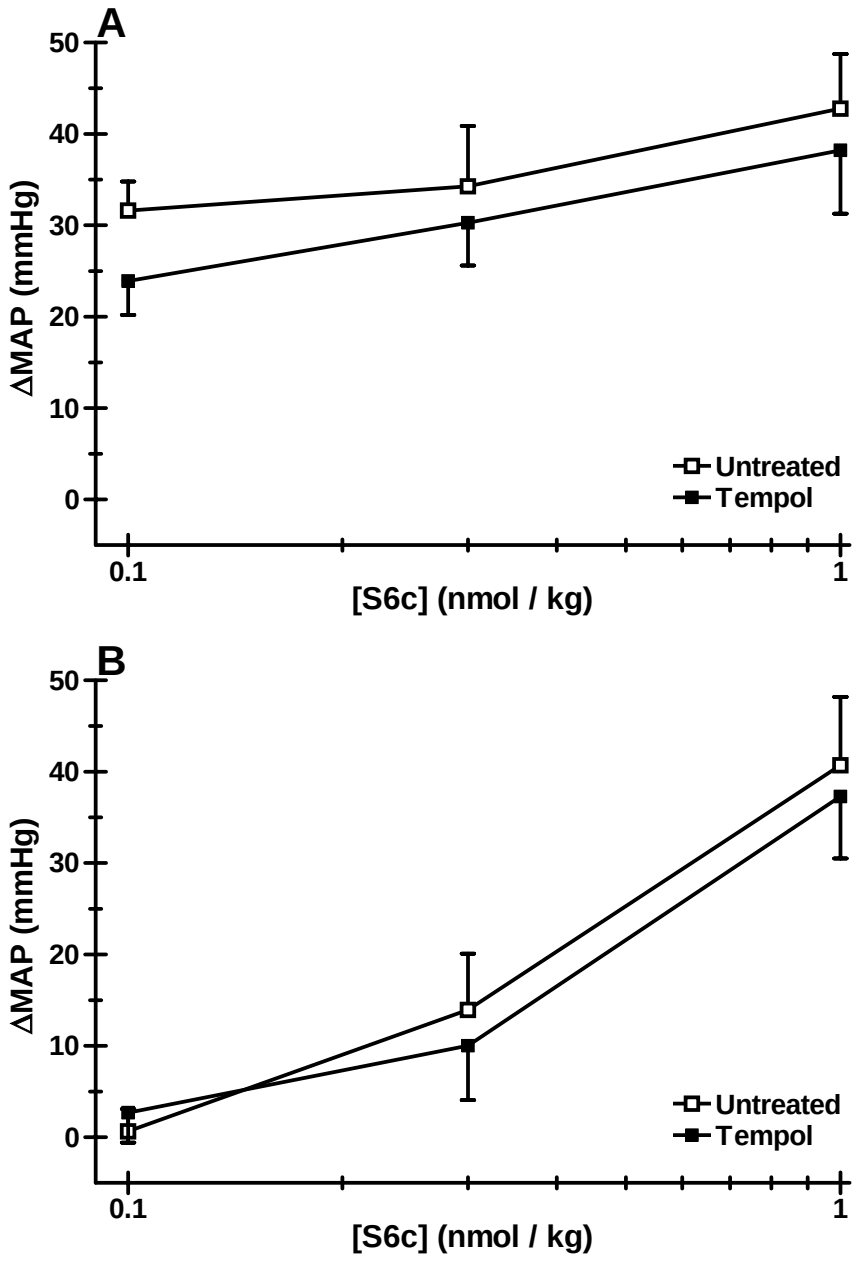

Figure S4: Effect of the free radical scavenger, tempol, on whole animal peak (A) and steady-state (B) pressor response to the ET_B receptor selective agonist sarafotoxin 6c (S6c) (*n*=5-6) in anesthetized, pre-hypertensive Dahl salt-sensitive rats in the presence of autonomic ganglionic blockade using chlorisondamine (5 mg/kg). Animals were either untreated or given tempol (1 mM in the drinking water mg/kg/day) for 3 days.

FIGURE S5

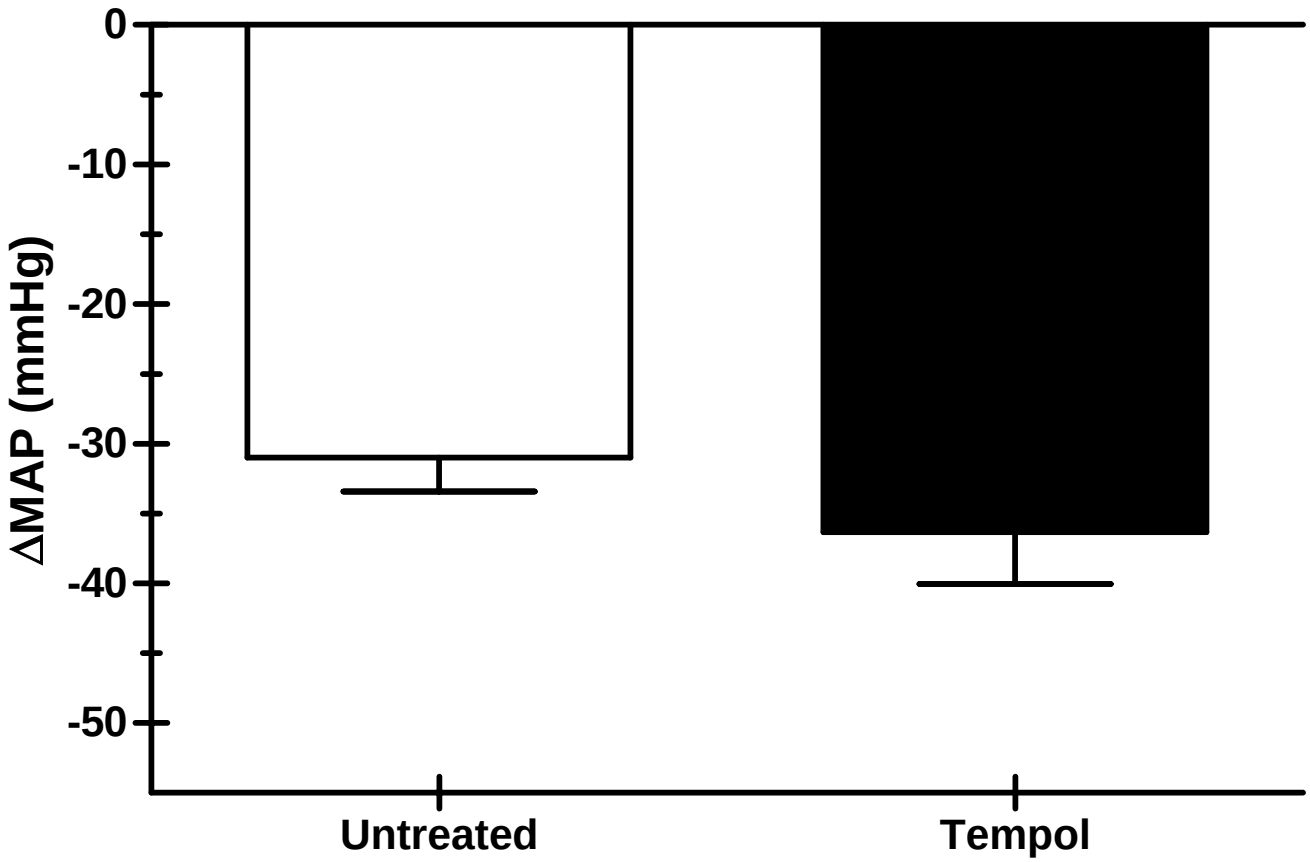

Figure S5: Effect of autonomic ganglion blockade with chlorisondamine (5 mg/kg) on baseline arterial pressure in pre-hypertensive Dahl salt-sensitive rats. Animals were either untreated (*n*=4) or given tempol (*n*=3) (1 mM in the drinking water mg/kg/day) for 3 days.

FIGURE S6

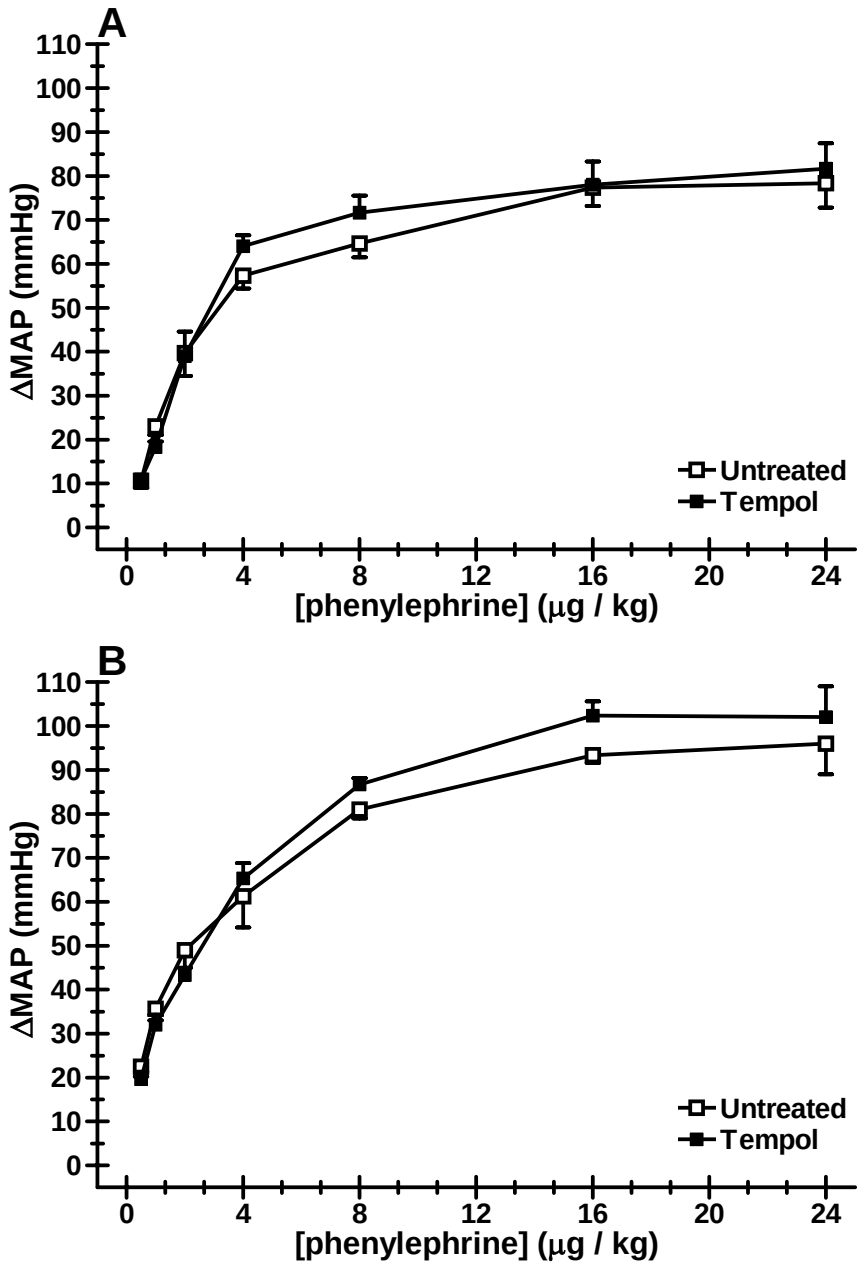

Figure S6: Effect of the free radical scavenger, tempol, on whole animal pressor response to exogenous phenylephrine (*n*=3-4) in anesthetized, pre-hypertensive Dahl salt-sensitive rats in the absence (A) or presence (B) of autonomic ganglionic blockade using chlorisondamine (5 mg/kg). Animals were either untreated or given tempol (1 mM in the drinking water mg/kg/day) for 3 days.