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

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Abstract

We previously reported that maternal separation (MS) sensitizes adult rats to angiotensin II (AngII)-induced hypertension. The aim of this study was to investigate the vascular reactivity to AngII and the role of renin-angiotensin system components, reactive oxygen species production and nitric oxide synthase (NOS) buffering capacity mediating the exacerbated AngII-induced responses. MS rats were separated from their mothers for 3 hours/day from day 2-14 of life. Controls (C) were non-handled littermates. At 12 weeks of age, aortic AngII-induced constriction was greater from MS rats compared to C ($p<0.05$), moreover, endothelial denudation abolished this difference. The response to other constrictors was unchanged. Angiotensin type 2 receptor (AT_2R) function was reduced in aortic AngII-induced constriction from MS rats compared to C. Angiotensin type 1 receptor (AT_1R) function was similarly abolished in both groups. However, protein expression of AT_1 and AT_2 receptors were similar in aortic rings from MS and C rats. Pre-incubation with superoxide inhibitor or scavenger attenuated the AngII-induced vasoconstriction in C, but not in MS rats. However, acute pre-incubation with a NOS inhibitor enhanced aortic AngII-induced constriction in aorta from C rats, but this effect was significantly reduced in MS rats compared to C. Accordingly, a further increase in AngII-induced hypertension due to chronic NOS inhibition (days 10-13) was blunted in MS rats compared to C. Similar NOS expression and activity was observed in C and MS rats. In conclusion, MS induces a phenotype with reduced endothelial NOS buffering capacity leading to dysfunctional endothelial AngII-mediated signaling and sensitization to AngII-induced vasoconstriction.

Keywords

maternal separation; vascular reactivity; AT_2 receptor; NOS buffering; angiotensin II

Within the last decade, a growing body of evidence links adverse behavioral or environmental stress during early life with chronic diseases in adulthood. The quality of

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Disclosures None

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early life, prenatal and/or postnatal, can influence mechanism(s) of metabolic and mental disorders as well as cardiovascular disease in both humans [1-3] and animal models[4-6]. Significant alteration in maternal care is considered a stressful experience associated with modifications in the endocrine response[7, 8] leading to enhanced acute stress-related responses in the offspring later in life. Maternal separation (MS) is a widely used model of early life behavioral stress in rodents (for review see [9]). Several studies reported that exposure to MS permanently alters the "programming" of the CNS, endocrine and immune systems[10-12].

Recently, we reported that MS sensitizes adult rats to angiotensin II (AngII)-induced hypertension and vascular inflammation[13]. These compelling experimental data led us to reason that early life stress, specifically MS, may induce defects in the renin-angiotensin system (RAS).

The RAS is comprised of classical and non-classical pathways. Classically, angiotensin-converting enzyme (ACE1) metabolizes AngI to AngII, while ACE2 and neprilysin, components of the non-classical pathway, metabolize various other angiotensin peptides[14]. AngII type 1 (AT_1; AT_{1a} and AT_{1b} in the rat) receptor activation mediates vasoconstriction, vascular hypertrophy and remodeling, promotes inflammatory cell activation, as well as sodium and water retention[15, 16]. It is well-established that activation of NADPH oxidase and production of reactive oxygen species (ROS) is a major AT_1-mediated pathway in many cell types [17]. AngII type 2 (AT_2) receptor activation is generally considered to be a counter-regulatory receptor to AT_1 receptor activation [18]. Both AT_1 [19, 20] and AT_2 [21] receptors stimulate NO synthase (NOS) activity and NO production, which blunts the vasoconstrictor actions of the AT_1 receptor [19].

We hypothesized that MS induces a dysfunctional vascular phenotype, specifically an imbalance in AT_1 and AT_2 receptor activation that may exacerbate ROS production and/or reduce the NOS buffering capacity ultimately leading to vascular dysfunction and promoting cardiovascular disease. ROS are linked to the mechanism underlying reduced NOS buffering capacity, given that superoxide can react with NO and diminish NO bioavailability[22, 23]. Thus, the aim of this study is to investigate AngII-mediated vascular reactivity and the role of the endothelium, RAS components, ROS production and NOS buffering capacity mediating the exacerbated AngII-induced responses. Receptor mediated mechanism(s) as well as expression and localization of aortic RAS pathway components in MS and control rats will also be determined. In addition, we designed studies to elucidate the role of NOS buffering capacity *ex vivo* and *in vivo* mediating AngII-induced vasoconstrictor responses by using a NOS non-selective inhibitor, L-NAME.

Methods

Detailed methods are provided in the online Data Supplement (http://hyper.ahajournals.org).

Animal model

All animal use protocols received prior approval by the institutional animal care and use committee at the Georgia Health Sciences University. Using Wistar Kyoto (WKY) rats, maternal separation protocol was performed as previously described[13]. Briefly, approximately half of the male pups were separated from their mothers (MS) and littermates by transferring the pups to a clean cage in an incubator (30±1° C) for 3 hours from day 2 to day 14 of life. Normally-reared non-handled littermates served as the control group. Each experimental group of rats originated from a minimum of 3 different litters.

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Aortic vascular reactivity

Thoracic aortic tissue was isolated as previously described[24]. Cumulative concentration-response curves (CRC) were generated for AngII, endothelin (ET-1, from 1×10^{-12} to 3×10^{-6} M), phenylephrine (PE, from 1×10^{-9} to 3×10^{-5} M), and KCl (4.7 mM to 100 mM). The following compounds were utilized to pre-incubate (30 min) aortic rings prior to AngII-induced constriction: candesartan (AT_1 receptor antagonist; 10^{-5} M) and PD123,319 (AT_2 receptor antagonist; 10^{-6} M), L-NAME (NOS inhibitor; 10^{-4} M), apocynin (NADPH oxidase inhibitor; 3×10^{-4} M) and PEG-SOD (cell permeable superoxide dismutase; 1000 U/ml).

Protein expression

Whole tissue homogenates (NOS isoforms) and membrane preparations (AT receptors, see supplement for detailed protocol) were obtained from aortic tissue. Equal protein amounts were loaded and run in SDS-PAGE analysis followed by immunoblotting on PVDF blots. Two-color immunoblots were analyzed using polyclonal primary antibodies to AT_1, AT_2, NOS1, NOS2 and NOS3 and detected by immunofluorescence. Densitometric results were reported normalized to β-actin.

NOS enzymatic activity

NOS enzymatic activity and expression were performed as previously described[25]. NOS activity was normalized to total protein concentration (Bradford method, BioRad) and expressed as pmoles citrulline/mg protein/30 min.

Immunohistochemical analysis of aortic tissue

Rats were anesthetized with pentobarbital and aortic rings were removed, rinsed in saline, and immersed in 10% buffered formalin overnight at room temperature and paraffin-embedded as previously described[13]. Following staining, 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).

Chronic inhibition of NOS

L-NAME was administered in the drinking water 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[13]. Blood pressure was expressed as a 24hr average at baseline, day 7, 14 and 28. A separate group of telemetry-implanted rats were infused with AngII. On days 10-13 of AngII infusion, rats were treated with L-NAME in drinking water (100 mg/kg/day).

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.

Results

Aortic vasoconstriction

Maximal AngII-induced vasoconstriction was significantly greater in aortic rings from MS than control rats (figure 1A; table S1). No differences in EC_{50} were observed between MS and control rats (table S1). Absolute tension in response to AngII (table S2) was also significantly higher in aortic tissue of MS rats (44.5±2.4 mN) compared to control rats

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(34.3±1.8 mN, $p<0.05$). Denudation of the endothelium revealed that AngII-induced a similar response in aortic rings from MS and control rats (figure 1B).

Pre-incubation with an AT_1 receptor blocker (candesartan; 10 μM) abolished the AngII-induced constriction in endothelium-intact aorta from both MS and control rats (figure 2A). Selective AT_2 receptor blockade (PD123,319; 1 μM) significantly increased the AngII-induced constriction in aortic rings from control rats (figure 2B), while pre-incubation with the AT_2 receptor blocker did not display a functional change in vasoconstriction in aortic rings of MS rats. EC_{50} in the presence and absence of receptor blockers was similar in MS and control rats (table S1).

To elucidate whether the enhanced AngII-mediated constriction is specific for AngII, we analyzed the vasoconstrictor responses to phenylephrine (PE), potassium chloride (KCl) and endothelin (ET-1) in aortic rings from MS and control rats (figure S1). No significant differences between groups were found in % max contraction or $-\log EC_{50}$ (table S1 and S2).

Aortic AT_1 and AT_2 receptor expression and immunolocalization

Protein expression of AT_1 (43 kDa) and AT_2 (band at 50 kDa) receptors were similar in membrane preparations of aortic tissue of control and MS rats (figure 3A). Immunolocalization of AT_1 (figure 3B and 3C) and AT_2 (figure 3E and 3F) receptors are similar in the endothelium and smooth muscle from control and MS rats, respectively. Specificity of the AT_1 and AT_2 receptor immunoreactivity was verified with antigen blockade for immunohistological analysis (figure 3D and 3G). Aortic mRNA expression of RAS components was assessed by quantitative real-time PCR from MS and control rats (figure S2). AT_1 receptor, mas receptor, ACE1, ACE2, and neprilysin mRNA expression were similar between groups. AT_2 receptor mRNA expression was significantly reduced in aorta from MS rats compared to control rats ($p<0.05$).

Aortic AngII-mediated ROS and NOS signaling during vasoconstriction

Pre-incubation with apocynin (300 μM; NADPH oxidase inhibitor) or PEG-SOD (1000 Units/ml; superoxide scavenger) significantly blunted the AngII-induced vasoconstriction in intact aortic rings from control rats (figure 4A and 4C). Surprisingly, these compounds did not modify the AngII-induced response in aortic rings from MS rats (figure 4B and 4D).

Pre-incubation with the NOS inhibitor (L-NAME; 100 μM) elicited a significantly greater AngII-induced constriction in intact aortic rings compared to untreated aortic rings from control rats (figure 5A). L-NAME treatment also produced an increased AngII-induced constriction in intact aorta from MS rats (figure 5B), although the response was significantly less than the response in control rats (figure 5C). Maximal vasoconstriction in the presence of L-NAME was increased in intact aortic tissue from MS rats compared to control rats (figure S3). The AngII-induced constriction in the presence of L-NAME was similar in endothelium-denuded aortas from control and MS rats (figure S4).

Blood pressure during chronic *in vivo* NOS inhibition

MAP at day 10 of AngII infusion, prior to NOS blockade, was significantly greater in MS rats (142.0±0.9 mmHg) compared to control rats (132.1±2.8 mmHg, $p<0.05$; figure 6A) confirming our previously published results[13]. L-NAME treatment, during AngII infusion, significantly increased MAP within 24 hours in control rats, while MS rats displayed a blunted response (figure 6B). The total pressor response in AngII-infused rats treated with L-NAME for 3 days (days 10-13 during AngII infusion) was significantly greater in control rats (158.3±2.5 mmHg) compared to MS rats (146.4±2.9 mmHg, $p<0.005$) (figure S5). The

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heart rate increase in response to chronic NOS inhibition during AngII infusion was severely attenuated in MS rats (figure S5). Chronic *in vivo* administration of L-NAME (in the absence of AngII infusion) induced a dose-dependent increase in blood pressure in control and MS rats (figure S6). Analysis of the slope revealed significantly attenuated effects of L-NAME on blood pressure in MS rats (figure S6; p=0.028). Conversely, chronic L-NAME administration, in the absence of AngII infusion, induced similar decreases in heart rate in control and MS rats (figure S6).

Vasorelaxation, NOS activity, and NOS expression

Endothelium-dependent and -independent vasorelaxation in aortic rings from MS and control rats were determined by analyzing responses to acetylcholine and sodium nitroprusside. Responses were similar from both groups (figure 7A, 7B and table S1). Total and isoform-specific NOS activity was determined in homogenates of aortic tissue from MS and control rats. No significant differences were observed in total or isoform-specific NOS activity (figure 7C). Aortic NOS1 (155 kDa) and NOS3 (135 kDa) expression by Western blot were found to be similar in both groups of rats (figure 7D). NOS2 expression was below detection (data not shown). In addition, NOS3 expression was determined by immunohistochemical analysis. NOS3 was highly expressed in the aortic endothelium in both MS and control rats (figure S7). Additional staining in the absence of primary antibody served as a negative control and confirmed the absence of immunoreactivity (figure S7).

Discussion

This study revealed that maternal separation, a model of early life stress, induces a dysfunction in endothelial AngII receptor signaling and NOS buffering. Specifically, aortic tissue from MS rats demonstrated a significantly increased AngII-induced constriction compared to control rats that results from a loss in endothelial- and AngII-mediated NO production. In addition, aortic tissue from MS rats showed a loss of functional AT_2 receptor activation, which appears to account for the increased AngII-induced constriction. Furthermore, the blood pressure response to chronic *in vivo* NOS inhibition was significantly attenuated in MS rats indicating that early life stress induces a phenotype with reduced NOS buffering capacity.

Maternal separation is considered a chronic behavioral stress model replicating the adverse environment during childhood in humans[26]. Therefore, maternal separation has been used to study behavioral consequences in stress-related responses, however, very few of these investigations have probed cardiovascular outcomes. Studies employing animal models using glucocorticoid exposure[27, 28] or low protein diet [29] during the prenatal period, demonstrate that these fetal programming paradigms affect the sensitivity of the RAS pathway directly[29, 30]. We recently reported that control and MS rats have similar circulating levels of AngII, Ang1-7, aldosterone and plasma renin activity in baseline conditions, yet MS rats were found to be more sensitive to chronic AngII-infused hypertension and vascular inflammation[13]. The current results, taken together with the previous findings, support our hypothesis that early life stress induces a vascular phenotype that predisposes the adult rat to endothelial dysfunction, a hallmark of cardiovascular disease. Furthermore, this study extends our knowledge of potential RAS-dependent mechanisms focused on the role of the vascular endothelium as a target affected by early life stress.

Both AT_1 and AT_2 receptor activation includes stimulation of the NO pathway, however, specific intracellular signaling pathways and subcellular localizations are yet to be clarified. Endothelial AT_1 receptor activates NO production via stimulation of protein kinase B (Akt) and phosphorylating NOS3[31]. Siragy *et al.*[32] have reported that a chronic infusion of AngII

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failed to increase NO release in AT_2 null mice. In addition, Tsutsumi *et al.*[33] have shown that the over-expression of AT_2 receptors in the vasculature attenuates AngII-induced hypertension. Alterations in the AT_1/AT_2 ratio has been suggested as a possible mechanism leading to increased blood pressure[34, 35]. Our study did not observe any differences in immunolocalization of the AT_1 and AT_2 receptors within the vasculature nor any difference in protein expression of the receptors with a vascular membrane preparation, however, AT_2 mRNA expression was reduced in MS rats as well as reduced AT_2 receptor function. Given that we did not observe differences in the wall thickness of control and MS aortae [13] and that the dysfunction is localized to the endothelium, we have reasoned that the increased AngII reactivity may be intrinsic to angiotensin receptor signaling rather than expression. Further studies with isolated endothelial cells are necessary to elucidate the specific nature of the dysfunction in the AngII signaling pathway.

The role of reactive oxygen species (ROS) mediating AngII actions in the vasculature has been extensively reported to be via NADPH oxidase activation of superoxide[36, 37]. Accordingly, we probed this pathway and observed that a cell-permeable superoxide scavenger and an NADPH oxidase inhibitor significantly reduced the aortic AngII-induced constriction in control rats. To our surprise, neither PEG-SOD nor apocynin modulated the aortic AngII response in MS rats. Schuijt *et al.* reported that acute AngII stimulation in human and porcine arteries is not mediated via superoxide generated through NADPH oxidase and/or xanthine oxidase activation, but rather results in the generation of the vasodilator H_2O_2[38]. At this point, it is unclear whether AngII activates other ROS dependent pathways or not and further experiments are planned to determine the status of these pathways. We propose that maternal separation induces a vascular phenotype that uncouples AngII-mediated activation of NADPH oxidase-mediated superoxide.

In vivo, NOS inhibition during chronic AngII infusion and in the absence of AngII infusion revealed a reduced sensitivity to increase blood pressure in MS rats compared to control rats. L-NAME induces a dramatic increase in blood pressure in control rats especially during chronic AngII infusion, but elicited a minimal change in MS rats. In addition, we observed that control rats displayed tachycardia as a consequence of NOS inhibition, whereas the heart rate was blunted in the MS rats in response to L-NAME. These data further support reduced NOS buffering capacity and suggests differences in the NOS pathway in cardiac function in MS rats. Others have reported that rats treated chronically with L-NAME develop hypertension [39, 40]. Noteworthy, most of the reports suggest that NOS inhibition in the central nervous system may increase the sympathetic activity and vagal suppression as well[41], which could be responsible, at least in part, for the increase in arterial pressure occurring in the L-NAME hypertensive model [40, 41]. Thus, further investigation is necessary to determine the role that early life stress exerts on the brain, kidneys, and heart in the regulation of long-term blood pressure.

Classically, endothelial dysfunction has been defined as blunted acetylcholine-induced vasorelaxation. Surprisingly, maternal separation did not influence endothelium-dependent or -independent vasorelaxation pathways, NOS activity, NOS expression, or NOS immunolocalization. Interestingly, we compared AngII-induced maximal constriction in the presence of L-NAME and found that the vasoconstriction is higher in MS than in control rats. Therefore, MS rats display an increased AngII reactivity regardless of the status of NOS. These data suggest that early life stress mediates a dysfunctional vasoconstriction rather than vasorelaxation. Thus, the endothelial AT_1 and AT_2 intracellular signaling pathways require further investigation, specifically, regarding the role of NOS. Taken together, our findings point to dysfunctional AngII signaling to NOS during the contractile response as a mechanism by which early life stress induces permanent changes in the adult phenotype increasing the risk to develop cardiovascular disease. Future research will test the

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hypothesis that increased AngII vasoconstriction in MS rats is mediated through reduced endothelial AT/NOS molecular signaling.

Perspectives

There is a consensus that adverse environment during early life programs biological consequences associated with stress and inflammatory response mechanisms in adulthood [42]. Indeed, this study distinguishes the impact of adverse early life environmental stressors on vascular function. New mechanistic strategies for the study of long-term consequences of early life stress in the development of cardiovascular disease are required.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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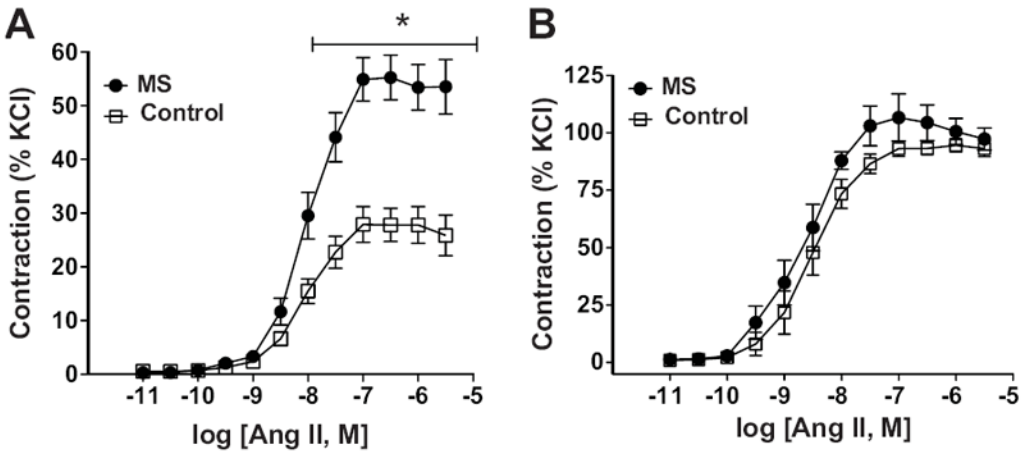

Figure 1.

Vascular reactivity in intact thoracic aortic rings. A) Cumulative concentration-response curves of AngII from MS (n=17) and control (n=17) rats. B) AngII-induced constriction in endothelium denuded rings of MS (n=6) and control (n=6) rats. * $p < 0.05$.

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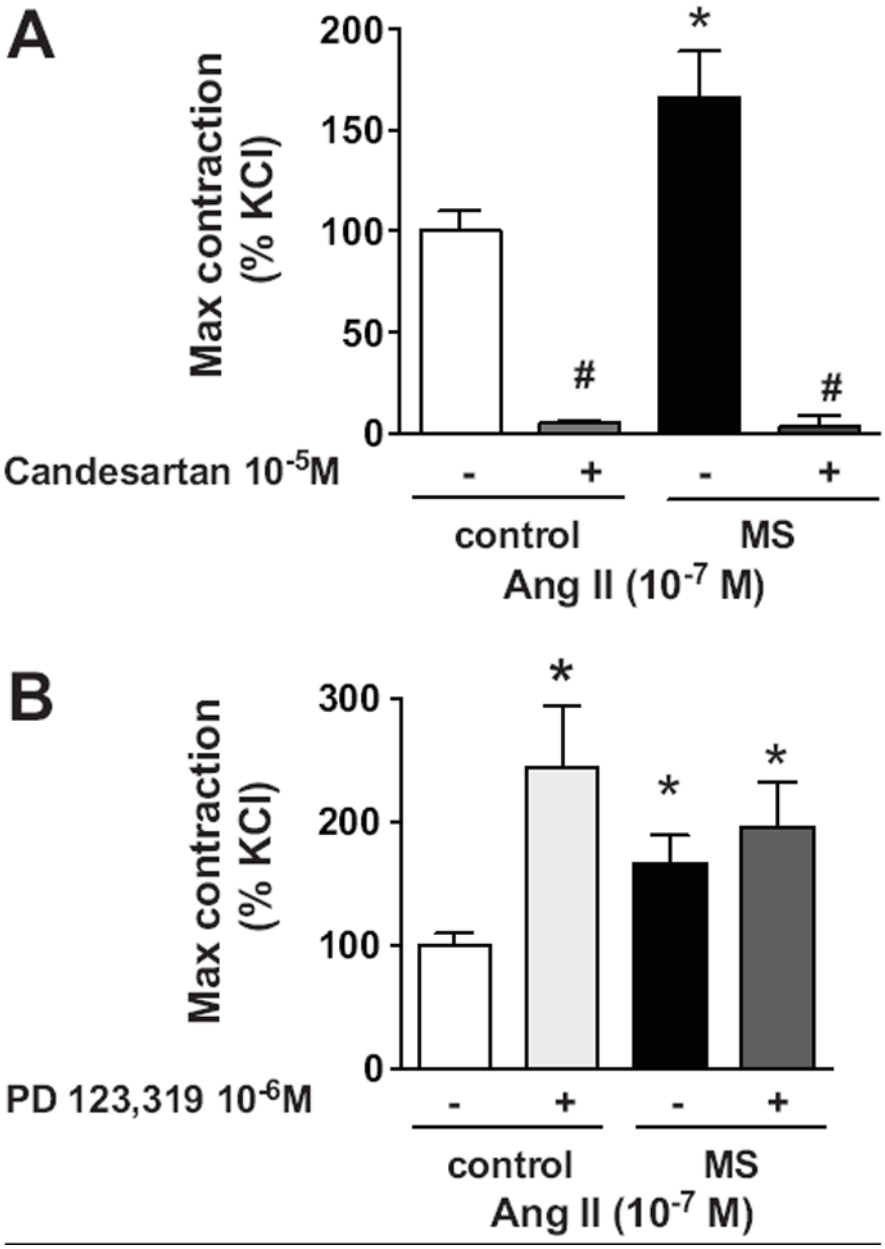

Figure 2.

AngII receptor pharmacological blockade. A) Pre-incubation with candesartan (specific AT_1 receptor blocker, 10^{-5} M) blunted AngII-induced constriction in MS (n=4) and control (n=4) rats. B) Pre-incubation with PD123,319 (specific AT_2 receptor blocker, 10^{-6} M) increased the response to AngII in control rats (n=7), but had no effect in MS rats (n= 7). Data are expressed in percentage of increase from the maximal response to AngII 10^{-7} M. Values from the control group represent the baseline (100%). * $p<0.05$ vs. control.

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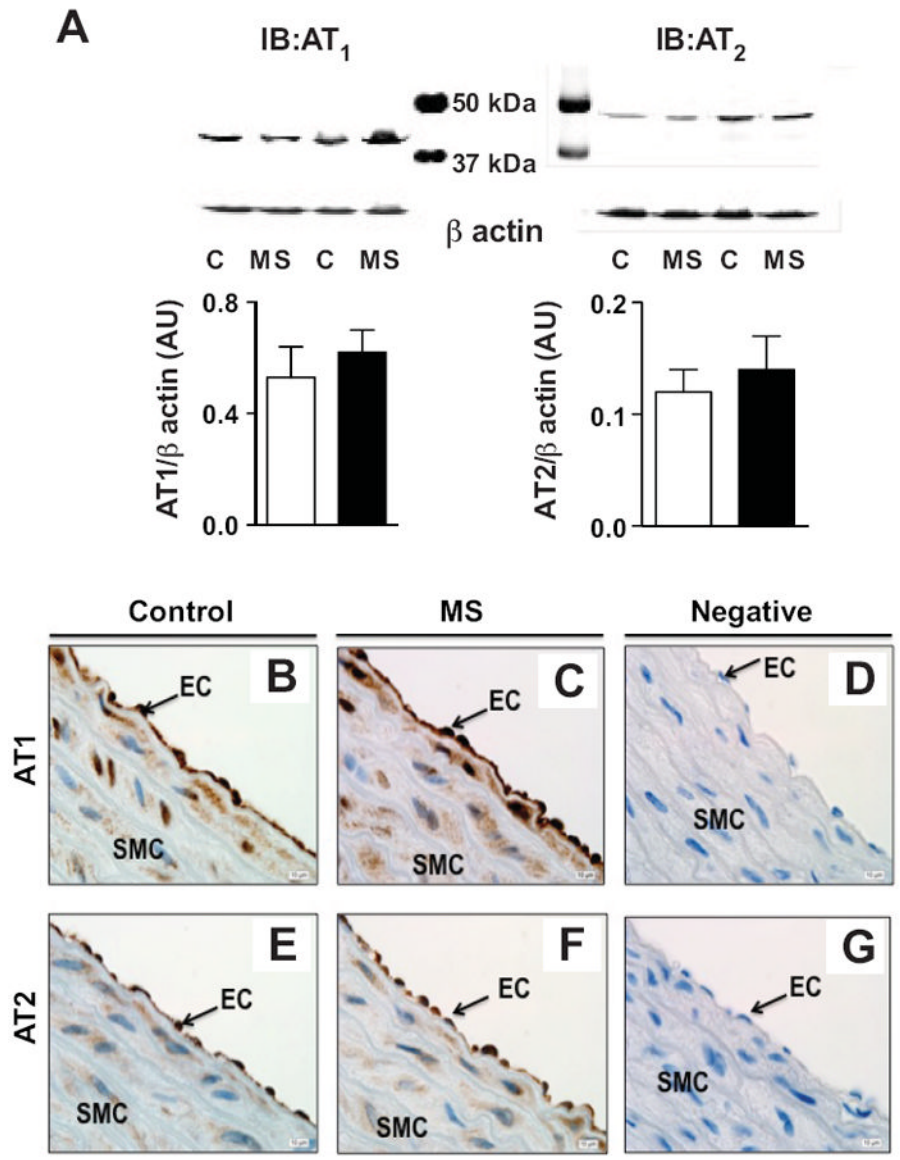

Figure 3.

Expression and localization of AT_1 and AT_2 receptor. A) Representative Western blot of AT_1 and AT_2 protein expression in aortic tissue of control and MS rats with densitometric analysis (n=5-6). Representative immunolocalization of AT_1 receptor (B and C) and AT_2 receptor (E and F) in control and MS aortae. Negative control was assessed by antigen blockade (D and F). Magnification=100X.

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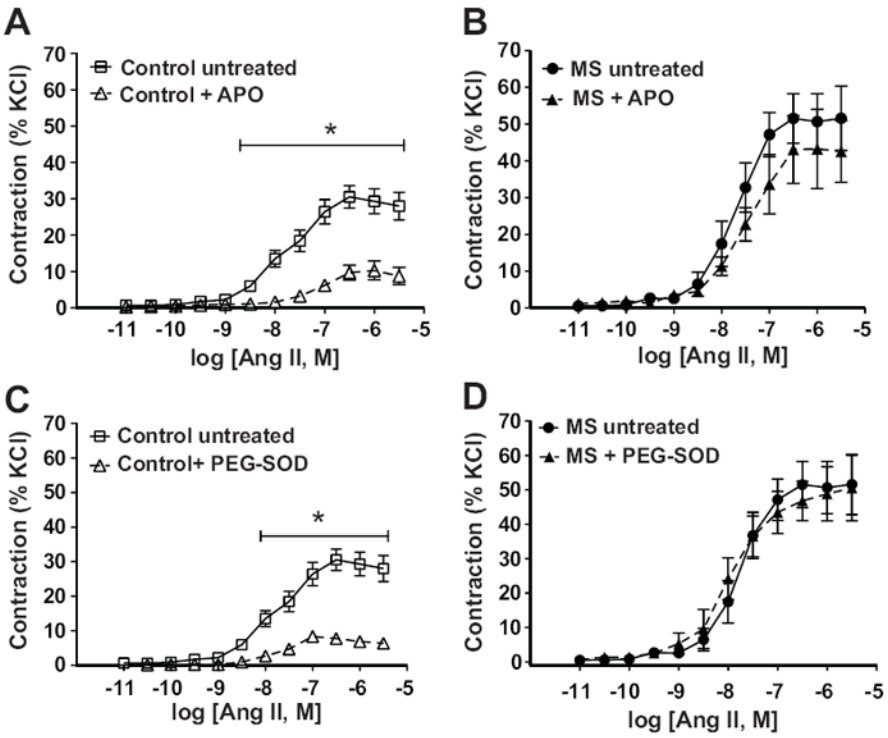

Figure 4.

Participation of ROS in AngII-induced aortic constriction. A) AngII-induced aortic constriction in control rats (n=12) was reduced after pre-incubation with apocynin (300uM, n=7), B) MS rats showed similar AngII-induced constriction in the presence (n=5) or absence (n=12) of apocynin. C) AngII-induced aortic constriction in control rats (n=12) was reduced after pre-incubation with PEG-SOD (1000 Units/mL, n=6), D) MS rats (n=12) showed similar AngII-induced constriction using PEG-SOD. * $p<0.05$ vs. untreated.

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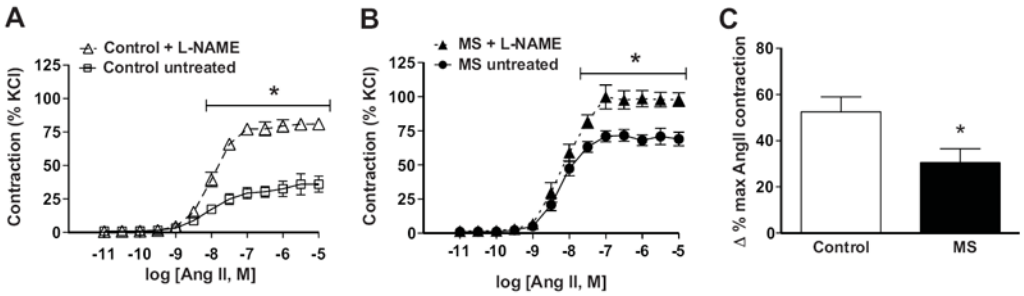

Figure 5.

AngII-induced aortic constriction during NOS blockade. Pre-incubation of intact aortic rings with a NOS inhibitor (L-NAME, 100uM) enhanced AngII-induced constriction in both A) control (n=7) and B) MS (n=6) rats. C) The increase in the percentage of contraction in the L-NAME-treated rings was significantly less in MS compared to control rats. * $p<0.05$ vs. control.

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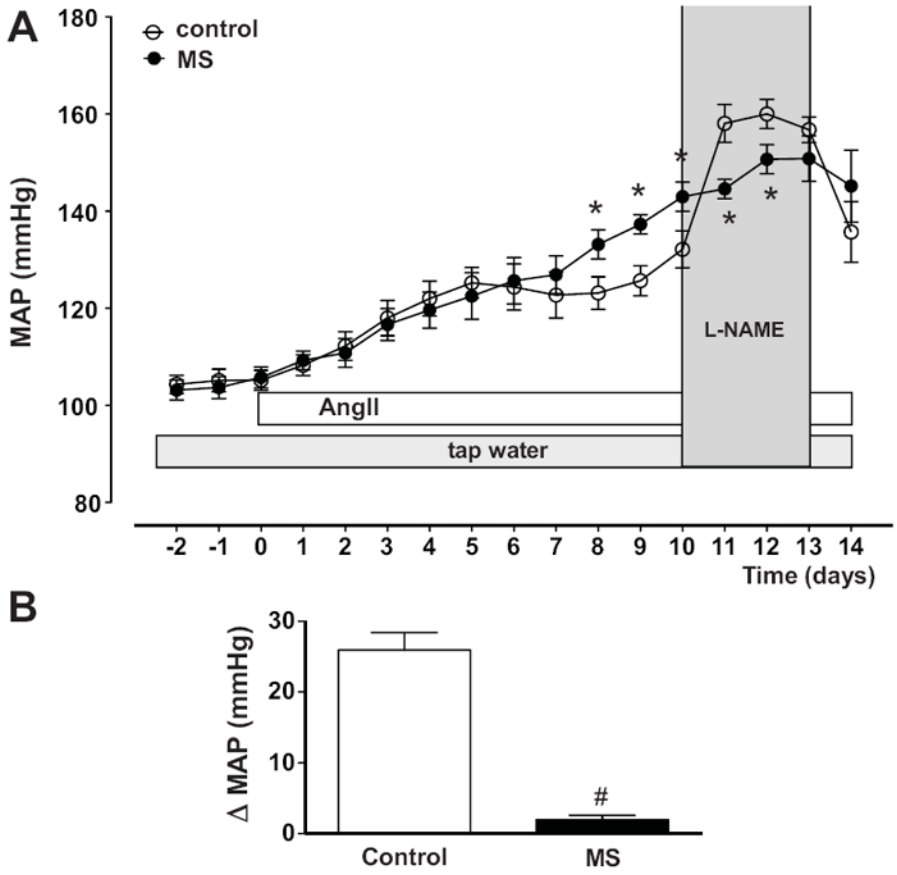

Figure 6.

MAP responses to chronic L-NAME treatment during AngII infusion. MS rats (n=6) have exaggerated MAP to chronic AngII infusion and a blunted pressor response to chronic L-NAME compared to control rats (n=6). A) MAP expressed as 24 hr average. B) Change in the MAP in response to L-NAME treatment after 24 hours. * $p<0.05$ vs. AngII-infused control rats, # $p<0.05$ vs. AngII-infused+L-NAME control rats.

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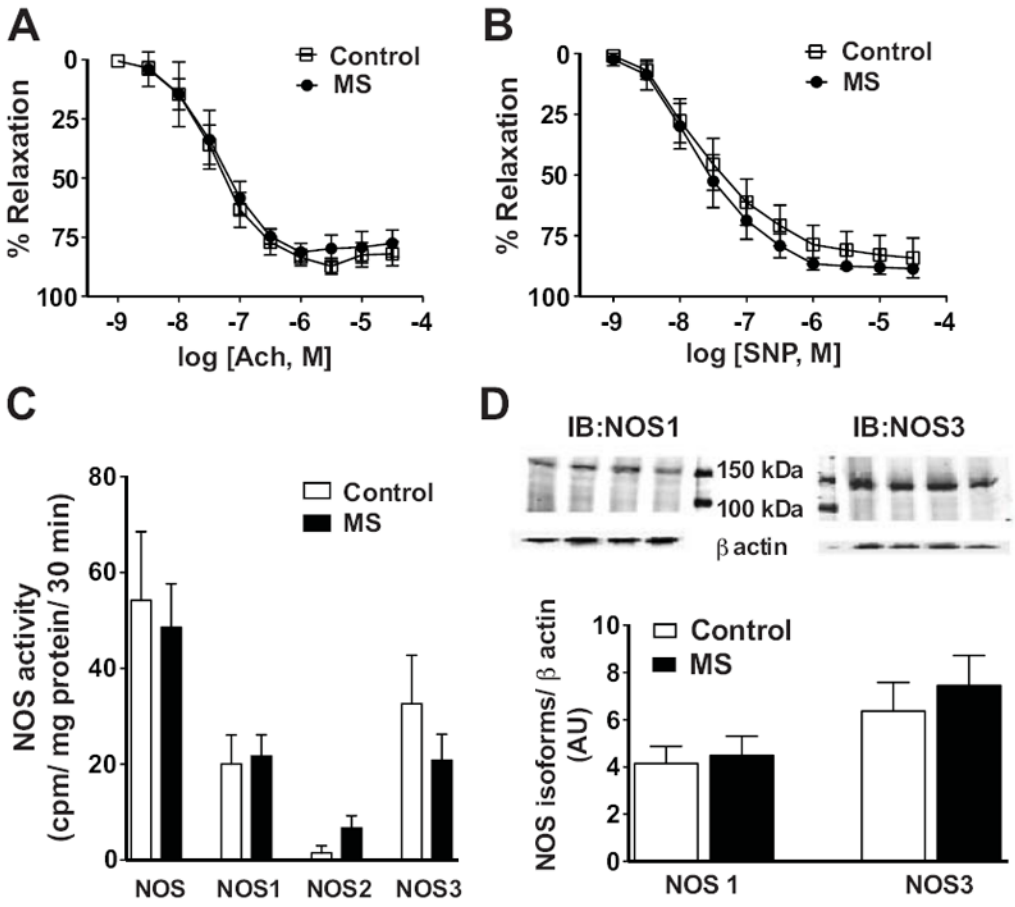

Figure 7.

Endothelial-dependent and –independent vasorelaxation of aortic rings. A) ACh and B) SNP-induced relaxation were similar in control (n=5) and MS (n=9) rats. C) Total NOS and NOS isoform enzymatic activity were similar in MS (n=7) and control (n=7) rats. D) Representative Western blots for NOS1 and NOS3 from aortic tissue of MS and control rats (top) and densitometric analysis of NOS1 and NOS3 immunoreactivity revealed similar expression in aortic tissue between MS and control rats (bottom, n=4).