Early Life Stress Sensitizes Rats to Angiotensin II–Induced Hypertension and Vascular Inflammation in Adult Life

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

Abstract—Maternal separation during early life is an established chronic behavioral model of early life stress in rats. It is known that perinatal adverse environments increase activity of the renin-angiotensin (Ang) system, specifically Ang II, in adulthood. The aim of this study was to investigate whether the effects of early life stress augment the sensitivity of the Ang II pathway. Using Wistar Kyoto rats, the maternal separation (MS) protocol was performed by separating approximately half of the male pups from their mother 3 h/d from days 2 to 14 of life. Pups remaining with the mother at all times were used as controls. Maternal separation did not influence the plasma basal parameters, such as blood glucose, insulin, Ang II, Ang 1-7 and plasma renin activity. Furthermore, body weight, blood pressure, and heart rate were similar in MS and control rats. The acute pressor response to Ang II was not different in anesthetized MS and control rats. However, the chronic infusion of Ang II (65 ng/min SC) elicited an exaggerated hypertensive response in MS compared with control rats (P<0.05). Surprisingly, HR was dramatically increased during the second week of Ang II infusion in MS compared with control rats (P<0.05). This enhanced Ang II sensitivity was accompanied by a greater vascular inflammatory response in MS versus control rats. Chronic Ang II infusion increased vascular wall structure in both groups similarly. These data indicate that early life stress sensitizes rats to an increased hemodynamic and inflammatory response during Ang II–induced hypertension. (Hypertension. 2010;55[part 2]:494-499.)

Key Words: maternal separation ■ Ang II–induced hypertension ■ vascular inflammation ■ behavioral stress ■ adverse environment

Traditionally, cardiovascular and metabolic disorders are thought to result from lifestyle risk factors acting on a certain genetic background. More recently, investigators have described a phenomenon whereby adverse stimuli affect the fetal environment altering the development of organs or tissues increasing the susceptibility to disease later in life, known as “early” or “fetal” origins of adult disease.1–3 In humans, adverse experiences in early childhood create and/or interact with genetic-based vulnerabilities, which are correlated with a greater incidence of endocrine, cardiovascular, and metabolic diseases in adulthood.4–6 In animal models, it is well accepted that environmental factors during development influence the risk for multiple forms of chronic adult diseases, such as depression, diabetes mellitus, and heart disease.7–9

The renin-angiotensin (Ang) system (RAS) is an important regulator of arterial pressure and body fluid balance through both the systemic and the intrarenal actions of Ang II.10 Excessive activation of the RAS also stimulates the sympathetic nervous system and mediates vascular hypertrophy and proinflammatory pathways. These Ang II–dependent mechanistic pathways are known to contribute toward cardiovascular dysfunction, especially hypertension, in adults. Animal models of fetal programming, such as a maternal low-protein diet or early gestational exposure to glucocorticoid, lead to alterations in RAS activity and adult onset of hypertension and renal dysfunction.11,12

Maternal separation is a widely used animal model of chronic behavioral stress during early life.13,14 Variations in maternal care during early life are considered a stressful experience associated with alterations in the endocrine response.15,16 Male rat pups separated from their mothers daily for a prolonged period (180 minutes) show, as adults, abnormal anxiety-related behavior and exaggerated endocrine responses to an acute stress, whereas females display normal or minimally exaggerated responses.14,17,18 In a study of >17 000 adults, early life stress or adverse childhood events are reported to be highly correlated with ischemic heart disease in adulthood more so than the traditional risk factors of smoking, obesity, and hypertension.4 Despite these dramatic observations, very little research has been conducted to determine whether early life behavioral stress leads to cardiovascular dysfunction and/or activation of the RAS system. We hypothesized that early life stress may activate the RAS signaling pathway leading to cardiovascular dysfunction in adults. This study was designed to investigate whether the rat model of early life stress, maternal separation, influences basal parameters of cardiovascular function, such as blood...
pressure, heart rate, vascular morphology, and vascular inflammation in male rats, and elucidate whether maternal separation sensitizes male rats to Ang II–induced cardiovascular dysfunction by monitoring the same parameters.

Methods

Early Life Stress: Maternal Separation Protocol
Close to delivery, pregnant Wistar Kyoto rats were carefully observed to determine the exact day of birth (postnatal day 0). Approximately half of the male pups from each litter were removed from the mother’s cage, and their tails were snipped and cauterized with silver nitrate for identification as maternally separated (MS). From postnatal days 2 to 14 of life, MS pups were separated from their mothers and littermates at the same time of day by transferring the pups to a clean cage in an incubator (30 ± 1°C) for 3 hours. The control group consisted of counterparts that remained with their mother and were nonhandled. Routinely, each breeding cycle included 5 breeding pairs at 1 time. Therefore, we included rats from ≥3 different litters in each experimental group. Weaning was performed at postnatal day 28. Body weights were determined every 2 days on postnatal days 2 to 14 and weekly until the rats were 12 weeks old.

Plasma Assays
Plasma glucose, insulin, plasma renin activity, Ang II, and Ang (1-7) peptide concentrations were assessed in baseline conditions. Additional methods are provided in the online Data Supplement (available at http://hyper.ahajournals.org).

Whole Animal Pressor Responses
Rats were anesthetized with thiobutabarbital (inactin; 65 mg/kg IP), and the right femoral artery and vein were isolated and cannulated with polyethylene 50 for monitoring mean arterial pressure (MAP) and heart rate (HR), respectively. Rats were allowed to equilibrate for 40 minutes, and Ang II (Phoenix Pharmaceutical Inc) was given as an IV bolus (0.04, 0.08, 0.16, and 0.32 μg/kg) in randomized order. Increments of MAP (24-hour average) in response to the Ang II bolus as well (Figure 1B). Chlorisondamine administration (5 mg/kg) abolished the HR change in HR (P < 0.05); thus, we consistently obtained 1 group of control rats (n = 9 to 12) along with a group of separated rats (n = 9 to 12). Subsequent treatment groups were divided among breeding pairs, as well as across breeding cycles. Therefore, we included rats from ≥3 different litters in each experimental group. Weaning was performed at postnatal day 28. Body weights were determined every 2 days on postnatal days 2 to 14 and weekly until the rats were 12 weeks old.

Acute Ang II Administration in Anesthetized Rats
The blood pressure (MAP) and HR responses to acute intravenous bolus of Ang II in anesthetized rats was not different in MS and control rats (Table S1A and S1B, respectively). Figure 1A represents the change in MAP (ΔMAP) in response to the Ang II bolus. Increments of change in HR (ΔHR) were not different in MS and control rats in response to the Ang II bolus as well (Figure 1B). Chlorisondamine administration (5 mg/kg) abolished the HR responses to Ang II as expected (Table S1B and Figure 1B).

Responses to Chronic Ang II Infusion
In parallel, we tested the blood pressure, HR, and locomotor activity changes in response to a chronic infusion of Ang II in telemetry-implanted rats. MAP (24-hour average) in MS (100.9 ± 3.0 mm Hg) and control rats (104.9 ± 3.2 mm Hg) was similar before infusion of Ang II (Table and Figure 2A). After 3 days of Ang II infusion, MAP increased significantly in MS rats compared with control rats (117.1 ± 2.1 versus 111.2 ± 1.7 mm Hg; P < 0.05, respectively). This enhanced
blood pressure response in MS rats continued during the second week of Ang II infusion (Figure 2A).

As a normal baroreflex response to an increase in BP, HR decreased significantly during the first week of Ang II infusion in control rats (Figure 2B; 371.2±7.8 bpm baseline versus 334.8±7.0 bpm at 7 days; *P<0.05*). Furthermore, HR was maintained at a reduced level compared with the baseline (320.0±4.5 bpm at 14 days; *P<0.05*) during the second week of Ang II infusion in C rats. MS rats have a similar HR at baseline (Figure 2B; 373.0±7.0 bpm at 7 days; *P<0.05*) during the first week of Ang II infusion (Figure 2A and 2B). The circadian rhythm of the MAP and HR was similar in both groups at baseline and during the Ang II infusion (Figure 2B). The circadian rhythm of the MAP and HR was significant in both groups at baseline and during the Ang II infusion (Figure 2A and 2B).

Locomotor activity was similar between control and MS rats at baseline conditions (Figure S2). Interestingly, locomotor activity in MS rats infused with Ang II was significantly (*P<0.05*) during the active cycle (6:00 PM to 6:00 AM) but not during the inactive cycle (6:00 AM to 6:00 PM; Figure S2). However, the activity increase did not correlate with the progressive augmentation in blood pressure or the changes in HR observed with chronic Ang II infusion in C rats. MS rats have a similar HR at baseline conditions (Figure S2). Interestingly, locomotor activity in MS rats infused with Ang II was significantly (*P<0.05*) during the active cycle (6:00 AM to 6:00 PM; Figure S2). However, the activity increase did not correlate with the progressive augmentation in blood pressure or the changes in HR observed with chronic Ang II infusion in C rats. MS rats have a similar HR at baseline conditions (Figure S2).

Our study of aortic morphology showed that the aortic thickness, OD, wall area, and cell density were not different between MS and control rats at baseline (Table S2). Ang II infusion significantly increased aortic wall thickness, OD, and wall area in both MS and control rats, similarly (Table S2). Immunolocalization of inflammatory cells in the aortas from MS and control rats demonstrated that monocytes/macrophages (CD68 marker) and T cells (CD3 marker) were associated with the endothelium and the perivascular adventitia at baseline conditions and after chronic Ang II infusion (Figure 3). No immunostaining for CD68 or CD3 cells was detected in the smooth muscle layer (data not shown). At baseline, the numbers of CD68+ (Figure 4A) and CD3+ (Figure 4B) cells were similar in the aortas from MS and control rats. Chronic Ang II infusion resulted in a significant increase in the number of CD68+ and CD3+ cells in both the endothelium and perivascular adventitia of aortas from MS and control rats (Figure 4). Furthermore, the increase in inflammatory cells in aortas from MS rats was significantly higher than in aortas from control rats (Figure 4).

**Discussion**

Foremost, our findings demonstrate that early life stress, specifically maternal separation, augments the responses to activation of the Ang II pathway in adult male rats. Our findings show that maternal separation does not induce hypertension as observed in other models of fetal programming; however, the consequent increased Ang II sensitivity may contribute to the pathogenesis of hypertension and cardiovascular disease. Specifically, maternal separation during the postnatal period sensitizes rats to Ang II-dependent
hypertension, increased HR, and vascular inflammation. Taken together, these data support a role for early life stress in the genesis of a risk factor mediating enhanced cardiovascular reactivity to Ang II.

Interestingly, rats display a hyporesponsive period to stress during neonatal life, as evidenced by a markedly attenuated response of the hypothalamus-pituitary-adrenocortical axis to environmental stressors.21,22 It has been shown that imposition of a stressor during the hyporesponsive period can promote changes in adult behavior and neuroendocrine function.17,18 Coincidently, the maternal separation protocol was conducted during this stress hyporesponsive period. We sought to elucidate the cardiovascular phenotype of adult rats that were exposed to maternal separation under baseline conditions and in response to exogenous Ang II. Our results have led us to hypothesize that early life stress elicits a sensitization of Ang II signaling in male rats. The sensitization of Ang II signaling may involve modulation of neuroendocrine mechanisms and/or it may represent a change in postnatal organ development modulating the susceptibility to cardiovascular disease. Typically, chronic infusion of an acutely subpressor dose of Ang II increases blood pressure after several days23,24 through mechanisms involving vascular dysfunction, a dysfunctional pressure/natriuresis relationship, and/or elevated sympathetic activity. At this point, it is not clear whether the mechanism of Ang II–induced hypertension is similar in the MS and control rats. Additional research is needed to further elucidate the mechanisms related to early life stress–induced sensitization of Ang II signaling.

Very few studies have explored the effects of maternal separation on cardiovascular parameters and/or function. Sanders and Anticevic25 examined the effects of early life stress on cardiovascular function in borderline hypertensive rats. They also report that baseline MAP and HR are similar between control and MS rats; nevertheless, MS rats subjected to restraint stress demonstrated an increased HR response, although they reported no significant differences in blood pressure responses. These data, in conjunction with our study, would indicate that early life stress does not elicit an obvious cardiovascular pathological phenotype in adulthood unless provoked by exogenous stimuli.

Interestingly, the HR responses to chronic Ang II infusion in the MS rats showed a dramatic and rather unexpected biphasic response. The MS rats appeared to maintain a normal baroreflex response during the first week of Ang II infusion, yet during the second week they developed tachycardia. Our finding may suggest that chronic Ang II sensitization in the MS rats provokes actions in the forebrain and/or the heart that increase sympathetic nerve activity to override the reflex bradycardia.26 Acute handling (3 min/d) of neonatal rats was found to alter autonomic controls of HR in preweanling spontaneously hypertensive and Wistar Kyoto

Figure 3. Localization of inflammatory cells in aortic tissue from Ang II–infused rats. A and B, Representative micrographs of CD68⁺ cells in Ang II–infused control (A) and MS (B) rats, respectively. C and D, Representative micrographs of CD3⁺ cells in Ang II–infused control (C) and MS (D) rats, respectively. Arrows point to CD68⁺ or CD3⁺ cells in the endothelium (En) or adventitia (Adv).

Figure 4. Quantitation of inflammatory cells in the thoracic aorta from vehicle and Ang II–infused rats. A, CD68⁺ cells in Ang II–infused control (C; n=5) and MS (n=9) rats and vehicle–infused C (n=4) and MS (n=4) rats. B, CD3⁺ cells in Ang II–infused C (n=6) and MS (n=5) rats and vehicle–infused C (n=4) and MS (n=4) rats. E indicates endothelium; Adv, adventitia.

*P<0.05 vs C; **P<0.05 vs C.
models of fetal programming. Yet, maternal separation renders the adult rats susceptible to activation of Ang II–dependent hypertension, increased HR, and vascular inflammation, which may contribute to the pathogenesis of cardiovascular disease. Overall, our findings support a role for early life stress in the genesis of a risk factor mediating enhanced cardiovascular reactivity to Ang II and provide justification for further investigation.

Perspectives

Chronic stressors are known to sensitize humans to risk factors for cardiovascular disease. This study used a model of chronic stress during early life and demonstrating a sensitization to Ang II–dependent responses. A complete understanding of the mechanisms involved in the Ang II–dependent chronic stress responses may disclose future therapeutic approaches and preventative measures for minimizing the risk of hypertension.

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Disclosures

None.

References


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Supplementary Information

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

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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-diaminetetraacetic acid (Sigma Chemical Co., St. Louis, Mo), 0.44 mmol/L 1,20-orthophenanthroline 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 O2 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

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

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<tr>
<th>Rat</th>
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<tr>
<td></td>
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<td>0.04</td>
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<tr>
<td>C</td>
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<td>114±2</td>
<td>117±2</td>
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<td>MS</td>
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<td>Chlorisondamine (5 mg/kg) + Ang II (µg/kg)</td>
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<tr>
<td>C</td>
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<td>87±3</td>
<td>95±4</td>
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<td>85±3</td>
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B) Heart Rate (HR), bpm

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<td>MS</td>
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<td>Chlorisondamine (5 mg/kg) + Ang II (µg/kg)</td>
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Table S2. Aortic tissue morphology in chronic ang II-infused control (C) and maternally separated (MS) rats, n=4. * p<0.05 vs. vehicle

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<td>AngII-infused</td>
<td>vehicle</td>
<td>AngII-infused</td>
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<tr>
<td>Thickness, μm</td>
<td>105.45 ± 3.37</td>
<td>133.13 ± 4.34*</td>
<td>100.11 ± 3.37</td>
<td>122.69 ± 3.16*</td>
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<tr>
<td>Aorta External Diameter, mm</td>
<td>1.64 ± 0.01</td>
<td>1.79 ± 0.02*</td>
<td>1.65 ± 0.02</td>
<td>1.76 ± 0.01*</td>
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<tr>
<td>Wall Area, x 10000 μm²</td>
<td>50.42 ± 0.9</td>
<td>66.45 ± 3.4*</td>
<td>54.33 ± 1.6</td>
<td>68.0 ± 0.3*</td>
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<tr>
<td>Cell density, nuclei per 100 μm²</td>
<td>21.34 ± 1.02</td>
<td>20.58 ± 1.27</td>
<td>21.83 ± 0.91</td>
<td>20.29 ± 0.60</td>
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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.