Early life stress in male mice induces superoxide production and endothelial dysfunction in adulthood

Dao H. Ho, Mariah L. Burch, Benjamin Musall, Jacqueline B. Musall, Kelly A. Hyndman, and Jennifer S. Pollock

1Cardio-Renal Physiology and Medicine, Division of Nephrology, Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama; and 2Department of Medicine, Augusta University, Augusta, Georgia

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Ho DH, Burch ML, Musall B, Musall JB, Hyndman KA, Pollock JS. Early life stress in male mice induces superoxide production and endothelial dysfunction in adulthood. Am J Physiol Heart Circ Physiol 310: H1267–H1274, 2016. First published February 26, 2016; doi:10.1152/ajpheart.00016.2016.—Early life stress (ELS) is a risk for cardiovascular disease in adulthood although very little mechanistic insight is available. Because oxidative stress and endothelial dysfunction are major contributors to cardiovascular risk, we hypothesized that ELS induces endothelial dysfunction in adult male mice via increased superoxide production. Studies employed a mouse model of ELS, maternal separation with early weaning (MSEW), in which litters were separated from the dam for 4 h/day [postnatal days (PD) 2–5] and 8 h/day (PD6–16), and weaned at PD17. Control litters remained undisturbed until weaning at PD21. When compared with control mice, thoracic aortic rings from adult male MSEW mice displayed significant endothelial dysfunction that was reversed by the superoxide scavenger, polyethylene glycol-superoxide dismutase (PEG-SOD). PEG-SOD-inhibitable superoxide production by aortae from MSEW mice was significantly greater than observed in control aortae, although unaffected by nitric oxide synthase inhibition, suggesting that uncoupled nitric oxide synthase was not responsible for the accelerated superoxide production. Aortic SOD expression, plasma SOD activity, and total antioxidant activity were similar in MSEW and control mice, indicating unaltered antioxidant capacity in MSEW mice. Increased expression of the NADPH oxidase subunits, NOX2 and NOX4, was evident in the aortae of MSEW mice. Moreover, endothelial dysfunction and superoxide production in MSEW mice was reversed with the NADPH oxidase inhibitor, apocynin, indicating increased NADPH oxidase-dependent superoxide production and endothelial dysfunction. The finding that MSEW induces superoxide production and endothelial dysfunction in adult mice may provide a mechanistic link between ELS and adult cardiovascular disease risk.

maternal separation with early weaning; early life stress; endothelial dysfunction; superoxide; NADPH oxidase

NEW & NOTEWORTHY

The present study provides compelling evidence that early life stress in mice programs increased superoxide generation in the vasculature, leading to endothelial dysfunction in adulthood. This translational study provides mechanistic insight into vascular dysfunction in adult humans who are exposed to adverse childhood experiences.

ADVERSE CHILDHOOD EXPERIENCES (ACEs), also known as early life stress (ELS), are associated with increased risk of cardiovascular disease (22). Retrospective studies strongly link chronic separation from parents during childhood with elevated systolic and diastolic blood pressures (2), as well as increased prevalence of ischemic heart disease and type 2 diabetes in late adulthood (3, 8). Moreover, our group recently reported that ACEs were associated with increases in pulse wave velocity, total peripheral resistance, and diastolic blood pressure in relatively healthy, young adults (35), indicating that vascular dysfunction may be a mediator in the ELS-induced increase in cardiovascular disease risk. Currently, there is a need for mechanistic studies to elucidate the relationship between ELS and vascular dysfunction.

In humans, ELS is associated with increased oxidative stress (33). Oxidative stress due to increased production of free radicals, reactive oxygen species (ROS), and/or loss of antioxidants has been shown to increase cardiovascular disease risk through induction of cellular damage in a wide range of organ systems, especially the vasculature (38). Components of the vasculature, such as endothelial cells, vascular smooth muscle cells, and resident monocytes and macrophages produce ROS and have the potential to contribute to overall oxidative stress in a biological system (38). Superoxide anion accumulation due to increased NADPH oxidase activity, nitric oxide (NO) synthase (NOS) uncoupling, and/or loss of antioxidant capacity, as well as the resulting decrease in NO bioavailability and endothelial dysfunction, are well-described mechanisms of vascular pathology (7). Indeed, endothelial dysfunction is associated with virtually every condition predisposing to cardiovascular disease (6) and is considered to represent an important mechanism of cardiovascular risk (11). Given that both ELS and oxidative stress induce vascular dysfunction and that ELS is associated with increased oxidative stress markers in humans (30), it is tempting to speculate that oxidative stress and/or endothelial dysfunction may contribute to the ELS-induced cardiovascular disease risk in adulthood.

In this study, experiments were performed to test the hypothesis that ELS induces endothelial dysfunction and/or increased oxidative stress. Maternal separation with early weaning in C57Bl/6J mice is a model of ELS involving extended maternal separation time and early weaning that generates robust effects on anxiety, hyperactivity, and behavioral despair in adulthood, of which all are contributors to the development and progression of cardiovascular disease (13, 41). Using this model, we elucidated whether ELS induces oxidative stress, as well as changes in cardiovascular physiological parameters such as endothelial function, blood pressure, heart rate, and blood glucose in adult mice.

METHODS

Mouse model of early life stress. Studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.
Western blot analysis. Thoracic aortae were cleaned of adherent fat and homogenized in 50 mM Tris (pH 7.4), 250 mM sucrose, 0.1 mM EDTA, 0.1 mM EGTA, 10% glycerol, 0.1% SDS, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% β-mercaptoethanol, and 0.01 mg/ml each of leupeptin, pepstatin, and aprotinin using a handheld motorized pestle. The samples then were freeze thawed, sonicated for 10 × 1-s bursts on ice, and incubated on a rocker at 4°C for 30 min. After centrifugation at 17,000 g at 4°C for 20 min, supernatant was collected and stored at −80°C. Samples were run on 8 or 15% SDS gels, transferred to polyvinylidene fluoride membranes (Immobilon-FL), and blotted using antibodies against NOS3 (No. 610297; BD Biosciences, San Jose, CA), pS1177 (No. 9571S; Abcam, Cambridge, MA), SOD1 (No. SOD-101; StressGen, Farmingdale, NY), SOD2 (No. SOD-111; StressGen), SOD3 (No. SOD-105; StressGen), and actin (Sigma-Aldrich). Secondary antibodies were used at a concentration of 1 μg/ml. Membranes were imaged using the Odyssey CLX Infrared Imaging System, and band intensities were quantified using Image Studio Software (LI-COR Imaging Systems, Lincoln, NE).

Quantitative real-time RT-PCR. Thoracic aortae were cleaned of adherent fat, flash frozen, and homogenized in TRIzol (Invitrogen, Grand Island, NY) using a glass mortar and Teflon pestle. RNA isolation was performed according to the TRIzol method established by Invitrogen, and reverse transcription of RNA to cDNA was performed using a commercial kit (QuantiTect Reverse Transcription Kit; Qiagen, Valencia, CA). Quantitative real-time RT-PCR was performed for detection of Cypb (encodes NOX2), Noxd, Ncf1 (encodes p47phox), Ncf2 (encodes p67phox), and Nos3 (encodes NO synthase 3) expression using commercial kits (QuantiFast SYBR Green RT-PCR Kit, QuantiTect primers; Qiagen).

Aortic superoxide production. PEG-SOD-inhibitable and apocynin-inhibitable superoxide production in thoracic aorta were assessed using the dihydroethidium (DHE) method previously described by Fink and colleagues (10). Briefly, aortae were cleaned of perivascular fat and cut into 2-mm rings. The rings were preincubated with either vehicle (Krebs-Henseleit buffer, KHB), PEG-SOD (100 U/ml), or apocynin (300 μM) for 20 min, and then DHE was added for an additional 40 min. Aortic rings were then rinsed in KHB, thoroughly homogenized, filtered, and processed for HPLC analysis (Elite LaChrome, Hitachi High-Technologies, Tokyo, Japan). To calculate PEG-SOD-inhibitable superoxide production, the value obtained for PEG-SOD-treated rings was subtracted from the value obtained for vehicle-treated rings. The same approach was used to calculate apocynin-inhibitable superoxide production. To assess the contribution of NOS activity to aortic superoxide production, aortic rings were incubated with NOS inhibitor Nω-nitro-l-arginine methyl ester (l-NNAME) (100 μM in KHB; Sigma-Aldrich) for 20 min before DHE incubation.

Statistical analyses. Data are expressed as means ± SE. Baseline parameters, superoxide production, antioxidant measurements, mRNA expression, and protein expression data were analyzed using unpaired t-tests (GraphPad Prism, La Jolla, CA). Differences in −logEC50 and Emax of cumulative concentration curves for vascular reactivity were analyzed using two-way ANOVA with subsequent Bonferroni post hoc analysis (GraphPad Prism). For real-time quantitative RT-PCR, the 2−ΔΔCt method of analysis was used. Statistical significance was defined as P < 0.05.

RESULTS

Body weight, blood pressure, and basal parameters. No significant difference in body weight between control and MSEEW mice was observed up to PD5 (values in grams reported as control vs. MSEEW, PD2: 1.6 ± 0.1 vs. 1.5 ± 0.1; PD5: 2.8 ± 0.1 vs. 2.6 ± 0.1, n = 14/group). At PD10, pups were easily distinguishable by sexually dimorphic features, and thus body weights were calculated accordingly. Body weights continued to show no significant differences between groups in both males (values in grams reported as control vs. MSEEW, PD10: 4.7 ± 0.2 vs. 4.3 ± 0.2; PD15: 6.3 ± 0.3 vs. 6.1 ± 0.3;
PD28: 11.4 ± 0.4 vs. 11.4 ± 1.2; n = 6–7/group) and females (values in grams reported as control vs. MSEW, PD10: 5.1 ± 0.2 vs. 4.5 ± 0.2; PD15: 6.8 ± 0.4 vs. 6.2 ± 0.3; PD28: 11.1 ± 0.3 vs. 11.9 ± 0.5; n = 7–8/group). Adult body weight, kidney and heart weight, blood pressure, heart rate, activity, and nonfasting blood glucose were comparable between control and MSEW male mice (Table 1). Also, female control and MSEW mice showed no significant difference in adult body weight (control: 19.9 ± 0.47 g vs. MSEW: 19.1 ± 0.11 g, n = 4–7/group), kidney weight (control: 0.11 ± 0.003 g vs. MSEW: 0.11 ± 0.004 g, n = 3/group), heart weight (control: 0.09 ± 0.008 g vs. MSEW: 0.09 ± 0.006, n = 3/group), and nonfasting blood glucose (control: 206 ± 14 mg/dl vs. MSEW: 202 ± 10 mg/dl, n = 5–7/group).

Aortic superoxide production and endothelial dysfunction. PEG-SOD-inhibitable superoxide production was significantly greater (~3-fold) in MSEW than control aortae (Fig. 1A). To determine the contribution of increased superoxide levels in MSEW aortae to endothelial dysfunction (defined as impaired NO-dependent relaxation), responses to cumulative increasing concentrations of ACh were assessed in the absence or presence of PEG-SOD. The maximum relaxation response to ACh was significantly blunted in the aortae of MSEW mice compared with control mice with no difference in \( -\log EC_{50} \) (Fig. 1B; Table 2). Preincubation of aortae with PEG-SOD reversed the blunted response to ACh in aortae from MSEW mice without significantly affecting SNP-induced vasorelaxation (Fig. 1, B and C; Table 2). SNP-induced vasorelaxation was similar in the MSEW and control groups (Fig. 1C; Table 2). ACh- or SNP-induced vasorelaxation in control aortae was not significantly affected by pretreatment with PEG-SOD (Fig. 1, B and C; Table 2). Pretreatment of aortic rings with PEG-SOD significantly decreased the level of preconstriction in response to 1 μmol/l phenylephrine; however, there was no difference between control and MSEW aortae in the degree of decrease in preconstriction [\( F_{\text{Treatment}} (1, 16) = 27.93, P < 0.0001, F_{\text{Group}} (1, 16) = 0.90, P = 0.36, F_{\text{Interaction}} (1, 16) = 0.41, P = 0.53 \)]. These results indicate that enhanced superoxide production significantly contributed to MSEW-induced endothelial dysfunction.

Aortae of control and MSEW female mice displayed no significant difference in maximum relaxation or \( -\log EC_{50} \) in response to ACh [values reported as control (n = 7) vs. MSEW (n = 4)]; \( E_{\text{max}}: 57.2 ± 6.87 \) vs. 51.9 ± 12.22, \( P = 0.69, -\log EC_{50}: 6.78 ± 0.187 \) vs. 7.19 ± 0.147; \( P = 0.17 \) or SNP (\( E_{\text{max}}: 99.3 ± 1.04 \) vs. 97.6 ± 2.55, \( P = 0.47, -\log EC_{50}: 8.42 ± 0.097 \) vs. 8.62 ± 0.108, \( P = 0.25 \), thus precluding them from further study.

Systemic and aortic antioxidant biomarkers. Plasma total antioxidant activity and SOD activity did not differ between groups (Fig. 1, D and E). In addition, aortic protein expression of SOD isoforms (SOD1, SOD2, and SOD3) were not different between groups [values (fold change relative to control) reported as control vs. MSEW, SOD1: \( 1.0 ± 0.1 \) vs. 0.9 ± 0.1; SOD2: \( 1.0 ± 0.01 \) vs. 1.0 ± 0.1; SOD3: \( 1.0 ± 0.2 \) vs. 0.9 ± 0.2; \( n = 4–6 \) group, \( P > 0.58 \)] Plasma NOx also did not differ between groups (Fig. 1F). Furthermore, no difference was found in expression of NO synthase 3 (NOS3) mRNA [values (fold change relative to control) reported as control vs. MSEW, control 1.0 ± 0.2 vs. MSEW 1.5 ± 0.4, \( n = 5–8 \) group, \( P = 0.49 \)] or NOS3 protein (control 1.0 ± 0.4 vs. MSEW 1.0 ± 0.1, \( n = 11–12 \) group, \( P = 0.91 \)) or phosphorylated Ser1177 NOS3 (control 1.0 ± 0.1 vs. MSEW 1.1 ± 0.2, \( n = 6 \) group, \( P = 0.55 \)) in aortae from control and MSEW mice.

Aortic NADPH oxidase activity and subunit expression. At the mRNA level, expression of the membrane-bound NADPH oxidase enzyme subunits, Cybb and Nox4, was upregulated in aortae of MSEW mice (Fig. 2A and B). In contrast, MSEW did not significantly alter aortic expression of Ncf1 and Ncf2, which encode cytosolic NADPH oxidase enzyme subunits, p47\( ^{phox} \) and p67\( ^{phox} \), respectively (Fig. 2, C and D). Preincubation of aortae with the NADPH oxidase inhibitor, apocynin, abolished the endothelial dysfunction in aortae from MSEW mice without affecting SNP-induced vasorelaxation (Fig. 2, F and G; Table 2). Neither ACh- nor SNP-induced vasorelaxation was significantly affected by apocynin treatment of control aortae (Fig. 2, F and G; Table 2). Pretreatment of aortic rings with apocynin significantly decreased the level of preconstriction in response to 1 μmol/l phenylephrine; however, there was no difference between control and MSEW aortae in the degree of decrease in preconstriction [\( F_{\text{Treatment}} (1, 16) = 33.39, P < 0.0001, F_{\text{Group}} (1, 16) = 0.07, P = 0.79, F_{\text{Interaction}} (1, 16) = 0.002, P = 0.96 \)]. Moreover, apocynin-inhibitable superoxide production was ~2.5 fold greater in aortae of MSEW mice compared with control (Fig. 2E). These results suggest that enhanced NADPH oxidase activity likely contributed to aortic endothelial dysfunction in MSEW mice. Additionally, L-NAME preincubation did not significantly influence superoxide production in aortae of MSEW mice (vehicle: 12.8 ± 3.10 vs. L-NAME: 18.6 ± 6.48 μmol/2-mm section, \( P = 0.49, n = 6–7 \) group), indicating that uncoupled NOS3 most likely is not the source of increased aortic superoxide production.

**TABLE 1.** Baseline parameters of 12-wk-old male mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>MSEW</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>25.7 ± 2.16</td>
<td>26.0 ± 1.58</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>0.13 ± 0.01</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>0.15 ± 0.02</td>
<td>0.15 ± 0.02</td>
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<tr>
<td>Blood glucose, mg/dl</td>
<td>208 ± 23</td>
<td>210 ± 34</td>
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<tr>
<td>12-hour mean arterial pressure, mmHg</td>
<td>120.7 ± 1.2</td>
<td>123.0 ± 1.0</td>
</tr>
<tr>
<td>Active period</td>
<td>108.5 ± 0.9</td>
<td>108.7 ± 1.2</td>
</tr>
<tr>
<td>Inactive period</td>
<td>135.2 ± 1.6</td>
<td>139.3 ± 1.2</td>
</tr>
<tr>
<td>12-hour systolic pressure, mmHg</td>
<td>123.2 ± 1.9</td>
<td>122.6 ± 1.9</td>
</tr>
<tr>
<td>Active period</td>
<td>106.3 ± 0.7</td>
<td>106.7 ± 2.8</td>
</tr>
<tr>
<td>Inactive period</td>
<td>93.8 ± 0.8</td>
<td>93.3 ± 2.6</td>
</tr>
<tr>
<td>12-hour diastolic pressure, mmHg</td>
<td>605 ± 11</td>
<td>601 ± 4</td>
</tr>
<tr>
<td>Active period</td>
<td>567 ± 14</td>
<td>557 ± 6</td>
</tr>
<tr>
<td>Inactive period</td>
<td>15.6 ± 1.4</td>
<td>14.5 ± 3.5</td>
</tr>
<tr>
<td>12-hour activity, counts/min</td>
<td>4.1 ± 1.1</td>
<td>3.5 ± 1.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. An unpaired, two-tailed \( t \)-test was performed for each parameter; \( n = 5–8 \) mice/group for body weight, heart weight, kidney weight, and blood glucose, and \( n = 4 \) mice/group for blood pressure, heart rate, and activity. MSEW, maternal separation with early weaning.
tion. Endothelial dysfunction, a hallmark of increased cardiovascular disease risk, may be due to a number of mechanisms that results in reduction of nitric oxide (NO) bioavailability (23, 34). The results of the present study indicate that endothelial dysfunction of the thoracic aorta in MSEW mice is likely due to increased NADPH oxidase-generated superoxide production and subsequent loss of NO bioavailability, rather than loss of antioxidant capacity (i.e., superoxide dismutase activity or NOS3 production), or increased superoxide generation resulting from NOS3 uncoupling.

NADPH oxidase is a predominant contributor to endothelial oxidative stress via production of ROS and has been shown to directly mediate vascular dysfunction (24, 25, 31). The NADPH oxidase complex consists of several components including membrane-bound subunits (NOX isoforms and p22phox) and cytosolic components (p67phox, p47phox, and rac), all of which are expressed in aortic tissue (5). The present study revealed that key components of the complex, NOX2 and NOX4, were upregulated in aorta at adulthood by MSEW. Studies indicate that overexpression of vascular endothelial NOX2 increases aortic superoxide and macrophage recruitment without altering blood pressure in mice (9), whereas overexpression of vascular NOX4 can either increase superoxide or hydrogen peroxide and induce protection or damage, depending on the vessel type and the nature of the stress condition under question [reviewed in Touyz and Montezano (39)]. More recently, NOX4 has been shown to produce predominantly hydrogen peroxide in vitro (27). Interestingly, in

![Figure 1](http://ajpheart.physiology.org/)

**A:** PEG-SOD-inhibitable superoxide production by aortae from control (n = 4) and MSEW (n = 4) mice. *P = 0.002. B: aortic relaxation in response to acetylcholine (ACh) in presence of vehicle (VEH) or PEG-SOD (100 U/ml). Refer to Table 2 for statistical analyses (n = 5–7 per group). *P < 0.05 vs. control VEH. †P < 0.05 vs. MSEW PEG-SOD. C: aortic relaxation in response to sodium nitroprusside (SNP) in the presence of vehicle (VEH) or PEG-SOD (100 U/ml). Refer to Table 2 for statistical analyses (n = 5–7 per group). D: total antioxidant capacity of plasma from control (n = 19) and MSEW (n = 17) mice. E: plasma SOD activity in plasma from control (n = 6) and MSEW (n = 6) mice. F: NOx levels in plasma from control (n = 12) and MSEW (n = 12) mice.
this study, inhibition of NADPH oxidase activity (apocynin treatment) or scavenging of superoxide (PEG-SOD treatment) restored endothelial function and reduced superoxide production to a greater extent in MSEW aortae, suggesting a prominent role of NOX-driven superoxide production in mediating MSEW-induced endothelial dysfunction. It must be noted that the use of apocynin as a selective NADPH oxidase inhibitor in vascular cells is controversial, and interpretation of its specificity must be approached with caution (15, 29). However, the observation of significant upregulation of two NADPH oxidase subunits in conjunction with the apocynin-reversible endothelial dysfunction as well as increased superoxide production in MSEW aortas lends credence to our conclusion that endothelial dysfunction in adult MSEW mice is largely due to an increase in NADPH oxidase-driven superoxide generation.

NADPH oxidase may drive superoxide generation from the transfer of electrons from NAPDH to oxygen or from downstream effector molecules. Previous studies from our laboratory and others have shown that NOS3 uncoupling is facilitated by activated NADPH oxidase and the resulting increase in superoxide levels (37); however, NOS inhibition did not alter superoxide production by aortae from MSEW mice, thus indicating that NADPH oxidase-derived superoxide generation did not potentiate superoxide production via NOS3 uncoupling in these mice. Additionally, MSEW mice had normally functioning systemic and aortic antioxidant capacity and plasma SOD activity, and they showed no changes in expression of SOD1, SOD2, or SOD3 in aortic tissue, suggesting a fully functional SOD system. To an extent, NO has the capacity to reduce oxidative stress and may play an important role in counterbalancing superoxide production. Neither NOS3 expression nor its phosphorylation status was affected by MSEW, indicating that the endothelial dysfunction is not likely due to reduced NOS3 expression or activity. These data further support the contention that the predominant mechanism underlying MSEW-induced endothelial dysfunction is the direct generation of superoxide by NADPH oxidase. Given that NADPH oxidase is expressed in endothelial cells, vascular smooth muscle cells, and vascular macrophages (38), additional studies are necessary to fully understand the cellular source of MSEW-driven superoxide production. In this study, we observed no change in endothelium-independent relaxation after treatment with an intracellular superoxide scavenger (PEG-SOD); thus, we propose that the endothelium is the likely source of MSEW-driven superoxide production and that the increase in intracellular superoxide reduces intracellular NO, rather than directly affecting vascular smooth muscle superoxide production.

The goal of this study was to investigate whether endothelial function was disturbed by ELS using a mouse model of MSEW. Flow-mediated dilation of the brachial artery, a non-invasive measure of endothelial function of a conduit vessel, is a popular choice of assessing endothelial function in humans (12). Results from the current study revealed that the MSEW mouse model may serve as an excellent model by which to investigate the mechanisms by which ELS may induce endothelial dysfunction and vascular disease of conduit vessels in humans. Studies have shown that correlation between endothelial function of conduit and resistance vessels is dependent on the condition or stressor under investigation (16, 40, 42); thus, we recognize that ELS may have varying effects on different vessel beds in the MSEW model and that this topic warrants further study.

Notably, MSEW triggered endothelial dysfunction in adult male mice without alterations in blood pressure, heart rate, activity, blood glucose, body weight, or organ weight. The absence of overt cardiovascular pathology in MSEW mice at 12 wk of age suggests that endothelial dysfunction is a mechanism by which early life stress increases the risk for cardiovascular disease in young adulthood. The current study is in agreement with our human studies showing that adolescents and young adults (13–29 yr old) exposed to ACEs exhibited relatively mild but significant vascular dysfunction without overt signs of cardiovascular disease (35). Additionally, in a longitudinal study, we found that blood pressure of people exposed to ACEs significantly deviated from people with no exposure to ACEs after 30 yr of age, indicating that underlying vascular dysfunction caused by ELS did not immediately manifest in blood pressure dysregulation but may still pose as a significant risk factor for disease (36).

In humans, it is well known that there exists prominent sex differences in the response to psychosocial stressors during childhood and adulthood [reviewed in Bale and Epperson (4)]. Also, rodent studies have shown that maternal separation has disparate sex-dependent effects on angiotensin II-mediated hypertension and renal function (21), behavioral reactivity to subsequent stressors (26), as well as neurogenesis (19). The

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### Table 2. Effect of APO and PEG-SOD on $-\log EC_{50}$ and $E_{\text{max}}$ of mouse thoracic aorta relaxation in response to ACh and SNP

<table>
<thead>
<tr>
<th>Agonist/Treatment</th>
<th>$-\log EC_{50}$</th>
<th>$E_{\text{max}}$</th>
<th>$-\log EC_{50}$</th>
<th>$E_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>MSEW</td>
<td>Control</td>
<td>MSEW</td>
</tr>
<tr>
<td>ACh</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEH</td>
<td>6.99 ± 0.12</td>
<td>73.2 ± 4.3</td>
<td>6.86 ± 0.07</td>
<td>53.5 ± 6.9*</td>
</tr>
<tr>
<td>APO</td>
<td>7.27 ± 0.11</td>
<td>87.0 ± 5.8</td>
<td>7.21 ± 0.09††</td>
<td>95.0 ± 2.1†††</td>
</tr>
<tr>
<td>PEG-SOD</td>
<td>7.20 ± 0.10</td>
<td>81.5 ± 4.3</td>
<td>7.14 ± 0.12</td>
<td>77.3 ± 4.7†</td>
</tr>
<tr>
<td>SNP</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>VEH</td>
<td>8.54 ± 0.13</td>
<td>101.8 ± 0.7</td>
<td>8.23 ± 0.11</td>
<td>101.5 ± 0.7</td>
</tr>
<tr>
<td>APO</td>
<td>8.48 ± 0.18</td>
<td>102.9 ± 1.1</td>
<td>8.17 ± 0.11</td>
<td>100.4 ± 0.7</td>
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<tr>
<td>PEG-SOD</td>
<td>8.41 ± 0.14</td>
<td>102.3 ± 0.7</td>
<td>8.30 ± 0.09</td>
<td>100.4 ± 0.5</td>
</tr>
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</table>

Values are means ± SE. Sensitivity ($-\log EC_{50}$) and maximal effect ($E_{\text{max}}$) values determined from %relaxation from phenylephrine preconstriction in response to agonist. Two-way ANOVA with Bonferroni post hoc analyses were performed for each agonist group [acetylcholine (ACh) or sodium nitroprusside (SNP)] with factors of group (Control, MSEW) and treatment [vehicle (VEH), apocynin (APO) or VEH, superoxide dismutase (PEG-SOD)]; $n = 5–7$ mice/group. $*P < 0.05$ compared with VEH Control; †$P < 0.05$, ††$P < 0.01$, and †††$P < 0.001$ compared with VEH MSEW.
present study is the first to show that MSEW caused adult endothelial dysfunction in male, but not female, mice. In our previous human cohort studies, we found that sex difference did not significantly affect ELS-mediated vascular dysfunction or blood pressure trajectory; however, endothelial function was not directly measured in these studies (35, 36). Results of the current study highlight the need to investigate the potential effect of sex difference on ELS-regulated endothelial dysfunction in humans.

ELS can significantly modulate components of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenal-medullary (SAM) system in rodents (20). Specifically, rodents that have been subjected to maternal separation before weaning show altered circulating plasma corticosterone levels, increased HPA axis responsiveness to stressors, altered hippocampal glucocorticoid receptor density, increased corticotropin-releasing factor expression in hypothalamus, and decreased serotonin transporter and tryptophan hydroxylase expression in

Fig. 2. MSEW induced NADPH oxidase subunit expression and activity in aorta of adult male mice. Quantitative RT-PCR analysis of Cybb (NOX2; A), Nox4 (NOX4; B), Ncf2 (p67phox; C), and Ncf1 (p47phox; D) in aorta of control (n = 3–5) and MSEW (n = 4–9) mice. *P < 0.05. E: apocynin-inhibitable superoxide production by aortae from control (n = 4) and MSEW (n = 4) mice. F: aortic relaxation in response to acetylcholine (ACh) in presence of vehicle (VEH) or apocynin (APO, 300 μM). Refer to Table 2 for statistical analyses (n = 5–7 per group). G: aortic relaxation in response to sodium nitroprusside (SNP) in presence of vehicle (VEH) or apocynin (APO, 300 μM). Refer to Table 2 for statistical analyses (n = 5–7 per group).
brain (1, 28, 32). Many of the components altered by ELS can play an important role in regulating vascular tone, senescence, and inflammation (14) and thus are possible mechanisms by which MSEE-induced superoxide in mouse aorta is orchestrated. Whether MSEE directly or indirectly mediates vascular NADPH oxidase-mediated superoxide production remains to be investigated. We propose future studies that test whether in vivo inhibition of NADPH oxidase activity or treatment with antioxidants during exposure to MSEE would abolish endothelial dysfunction in adulthood.

In conclusion, the results of the present study provide compelling evidence for ELS-induced endothelial dysfunction through NADPH oxidase-mediated superoxide production in adult male mice. The current findings indicate that psychosocial stress in early life acts to program the vasculature, and in particular the endothelium, toward higher susceptibility to the development of vascular disease through oxidative stress pathways. We propose that this mechanism may precede overt disease and may lead to a predisposition toward cardiovascular disease risk later in life when secondary stressors are present.

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Present address of D. H. Ho: Tripler Army Medical Ctr., Dept. of Clinical Investigation, 1 Jarrett White Rd., MCHK-CL, Bldg. 40, Rm. 216, Honolulu, HI 96859.

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AUTHOR CONTRIBUTIONS


REFERENCES


