ONLINE SUPPLEMENT

EARLY LIFE STRESS ENHANCES ANGIOTENSIN II-MEDIATED VASOCONSTRICTION BY REDUCED ENDOTHELIAL NITRIC OXIDE (NO) BUFFERING

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Aortic vascular reactivity

Thoracic aortic tissue was isolated and cleaned of adventitial fat prior to cutting into rings and performing vascular reactivity measurements as previously described[1]. Aortic rings were transferred to a vessel chamber filled with physiological saline solution (composition in mM: 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 11.1 dextrose), mounted, and allowed to equilibrate (Danish MyoTechnology). After verification of responsiveness and endothelium integrity, aortae were reequilibrated for 30 min, and a cumulative concentration-response curve (CRC) to AngII, endothelin (ET-1, from $1x10^{-12}$ to $3x10^{-6}$ M), phenylephrine (PE, from $1x10^{-9}$ to $3x10^{-5}$ M), and KCl (4.7 mM to 100 mM) were performed. A separate group of aortic rings were denuded of endothelium prior to performing the CRC to AngII. The aortic rings were mechanically denudated by gentle rubbing with curve forceps. We verify that the vessels were denuded by the absence of Ach-induced vasorelaxation (10^{-3} M) in pre-constricted vessels (PE, 5ul, 10^{-3} M), We pre-incubated (30 min) aortic rings in the presence of inhibitors or antagonists prior to AngII-induced constriction. The following compounds were utilized: candesartan (AT1 receptor antagonist; 10^{-5} M) and PD123,319 (AT2 receptor antagonist; 10^{-6} M), L-NAME (NOS inhibitor; 10^{-4} M), apocynin (NADPH oxidase inhibitor; $3x10^{-4}$ M) and PEG-SOD (cell permeable superoxide dismutase; 1000 U/ml). We also completed CRC to acetylcholine ($1x10^{-9}$ to $1x10^{-5}$ M) and sodium nitroprusside (100 pmol/l to 31.6 µmol/l) after precontraction with PE (10^{-5} M). Vasoconstriction responses to AngII, PE, and KCl were reported as normalized to the % maximum response to KCl. In figure 2, CRC are expressed in percentage of increase from the maximal response to AngII 10^{-7} M. Values from the control group as the baseline (100%), and expressed the changes in % from this value. Responses to Ang II and ET-1 were reported as the force generated (absolute tension) above the baseline (28 mN). Vasorelaxation responses were reported as normalized to the % maximum relaxation from PE pre-constriction. Maximum responses and EC_{50} calculations were analyzed by Prism version 4.03 (GraphPad Software, Inc.).

Quantitative real time PCR

RNA was extracted from dissected aortic tissue using RNeasy mini kit (Qiagen, CA). Genomic DNA elimination and reverse transcription was performed using a QuantiTaq kit (Qiagen, CA). RNA quantification and $A_{260/280}$ ratio was determined by spectrophotometric analysis (NanoDrop ND-1000, Thermo Scientific, LLC, De). Forward (sense) and reverse (antisense) primers for GAPDH (Entrez ID: 35728), AT_1 receptor (Entrez ID: 24182), AT_2 receptor (Entrez ID: 24180), mas receptor (Entrez ID:404652), ACE1 (Entrez ID:24310), ACE2 (Entrez ID:302668), and neprilysin (Entrez ID:24590) were analyzed using real-time PCR Systems (Step One Software version 2.0. Applied Biosystems, CA). The calibrator was the average ΔC_T value of the control group samples. To calculate the relative expression levels in each sample, the C_T value for GAPDH was subtracted from the C_T value of the gene of interest to give a ΔC_T value. The ΔC_T value of the calibrator was then subtracted from each individual sample to give a $\Delta\Delta C_T$ value. This number was then inserted into the formula $2^{-\Delta\Delta C}{}_T$ to give the expression level relative to the calibrator.

Membrane fraction isolation.
Thoracic aorta arteries (50-100 mg) were snap-frozen and homogenized in ice-cold homogenizing solution (250 mM sucrose, 50 mM Tris HCl, pH 7.4, 5 mM EDTA, and 15 uM, phenylmethylsulfonyl fluoride (PMSF) in a glass/glass homogenizer. The homogenate was centrifuged at 100,000 g for 45 min at 4°C. This supernatant was removed, and the pellet was resuspended in one-half the initial amount of homogenization buffer. Protein concentration was determined using the Bradford method (Bio-Rad; Hercules, CA).

Receptor protein expression
Western blot technique was performed using membrane preparations of aortic tissue. Primary AT1 and AT2 antibody (Alomoni, 1:500) was detected with a secondary goat anti-rabbit (1:1000). Bands were normalized by alpha-actin (Santa Cruz, 1:10000).
Tissue was snap-frozen and homogenized in cold homogenization buffer (20.0 mmol/l Tris·HCl with pH 7.4, 137.0 mmol/l NaCl, 10% glycerol, and 1% NP40) containing protease inhibitors. Protein concentrations were determined by standard Bradford assay, and Western blotting on PVDF blots and low-temperature SDS-PAGE were performed as described previously[2, 3]. Two-color immunoblots were analyzed by using polyclonal primary antibodies to AT1 (1:500, Alomoni, Jerusalem, Israel), AT2 (1:500, Alomoni, Jerusalem, Israel), NOS1 (1:250; BD Transduction Laboratories, San Jose, CA), NOS2 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), NOS3 (1:500; BD Transduction Laboratories), and β-actin (A1978, 1:10000; Sigma, St Louis, MO). After incubation with secondary antibody (IRDye 800-conjugated affinity-purified anti-mouse IgG, 1:2,000 or AlexaFluor 680-conjugated affinity-purified anti-rabbit IgG, 1:2,000), the blot was scanned and the intensity of specific bands was quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Densitometric results were reported normalized to β-actin.

NOS enzymatic activity and expression
NOS enzymatic activity and expression were performed as previously described[4]. Briefly, thoracic aortae were dissected as quickly as possible in ice-cold homogenization buffer in the presence of protease inhibitors and frozen in liquid nitrogen. Frozen aortae were then pulverized and homogenized in buffer with the homogenate immediately used in NOS activity measurements and Western blotting. Total NOS activity was defined as the conversion of [^{3}H]arginine to [^{3}H]citrulline inhibited by the nonselective NOS inhibitor *N* -nitro-L-arginine (L-NNA; 1 mM). The activity of each NOS isoform was determined based on the impact of isoform-selective inhibitors (VNIO, NOS1-selective, 1 μM and 1400W, NOS2 selective, 100 nM). NOS3 activity was calculated as total NOS activity minus the sum of NOS1 and NOS2 activities. NOS activity was normalized to protein concentration (Bradford method, BioRad) and expressed as pmoles citrulline/mg protein/30 min.

Immunohistochemical analysis of aortic tissue

Aortic rings were cleared of blood under pentobarbital anesthesia and immersed in 10% buffered formalin overnight at room temperature and paraffin-embedded as previously described[5]. Rings were sectioned at a thickness of 4 μm onto Superfrost plus slides. Endogenous peroxidase was blocked by exposure to 3% H_2O_2 for 15 min followed by washing successively in deionized, distilled H_2O and phosphate-buffered saline for 5 min. Tissues were processed for specific staining by first utilizing Target Retrieval Solution (Dako Corp. Carpinteria, CA) and/or Rodent Decloaker (BioCare Medical in Concord, CA), rinsing in dH_2O and rinsed with phosphate-buffered saline (PBS) for 5 min. Slides were incubated in Rodent Block R (BioCare) for 15 min and rinsed with PBS. Slides were incubated in presence of primary antibody: AT1, 1:4000 (Alomone, Jerusalem, Israel); AT2, 1:2000 (Alomone, Jerusalem, Israel); or NOS3, 1:500 (BD biosciences, CA) in humidity chambers overnight at 4°C. Slides were rinsed with PBS followed by incubation with Mouse on Rat HRP Polymer (BioCare Medical Concord, CA) for 20 min, rinsed in PBS and visualized with diaminobenzamidine (DAKO Corp), rinsed in H_2O and counterstained with Mayers Hematoxilin. The stained sections were viewed with an Olympus BX40 microscope (Olympus America, Melville, NY) on brightfield setting fitted with a digital camera (Olympus DP12, Olympus America, Melville, NY). We performed additional experiments to determine the specificity of the AT1 and AT2 primary antibodies with antigen blockade in the immunohistochemical protocol. Antibody solutions were pre-incubated overnight in the presence and absence of peptide antigen (2 ul antibody, 30 ul peptide of 0.4 mg/ml and 468 ul of PBS), and diluted up to 4000 ml for AT1 and 2000 ml for AT2). Staining was present only in the samples in the absence of the peptide antigen. Staining was also performed in the absence of primary antibody in random samples to corroborate the loss of specific immunoreactivity.

Chronic inhibition of NOS

L-NAME was administered in the drinking water *ad libitum* from week 14-15 (10 mg/kg/day), week 16 (50 mg/kg/day) and week 17 of life (100 mg/kg/day). MAP was monitored by telemetry as previously described[5]. Blood pressure was expressed as a 24hr average at day 0, 7, 14 and 28.

Statistical analysis

Linear and logarithmic data are presented as means ± SE. Basal parameters in table S1 were compared using unpaired Student's t-test. Vascular reactivity data were compared between concentrations by unpaired Student's t-test or one-way ANOVA with Bonferroni's post hoc performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). A value of $p < 0.05$ was considered statistically significant.

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Table S1. Vascular reactivity in aortic rings from control and MS rats. *p<0.05.

A)

Vasorelaxation		
Treatments → Groups ↓	**Ach (% Max Relaxation)**	**SNP (% Max Relaxation)**
Control (n=7)	82.04±5.10	84.25±8.22
MS (n=10)	77.62±5.68	88.75±1.78
Vasoconstriction		
Treatments → Groups ↓	**AngII (% Max Constriction)**	**PE (% Max Constriction)**
Control (n=15)	29.22±5.68	119.79±5.61
MS (n=10)	55.27±4.26*	116.91±8.06

B)

Vasorelaxation		
Treatments → Groups ↓	**Ach (-log EC_{50})**	**SNP (-log EC_{50})**
Control (n=7)	8.12±0.29	7.67±0.27
MS (n=10)	8.09±0.19	7.20±0.25
Vasoconstriction		
Treatments → Groups ↓	**AngII (-log EC_{50})**	**PE (-log EC_{50})**
Control (n=15)	7.86±0.08	7.62±0.05
Control+PD (n=7)	8.4±0.1¥	Not determined
MS (n=10)	7.97±0.17	7.57±0.17
MS+PD (n=7)	8.8±0.3 π	Not determined

¥ p= 0.08 vs. control

π p= 0.25 vs. control

Table S2. Absolute tension in response to cumulative doses of ET-1 and AngII
*p<0.05 vs. control.

	Absolute Tension (mN)			
log [ET-1] → **Groups ↓**	**-12**	**-10**	**-8**	**-6**
control (n=7)	28.35±0.09	28.85±3.75	47.66±4.25	60.47±3.23
MS (n=10)	28.25±0.09	28.67±2.56	52.50±1.10	64.75±2.12
	Absolute Tension (mN)			
log [Ang II] → **Groups ↓**	**-12**	**-10**	**-8**	**-6**
control (n=15)	28.39±0.07	28.48±3.75	32.73±0.86	34.32±1.76
MS (n=10)	28.63±0.17	28.79±0.18	37.30±1.91*	44.53±2.36*

FIGURES

Fig S1

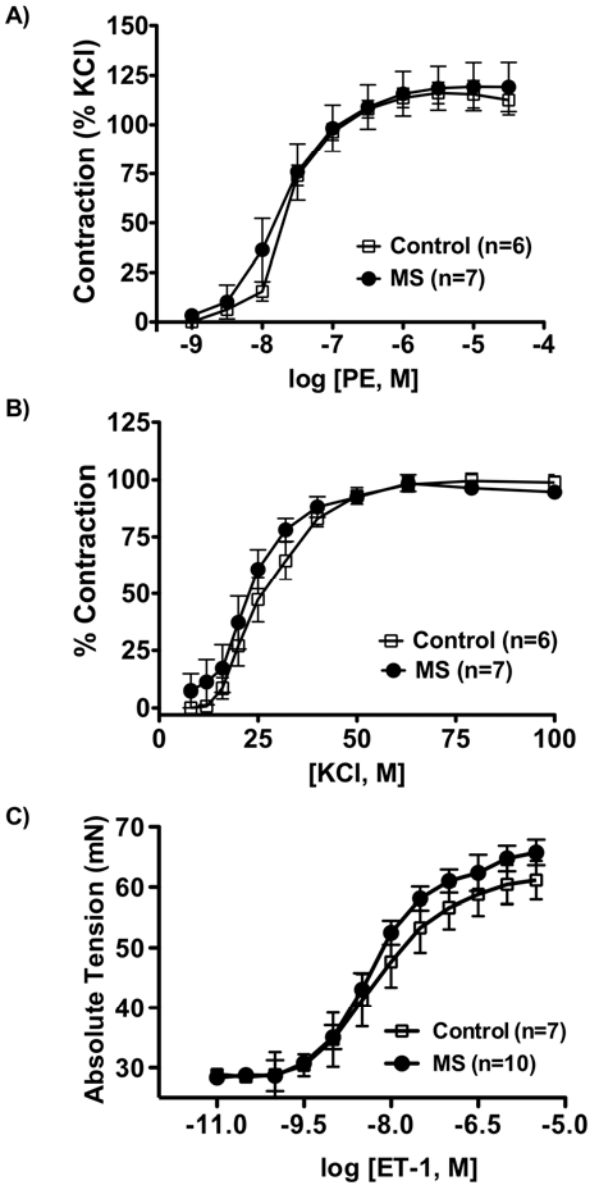


Figure S1. Aortic vasoconstriction to A) PE, B) KCl, or C) ET-1 from MS and control rats.

Fig S2
A)

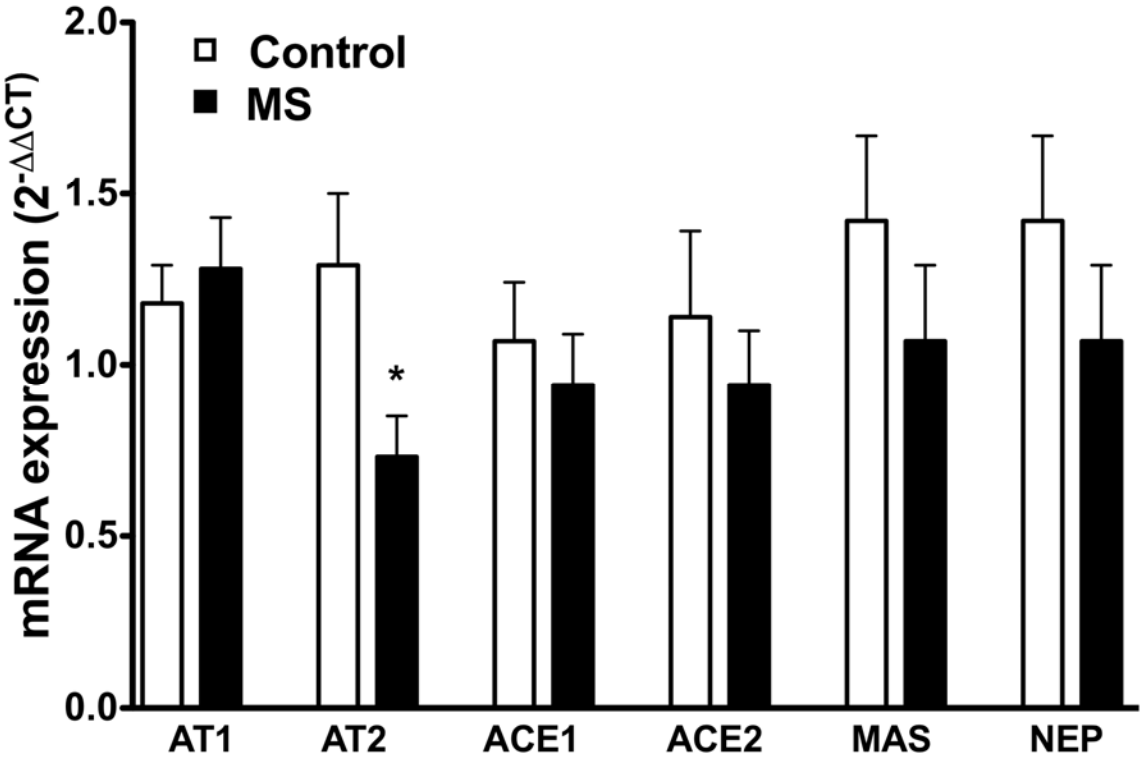


Fig S2. RAS components protein and mRNA expression in aortic tissue. A) RT-PCR analysis show a reduced expression of AT2 receptor ($p<0.05$, n=6-8).

Fig S3

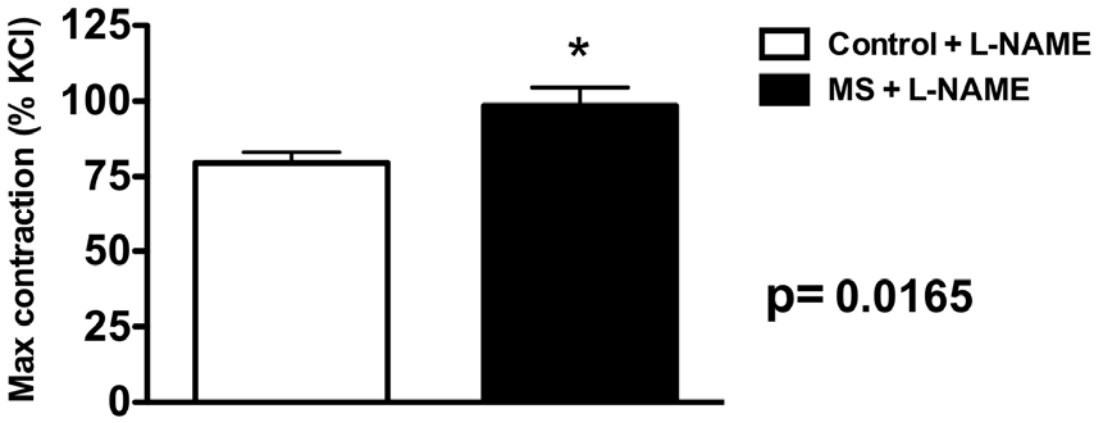


Figure S3. Maximal vasoconstriction in response to AngII in presence of L-NAME in aortic tissue from control (n=7) and MS (n=6) rats. *p<0.05.

Fig S4

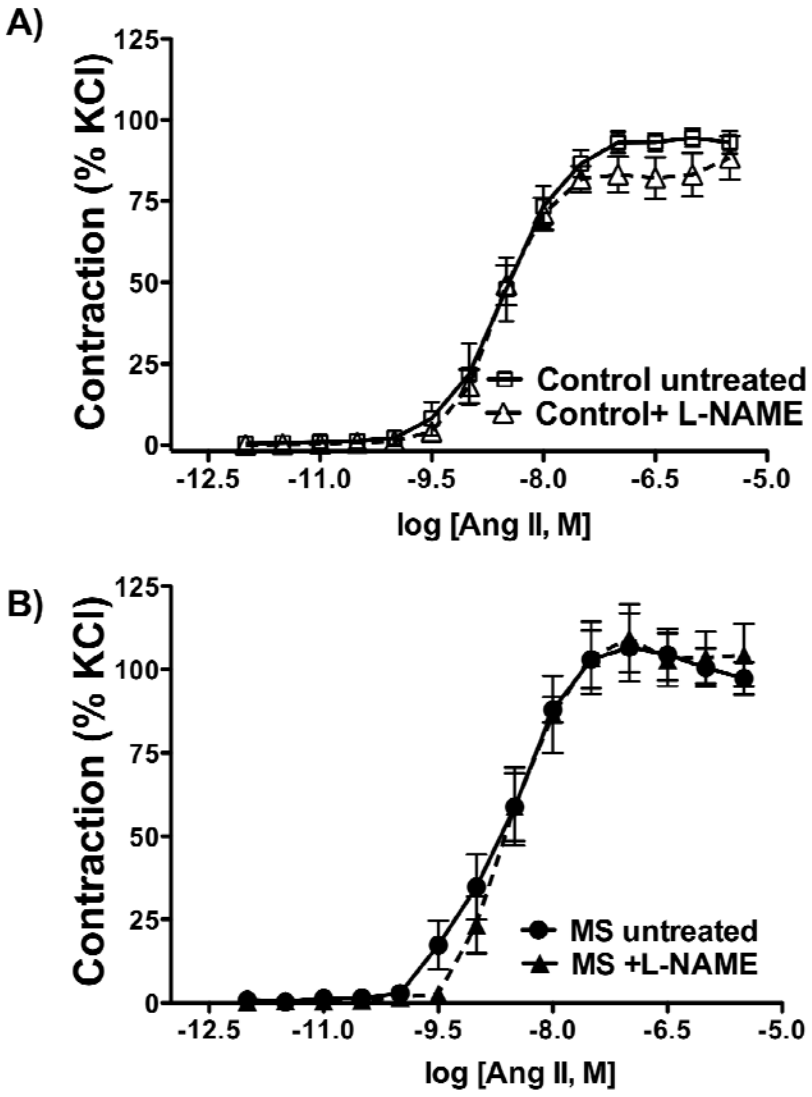
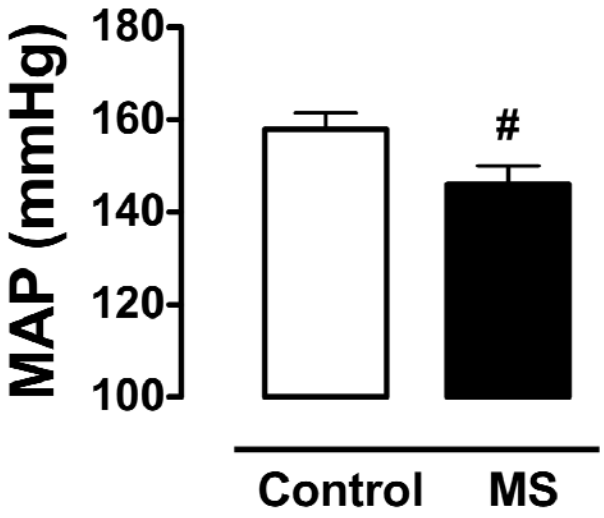


Figure S4. Endothelium-denuded aortic rings from A) control (n=7) and B) MS (n=6) rats in the presence and absence of L-NAME.

Fig S5

A)



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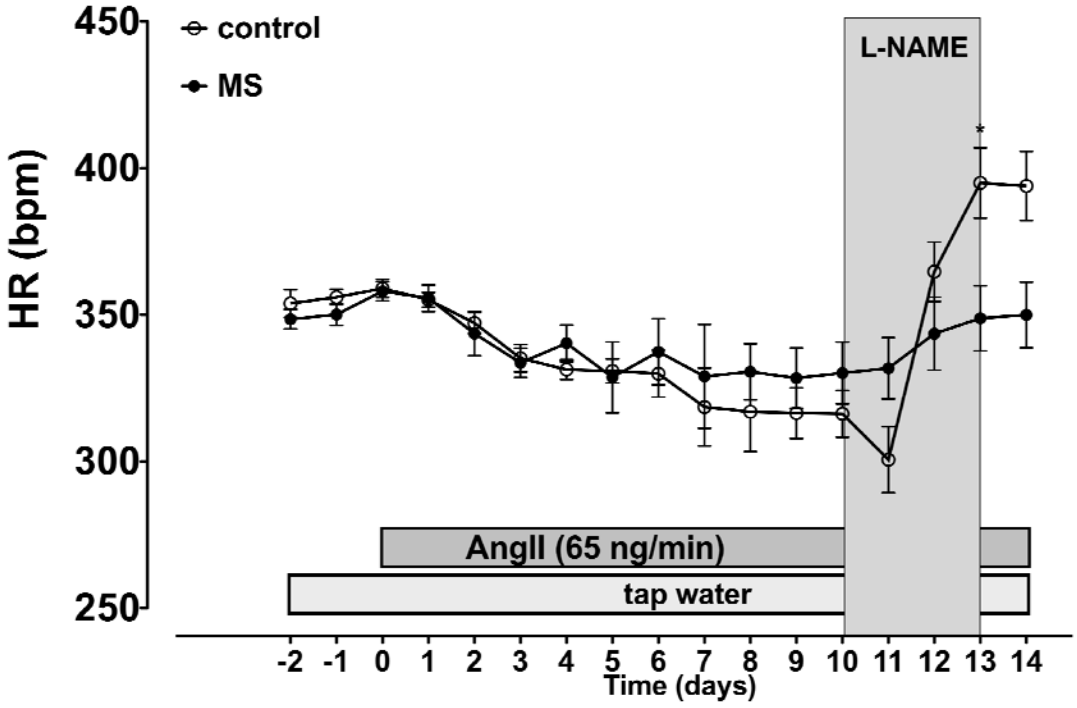
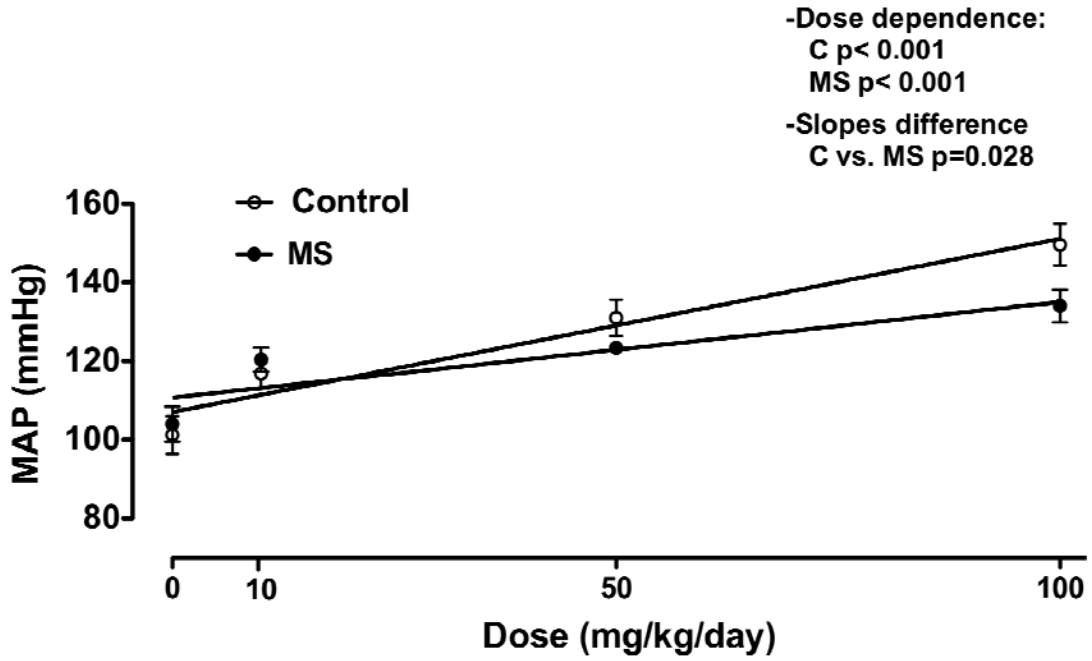


Figure S5. Cardiovascular response in AngII-infused rats treated chronically with L-NAME. A) Total pressor response (72 hr) in AngII-infused rats treated with L-NAME (day 10-13) is greater in control (n=6) than in MS (n=6) rats (#$p<0.05$). B) HR Changes in response to L-NAME administration in control and MS rats (n=6). * $p<0.05$.

Fig S6

A)



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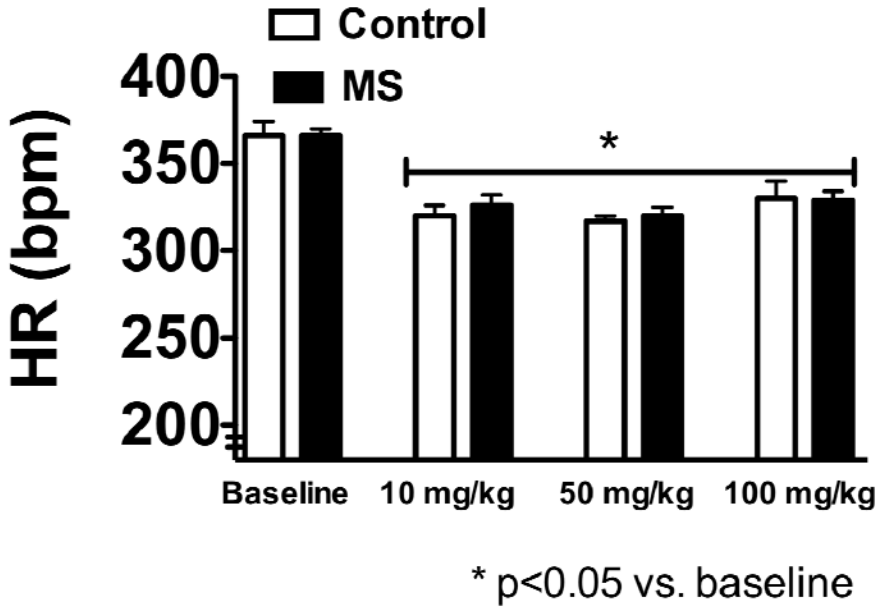

Figure S6. Blood pressure and vascular reactivity in rats treated chronically with L-NAME. A) MAP in response to increasing doses of L-NAME in MS (n=3) and control rats (n=3). B) Chronic NOS inhibition decreased HR similarly in control and MS rats. * p<0.05.

Fig S7

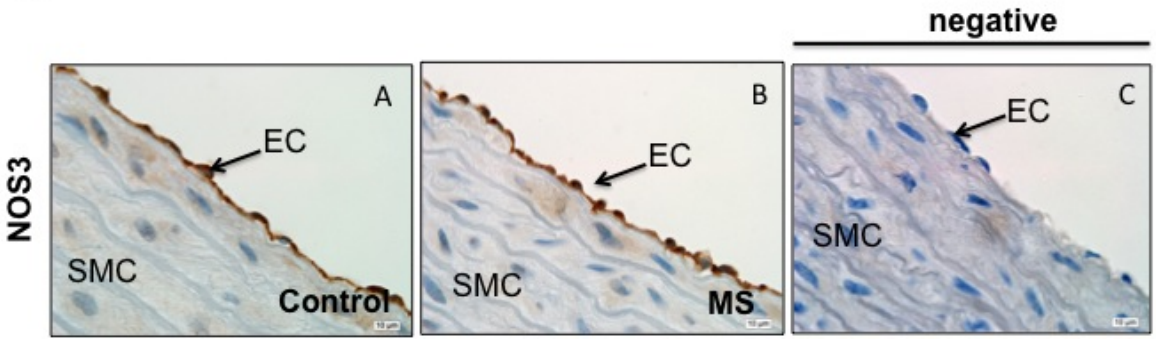

Figure S7. Immunohistochemical analysis of aortic rings (n=6). NOS3 immunolocalizaiton in aorta from A) control rats and B) MS rats. C) Immunostaining in the absence of primary NOS3 antibody.