Supplementary Information

Early life stress sensitizes rats to angiotensin II-induced hypertension and vascular inflammation in adult life

Analia S. Loria, David M. Pollock and Jennifer S. Pollock

Vascular Biology Center, Medical College of Georgia, Augusta, GA 30912

Correspondence to:

Jennifer S. Pollock, PhD

Vascular Biology Center, CB 3213

Medical College of Georgia.

1459 Laney Walker Blvd., Augusta, GA 30912

Telephone: 706-721-8514; Fax: 706-721-9799; E-mail: jpollock@mcg.edu

Detailed methods

Animals

Breeding pairs of Wistar Kyoto rats (WKY, 12-14 weeks old) were housed under standard conditions in the animal care facility at the Medical College of Georgia prior to use. All rats were housed in a temperature controlled (21±1°C) environment with 12:12 hr light cycle in the animal care facility. Rats had free access to water and normal rat chow (Harlan Teklad Rodent Diet, WI). All protocols received approval by the association for the Assessment and Accreditation of Laboratory Animal Care and Use Committee of the Medical College of Georgia.

Plasma Assays

Glycemia in fasted and randomized sample was determined in a peripheral blood sample (Accu-Check, Roche Diagnostics). Plasma samples were collected in pre-chilled tubes using EDTA 7.5% as anti-coagulant. Plasma insulin was determined using an enzyme immunoassay (SPI-BIO, Bertin technologies, France). Plasma concentrations of Ang II and Ang-(1–7) were determined by radioimmunoassay from blood collected into chilled tubes containing a mixture of 25 mmol/L ethylene-diamine-tetraacetic acid (Sigma Chemical Co., St. Louis, Mo), 0.44 mmol/L 1,20-orthophenanthrolene monohydrate, 1 mmol/L Na+ para-chloromercuribenzoate, and 3 µmol/L of WFML (rat renin inhibitor: acetyl-His-Pro-Phe-Val-Statine-Leu-Phe) (1). PRA was measured by radioimmunoassay (GammaCoat 125I Plasma Renin Activity Radioimmunoassay Kit; DiaSorin, Stillwater, MN).

Osmotic mini-pump and telemetry transmitter implantation

Rats were implanted with telemetry transmitters at 9-10 weeks old (Data Sciences, Inc., St. Paul, MN). Mean arterial pressure, heart rate and activity were continuously recorded throughout the study using the Dataquest ART Acquisition program (Data Sciences International, St. Paul, MN). After recovery (7-10 days) and a baseline period (7-10 days), rats were anesthetized with isoflurane (2% in O_2 at 1 L/min) and shaved in the interscapular region. Osmotic mini-pumps (model 2002 for 14-day infusion; Alzet, Palo Alto, CA) were implanted subcutaneously under sterile conditions according to the manufacturer's instructions preceeding implantation to assure immediate subcutaneous delivery of ang II.

Immunohistochemical analysis

Briefly, aortic rings were collected in formalin, and sectioned at a thickness of 4 µm onto Superfrost plus slides. Anti-CD68 (ED-1) antibody was used for staining monocytes/macrophages (1:100, Serotec, Kidlington, Oxford, UK) and anti-CD3 antibody (1:2000), Santa Cruz Biotechnology, Santa Cruz, CA) for T cell staining. Slides were incubated overnight in the absence (negative control) or presence of primary antibody in humidity chambers at 4°C overnight, followed by incubation for 30 min with peroxidase-conjugated anti-IgG (Serotec) for CD68 and ImmunoCruz Staining System for CD3 (Santa Cruz) at room temperature. Staining detection was performed with diaminobenzamidine (DAB) substrate kit for peroxidase (DakoCytomation), counterstained with Mayers hematoxylin, and coverslipped.

Image analysis of Immunohistochemical and structural parameters

The stained sections were viewed with an Olympus BX40 microscope (OlympusAmerica, Melville, NY) on bright-field setting fitted with a digital camera (Olympus DP70; Olympus America). For quantification of ED-1, the appropriate software (DPController, Olympus Optical) was used to convert the image. For each slide, rings were viewed at x40 magnification, with CD68 and CD3-positive cells counted in a blinded manner. Using the same system, areas and diameters were determined in 2 rings from each rat and averaged. Areas were analyzed using MetaMorph software (Meta Imaging Series, Molecular Devices, PA).

Reference

1. Ishiyama Y, Gallagher PE, Averill DB, Tallant EA, Brosnihan KB, Ferrario CM. Upregulation of angiotensin-converting enzyme 2 after myocardial infarction by blockade of angiotensin II receptors. *Hypertension.* 2004; 43:970-976.

Table S1. MAP (A) and HR (B) in response to an i.v. bolus of increasing doses of ang II in the presence and absence of chlorisondamine in anesthetized control (C) and maternally separated (MS) rats, n=5.

A) Mean Arterial Pressure (MAP), mmHg

	Ang II (µg/kg)				
Rat	**0**	**0.04**	**0.08**	**0.16**	**0.32**
C	103±5	114±2	117±2	132±5	160±4
MS	87±6	105±5	105±5	120±4	147±6
	Chlorisondamine (5 mg/kg) + Ang II (µg/kg)				
C	73±4	87±3	95±4	127±7	154±2
MS	64±1	79±4	85±3	122±3	152±4

B) Heart Rate (HR), bpm

	Ang II (µg/kg)				
Rat	**0**	**0.04**	**0.08**	**0.16**	**0.32**
C	317±5	322±11	330±5	310±5	303±15
MS	314±7	312±8	310±11	311±7	315±7
	Chlorisondamine (5 mg/kg) + Ang II (µg/kg)				
C	309±8	325±8	321±7	321±9	291±21
MS	316±7	321±13	314±15	327±8	342±5

Table S2. Aortic tissue morphology in chronic ang II-infused control (C) and maternally separated (MS) rats, n=4. * p<0.05 vs. vehicle

Tissue Morphology	**C**		**MS**	
	vehicle	**AngII-infused**	**vehicle**	**AngII-infused**
Thickness, μm	105.45±3.37	133.13 ±4.34*	100.11 ±3.37	122.69 ±3.16*
Aorta External Diameter, mm	1.64 ±0.01	1.79 ±0.02*	1.65 ±0.02	1.76 ±0.01*
Wall Area, x 10000 μm^2	50.42 ±0.9	66.45 ±3.4*	54.33 ±1.6	68.0 ±0.3*
Cell density, nuclei per 100 μm^2	21.34 ±1.02	20.58 ±1.27	21.83 ±0.91	20.29 ±0.60

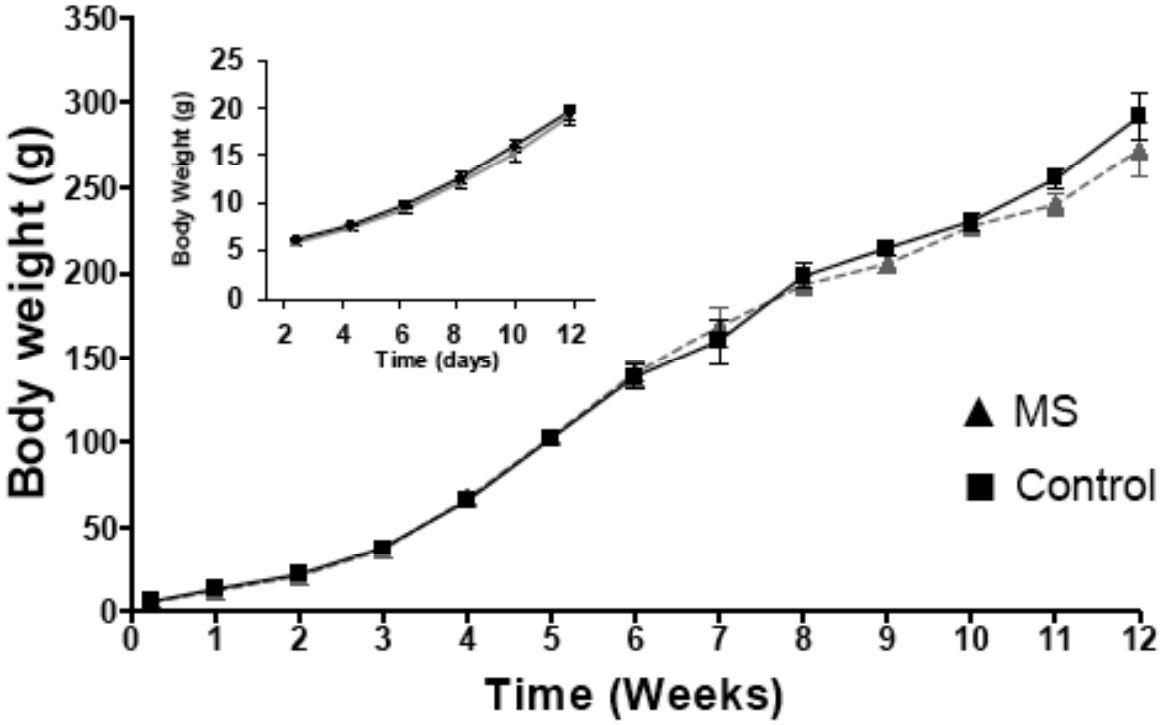

Figure S1. Body weight in male WKY rats from the postnatal day 2 until day 12 (inset) as well as until the age of the experiments (12 weeks old). There are no significant differences between maternally separated, MS (▲) and control, C (■) rats. N= 9-16.

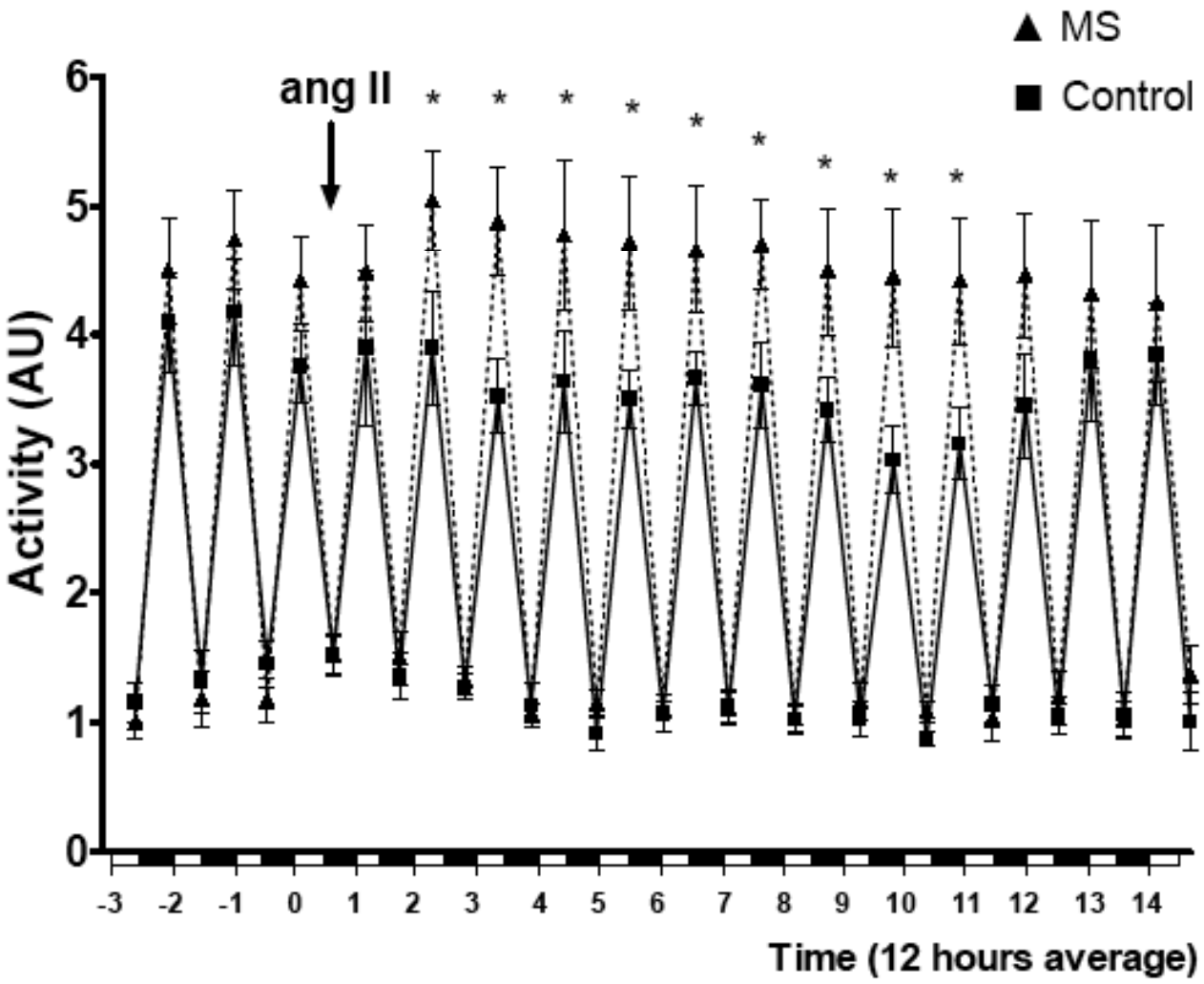

Figure S2. Locomotor activity expressed in 12 hour average in control, C (■, solid line, n=4) and maternally separated, MS (▲, dashed line, n=6) rats. On the x-axis: white bar represents 6:00 a.m. to 6:00 p.m. period; black bar represents 6:00 p.m. to 6:00 a.m. period. * $p<0.05$ vs. C rats.