Maternal-pup interaction disturbances induce long-lasting changes in the newborn rat pulmonary vasculature

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Maternal-pup interaction disturbances induce long-lasting changes in the newborn rat pulmonary vasculature. Am J Physiol Lung Cell Mol Physiol 309: L1186–L1198, 2015. First published September 4, 2015; doi:10.1152/ajplung.00044.2015. —The factors accounting for the pathological maintenance of a high pulmonary vascular (PV) resistance postnatally remain elusive, but neonatal stressors may play a role in this process. Cross-fostering in the immediate neonatal period is associated with adult-onset vascular and behavioral changes, likely triggered by early-in-life stressors. In hypothesizing that fostering newborn rats induces long-lasting PV changes, we evaluated them at 14 days of age during adulthood and compared the findings with animals raised by their biological mothers. Fostering resulted in reduced maternal-pup contact time when compared with control newborns. At 2 wk of age, fostered rats exhibited reduced pulmonary arterial endothelium-dependent relaxation secondary to downregulation of tissue endothelial nitric oxide synthase expression and tetrahydrobiopterin deficiency-induced uncoupling. These changes were associated with neonatal onset-increased ANG II receptor type 1 expression, PV remodeling, and right ventricular hypertrophy that persisted into adulthood. The pulmonary arteries of adult-fostered rats exhibited a higher contraction dose response to ANG II and thromboxane A2, the latter of which was abrogated by the oxidant scavenger Tempol. In conclusion, fostering-induced neonatal stress induces long-standing PV changes modulated via the renin-angiotensin system.

neonatal stressors; pulmonary vasomotor tone; renin angiotensin system

THE TRANSITION FROM FETAL to postnatal life is characterized by a rapid decrease in pulmonary vascular resistance (PVR), as a result of the birth-associated lung expansion, alveolar oxygenation, and CO2 removal (58). Infants with the so-called persistent pulmonary hypertension syndrome of the newborn (PPHN) either fail to show the physiological decline in PVR at birth or exhibit a postnatal increase in resistance (19). Neonatal stressors as one of the causative factors of this syndrome have been suggested (57), but their pathogenesis is poorly understood.

The renin-angiotensin (Ang)-aldosterone system (RAAS) plays an essential role in systemic blood pressure regulation and is a likely modulator of the stress-induced vasomotor tone. RAAS primarily depends on the renal cell generation of renin, which in turn, promotes the liver and fat-derived angiotensinogen conversion to ANG I. Ang-converting enzyme (ACE), mostly present in the lungs, converts ANG I into the vasoactive form ANG II that signals through two main receptors: ANG II receptor types 1 and 2 (AT1 and AT2, respectively) (42). In the regulation of vascular resistance, AT1 receptors are responsible for vasoconstriction and vessel-wall compliance, whereas AT2 receptors modulate vasodilation (25, 49, 51, 52). Adding to the renin-angiotensin system complexity, ACE2 promotes the conversion of ANG II into Ang-(1–7), which induces vasodilation via the Mas receptor (24). Downregulation of Ang-(1–7) and/or its Mas receptor is associated with systemic hypertension (24).

A prenatal, maternal low-protein diet results in late-onset systemic hypertension in rats via a mechanism involving RAAS (32, 34). This fetal programming is characterized by an enhanced vasomotor response to ANG II later in life, secondary to the increased renal and vascular tissue AT1 receptor expression (65). Such fetal programming is not limited to the systemic circulation, since a restrictive protein diet during gestation also induces an increase in lung ACE activity in adult mice (62).

Fetal sheep delivered by cesarean section have lower plasma ANG II levels when compared with animals born vaginally (11), suggesting that birth-related stress is associated with higher concentration of this metabolite in the immediate neonatal period. Limited studies, however, previously addressed the impact of postnatal stressors on the regulation of vascular tone. Maternal cross-fostering in the early postpartum period in rodents is associated with adult-onset systemic hypertension and metabolic dysfunction (43), as well as behavioral abnormalities (40). Changes in maternal pup-rearing behavior in rodents are known to promote neonatal humoral changes that result in altered stress response later in life (27). Thus there is reason to suspect that maternal cross-fostering via maternal-pup interaction disturbances results in neonatal stress. Yet, the mechanism accounting for these changes and whether they are limited to the systemic circulation are presently unknown and the main focus of the present study.

In hypothesizing that maternal cross-fostering promotes neonatal pulmonary vasomotor changes via RAAS, we comparatively evaluated control and fostered rat pups in the immediate postnatal period and later in life. The maternal behavior following cross-fostering was assessed continuously during the 1st wk postpartum, and the factors potentially affecting the pulmonary vasomotor regulation were evaluated. Cross-fostering promotes RAAS-dependent endothelial dysfunction, which results in short- and long-term PV changes.

MATERIALS AND METHODS

Animals

Time-bred Sprague-Dawley rats were studied from birth until adulthood. All procedures were conducted according to criteria established by the Canadian Council on Animal Care and were approved by...
the Animal Care Committee of The Hospital for Sick Children Research Institute.

Cross-Fostering and Maternal Behavior Monitoring

Cross-fostering. Female rats were bred and their litters culled to 12 pups, randomly assigned in the immediate postpartum period to be either raised by their biological mothers (control group) or by a foster doe. Cross-fostering was conducted as follows: two female rats were time bird such that their litters were born within <24 h of each other. On the morning of the second postpartum day, the mothers were swapped, such that they were no longer raising their biological pups. Daily pup body weight was obtained during the first 14 days of life by placing each pup on a laboratory scale with a 0.1-g precision accuracy. The mother and litter were maintained in a 12-h timed light-dark cycle quiet room, and the cages were cleaned twice/week. The mothers were fed a regular pellet diet, and water was provided ad libitum.

Maternal behavior. In a subset of animals, maternal behavior was videotaped continuously for the first 7 postpartum days. Footage was recorded continuously using two cameras positioned at different angles to ensure constant maternal-behavior tracking. The recorded videos were later scored by one of the investigators (S. Sadeghi) without knowledge of the group assignment. Maternal behavior was scored, as proposed by others (14), in one of five categories and the precise duration of each category recorded. The distinct maternal behavior categories used in this study were as follows: 1) arched-back nursing; 2) blanket nursing (laying over the pups in a blanket position); 3) passive nursing (passive nursing while in a supine or side position); 4) grooming the pups; 5) no pup contact. The distinct maternal behaviors are reported as a percentage of the total observation time over a 24-h period.

Organ Bath Studies and Fulton Index Determination

The rats were studied at 7, 14, and 60–90 (adult) days of age. The animals were euthanized with an overdose of pentobarbital sodium (50 mg/kg ip). The lungs were quickly removed and maintained on an ice bed; the heart was excised and maintained in 4% buffered paraformaldehyde for future determination of the Fulton index.

The near-resistance (third to fourth generations) intrapulmonary arteries were dissected free and mounted on a wire myograph (Danish Myo Technology A/S, Aarhus, Denmark). The muscle bath was filled with Krebs-Henseleit buffer solution (NaCl, 115 mM; NaHCO3, 25 mM; NaHPO4, 1.38 mM; KCl, 2.51 mM; MgSO4·7H2O, 2.46 mM; CaCl2, 1.91 mM; and dextrose, 5.56 mM), bubbled with air/6% CO2, and maintained at 37°C. After 1 h of equilibration, the optimal vessel resting tension was determined by repeated stimulation with 128 mM KCl until maximum active tension was reached. All subsequent force measurements were obtained at optimal resting tension.

PV muscle force generation was evaluated by stimulating with the thromboxane A2 mimetic U46619 or ANG II. Contractile responses were normalized to the tissue cross-sectional area as follows: (width × diameter) × 2, expressed as milli-newtons/square millimeter. The relaxant response to endothelium-dependent acetylcholine and the endothelium-independent nitric oxide (NO) donor S-nitroso-N-acetyl-penicillamine were determined after vascular muscle preconstriction with U46619 (10−6 M).

For the Fulton index determination, the hearts were dissected to obtain the right-ventricular (RV) wall and left-ventricle plus septum weights (4). The data are expressed as the RV/left-ventricular plus septum-weight ratio and used as a measure of RV hypertrophy.

Western Blot Analysis

Vascular endothelial cells were isolated from freshly dissected peripheral lung tissue by digesting with 1 mg/ml collagenase type II (Sigma-Aldrich, Oakville, Ontario, Canada) for 2 h at 37°C. The digest was then passed through a 70-μm cell strainer to remove tissue fragments, pelleted by centrifugation at 200 G for 10 min, and resuspended with 2% FBS (Gibco, Ontario, Canada) in PBS containing 5 μl biotinylated rat anti-mouse CD31 antibody (BD Pharmingen, San Diego, CA). After incubation on ice for 1 h, the endothelial cells were immobilized with streptavidin magnetic beads (New England BioLabs, Ipswich, MA). The endothelial cells were then placed on the EasySep magnet (Stemcell Technologies, Vancouver, BC, Canada) for 5 min and the unbound cells removed. Bead-bound endothelial cells were lysed in 10 mmol/l Tris-HCl, pH 7.4, lysis buffer containing 1% Triton X-100 and protease/phosphatase inhibitors (Roche Diagnostics Canada, Laval, Quebec, Canada) and centrifuged at 13,000 g for 30 min. Protein content was determined by the Bradford method using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Equal amounts of lysate proteins in Laemmli buffer were separated by SDS-PAGE, transferred onto polyvinylidene fluoride membrane, and immunoblotted using the following antibodies: mouse endothelial NO synthase (eNOS; 1:1,000; BD Biosciences, Franklin Lakes, NJ), mouse dihydrofuroate reductase (DHFR; 1:1,000; Cell Signaling Technology, Danvers, MA), mouse tubulin (1:5,000; Santa Cruz Biotechnologies, Santa Cruz, CA), anti-mouse IgG horseradish peroxidase (HRP) conjugated (1:10,000; Sigma-Aldrich), and anti-rabbit IgG HRP conjugated (1:5,000; Cell Signaling Technology). Detection was performed with the enhanced chemiluminescence reagent (PerkinElmer, Waltham, MA). Band intensities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD) and expressed relative to tubulin. Cell lysates from control and cross-fostered animals were run on the same gel for comparison, and nonessential lanes were removed during image processing.

eNOS Dimer/Monomer Ratio

To ascertain for the eNOS coupling state, lung tissue was dissected and homogenized immediately in cold lysis buffer containing 10 mmol/l Tris-HCl, pH 7.4, 1% Triton X-100, and protease/phosphatase inhibitors without boiling and separated on 4% lithium dodecyl sulfate-PAGE maintained at 4°C. Proteins were immunoblotted using eNOS antibody, as above described. The ratio of dimer over monomer expression was determined by measuring the Western blot-respective band densities using ImageJ software.

Lung H2O2 Content

The lung tissue H2O2 content was measured as a surrogate marker of superoxide generation. For this, lung tissue was homogenized in cold lysis buffer, followed by centrifugation at 13,000 g for 30 min, and divided into two groups. For the first group, the lysate H2O2 content was determined by the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Life Technologies, Carlsbad, CA), according to the manufacturer’s protocol. The second group of tissue homogenates was first preincubated with polyethylene glycol-superoxide dismutase (PEG-SOD; 250 U/ml) for 30 min. Absorbance of all samples was measured at ~560 nm using POLARstar Omega microplate reader (BMG LABTECH, Ortenberg, Germany). The difference between the absorbance of PEG-SOD-treated and untreated samples represents SOD-inhibitable H2O2 tissue content. Tissue total protein was determined by the Bradford method using the Bio-Rad protein assay (Bio-Rad Laboratories) and used for data normalization.
TurboIonSpray ionization probe source and coupled to an Agilent HPLC system (Agilent Technologies, Palo Alto, CA), was used. Heart tissue biopterin concentrations were determined by liquid chromatography electrospray tandem mass spectrometry. A triple quadrupole mass spectrometer API 4000 (Applied Biosystems/MDS SCIEX, Foster City, CA), operated in negative ionization mode with the Biopterin Measurements.

**Real-Time qPCR**

Total RNA was isolated from lung tissue using the PureLink RNA Mini Kit (Life Technologies), according to the manufacturer’s instructions. First-strand cDNA was synthesized from 1 μg RNA by SuperScript II RT (Life Technologies) and amplified by real-time quantitative (q)PCR using SYBR Select Master Mix (Life Technologies). Primers used for the RT-PCR assays are presented in Table 1.

**Bioprotein Measurements**

Tissue bioprotein concentrations were determined by liquid chromatography electrospray tandem mass spectrometry. A triple quadrupole mass spectrometer API 4000 (Applied Biosystems/MDS SCIEX, Foster City, CA), operated in negative ionization mode with the TurboIonSpray ionization probe source and coupled to an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA), was used. The method used a C18 column (150 × 2 mm id, 5 μm particle size, 110 Å pore size) with an isotropic solvent system of water with 0.002% formic acid at 0.3 mL/min and a run time of 13 min. Tissue tetrahydrobiopterin (BH4) and dihydrobiopterin (BH2) concentration were measured after differential iodine oxidation, as described previously (63).

**Echocardiographic Assessment of PVR Changes**

The inverse ratio of pulmonary arterial acceleration time (PAAT) to RV ejection time (RVET), as a surrogate of PVR, was measured by two-dimensional echocardiography/Doppler ultrasound, as described previously in detail (5, 22, 23). Briefly, a short axis view at the level of the aortic valve was obtained, and the pulmonary artery was identified using color-flow Doppler. The PAAT was measured as the time from the onset of systolic flow to peak pulmonary outflow velocity and the RVET as the time from onset to completion of systolic pulmonary flow.

**Lung ROS Generation**

Lung tissue homogenates were divided into two groups. Lucigemin was added to the first group of samples to a final concentration of 5 mM, and 50 ml of the homogenate-lucigemin mixture was transferred to individual wells of an opaque, white 384-well plate. To confirm further the specificity of the measurement for superoxide determination, the second group of tissue homogenates was preincubated with 0.1 mM N^2^-nitro-L-arginine methyl ester (L-NAME) for 30 min at 37°C before adding lucigemin. The reaction was started by the addition of 100 mM NADPH. Chemiluminescence readings were recorded every 3 min over a period of 30 min in a POLARstar Omega microplate reader (BMG LABTECH). Background signals from buffer were subtracted from homogenate signals, and the resulting value was further corrected by determining the L-NAME-inhibitable values and normalized for protein concentration.

**Cross-Fostering Affects Maternal-Newborn Interaction**

Cross-fostering had no impact on the experimental pups’ breast-milk intake, as judged by the nonsignificant differences observed between control and experimental groups.

**RESULTS**

**Cross-Fostering Affects Maternal-Newborn Interaction**

Cross-fostering had no impact on the experimental pups’ breast-milk intake, as judged by the nonsignificant differences observed between control and experimental groups.

**Table 2. Pup body weight at 7 and 14 days of age for control and experimental groups**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Fostered</th>
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<tr>
<td><strong>14 Days</strong></td>
<td></td>
<td></td>
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<tr>
<td>Body weight, g</td>
<td>27.9 ± 0.4 (24)</td>
<td>27.7 ± 0.7 (35)</td>
</tr>
<tr>
<td>RV/body weight, ×10^-3</td>
<td>1.00 ± 0.03 (8)</td>
<td>1.18 ± 0.03 (10)*</td>
</tr>
<tr>
<td>LV + sept/body weight, ×10^-3</td>
<td>3.12 ± 0.08 (8)</td>
<td>3.54 ± 0.06 (10)*</td>
</tr>
<tr>
<td><strong>Adult</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>219 ± 8 (41)</td>
<td>241 ± 5 (61)*</td>
</tr>
<tr>
<td>RV/body weight, ×10^-3</td>
<td>6.63 ± 0.02 (8)</td>
<td>7.76 ± 0.02 (10)*</td>
</tr>
<tr>
<td>LV + sept/body weight, ×10^-3</td>
<td>2.37 ± 0.05 (8)</td>
<td>2.71 ± 0.05 (10)*</td>
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Values are means ± SE (number of pups). RV, right ventricular; LV, left ventricular; sept, septum. *P < 0.01 and †P < 0.05 compared with control animal values.

**Statistical Methods**

Data were first evaluated to determine Gaussian distribution by Skewness, Kurtosis, and Omnibus testing. Normally, distributed data were analyzed by repeated-measures, two-way ANOVA with multiple comparisons obtained by the Tukey-Kramer test or unpaired Student’s t-test when appropriate. The Mann-Whitney U-test was used for nonparametric data. Statistical significance was determined at P < 0.05. All statistical analyses were performed with the Number Cruncher Statistical System software (NCSS, Kaysville, UT). Data are presented as means ± SE.
in pups’ body weight at 14 days of life (Table 2). Yet, significant changes in maternal-pup interaction were documented in fostered litters. As shown in Fig. 1, during the first 72-h postcross-fostering initiation, the experimental pups experienced significantly less maternal contact and grooming when compared with animals raised by their biological mothers.

**Cross-Fostering Induces Early-Onset PV Endothelium-Dependent Changes**

We next evaluated the cross-fostering effect on the RV wall mass by comparatively measuring the Fulton index at 14 days of age (Fig. 2A) and later in adulthood (Fig. 2B). At both ages, fostered pups exhibited higher index values when compared with same-age control animals. The RV/body weight ratio was also increased significantly in fostered when compared with control animals of both ages (Table 2). No sex-dependent group differences in either the Fulton index or RV/body weight ratio were observed. Lastly, the left ventricular + septum/body weight ratio was increased in the experimental animals when compared with control rats of both ages (Table 2).

Echocardiographic PVR assessment showed no group differences for 14-day-old and adult animals (Fig. 3, A and B, respectively). Adult-fostered rats, however, exhibited a significant reduction in TAPSE, indicative of RV dysfunction, when compared with same-age control animals (Fig. 3C). No significant group differences in endothelium-independent pulmonary arterial relaxation response at either age (Fig. 6, A and D).

To evaluate the factors accounting for the cross-fostering effect on the pulmonary vasculature, we proceeded to study the animals’ pulmonary arterial smooth muscle contraction and relaxation potentials. Following agonist-induced contraction, a significant age-dependent group difference in dose response was observed (Fig. 5, A and B). Whereas no group differences were observed at 14 days of age (Fig. 5, A and C), adult pulmonary arteries exhibited a significantly increased \( P < 0.01 \) dose response to ANG II and thromboxane \( A_2 \) analog U46619 when compared with control animals (Fig. 5, B and D). Tempol, a reactive oxygen species (ROS) scavenger, reduced the fostered adult animals’ U46619-induced dose response to a level comparable with control rat values (Fig. 5E).

A distinct age-dependent difference in pulmonary arterial relaxation potential was also observed between groups (Fig. 6). Compared with vessels from control-group animals, endothelium-dependent pulmonary arterial relaxation dose response was reduced significantly in 14-day-old fostered pups (Fig. 6A) but not adult animals (Fig. 6C). There was no significant group differences in endothelium-independent pulmonary arterial relaxation response at either age (Fig. 6, B and D).

**Fig. 1.** Cross-fostering effect on the maternal-pup interaction. Maternal behavior during the 72-h postcross-fostering \( (n = 3) \) or equivalent period for controls \( (n = 4) \) was determined by continuous video monitoring and offline scoring. Methodological details are provided in the text. Means ± SE. **\( P < 0.01 \)** compared with control group by unpaired Student’s \( t \)-test.

![Fig. 1. Cross-fostering effect on the maternal-pup interaction.](http://ajplung.physiology.org/)

![Fig. 2.](http://ajplung.physiology.org/)
Fostering is associated with lung eNOS expression downregulation and uncoupling, as well as increased ROS generation.

Given the abnormal endothelium-dependent pulmonary arterial relaxation response of 14-day-old fostered pups, we proceeded to investigate the mechanism responsible for this change. We first hypothesized that cross-fostering may induce age-dependent changes in lung vascular endothelial cell eNOS expression and/or uncoupling. Indeed, a marked decrease in lung endothelial cells’ eNOS expression was documented in fostered animals at 2 wk of age but not later in adulthood when compared with control group animals (Fig. 7).

We further evaluated the lung tissue ROS generation for control and fostered animals at both ages. When compared with control animals, a 14-fold increase in lung H₂O₂ content was documented in the 2-wk fostered pups, whereas no group difference was observed in adult animals (Fig. 8A). To confirm further that cross-fostering enhanced eNOS-dependent lung ROS generation, we measured tissue total and L-NAME-inhibitable superoxide levels at both ages using the lucigenin chemiluminescence assay (Fig. 8B). Compared with control animals, total chemiluminescence values were increased significantly in fostered animals at both ages. Yet, an L-NAME-inhibitable, lucigenin-derived signal was significantly higher in fostered pups at 14 days of age but not during adulthood.

These data led us to interrogate whether eNOS uncoupling was responsible for the increased ROS generation in fostered pups. Compared with control pups, the eNOS dimer/monomer...
ratio, a marker of eNOS uncoupling, was reduced significantly at 14 days of age in fostered animal (Fig. 9A).

Fostering-Induced eNOS Uncoupling Is Related to DHFR-Dependent BH4 Deficiency

Amongst other factors, reduced BH4 availability promotes eNOS uncoupling (17). To test whether cross-fostering-induced eNOS uncoupling was related to BH4 deficiency, we determined the lung tissue BH4/BH2 content ratio at 14 days of age in both groups. The lung BH4/BH2 ratio was significantly lower \( (P < 0.05) \) in fostered pups when compared with control animals at 2 wk of age (Fig. 9B), indicating tissue BH4 deficiency.

Since DHFR expression regulates tissue BH4/BH2 ratio by recycling BH2 into BH4 (33), we proceeded to interrogate whether the fostering-induced lung BH4/BH2 ratio changes were the result of a reduction in the PV endothelium DHFR content. Two-week-old fostered pups had significantly \( (P < 0.01) \) lower DHFR levels in lung vascular endothelial cells when compared with same-age control animals (Fig. 10A), whereas no significant group differences were found in adult animals (Fig. 10B).

Cross-Fostering Is Associated with Changes in RAAS Components Expression

Based on the previously reported RAAS involvement in the programming of systemic hypertension in rodents (47), we investigated whether a similar mechanism was operative in fostering-induced PV changes. Firstly, we evaluated mRNA expression of the ANG II receptors AT\(_1\) and AT\(_2\) and MAS. When compared with control animals, AT\(_1\) mRNA expression was increased significantly in fostered pups at 14 days of age and during adulthood (Fig. 11, A and B, respectively), whereas no group differences in AT\(_2\) (Fig. 11, A and B, respectively) and MAS were observed. Two-week-old fostered pups had significantly \( (P < 0.001) \) lower AT\(_2\) mRNA expression when compared with control animals (Fig. 11, A and B).
were documented. Sensitive decrease in lung ACE expression at 1 wk of age and AT1 receptor expression upregulation was a decrease in its tissue ANG II content. Since the lung ANG II levels are dependent on the expression/activity of ACE, we proceeded to measure the enzyme expression. The lung ACE mRNA expression was reduced significantly in fostered animals at 7 but not 14 days of age when compared with control pups (Fig. 12).

Given the ROS scavenger (Tempol) effect at reducing the U46619-induced, dose-response magnitude in fostered rats to a level comparable with control animals, we speculated whether the NADPH oxidase (NOX) pathway was involved in the increased ROS generation in the experimental group. We documented that NOX1, -2, and -4 isoforms of mRNA expression were increased significantly in adult-fostered animals compared with control animals by 2-way ANOVA and Tukey-Kramer multiple comparison testing. Possibly responsible for the fostering-induced lung AT1 receptor upregulation was a decrease in its tissue ANG II content. Since the lung ANG II levels are dependent on the expression/activity of ACE, we proceeded to measure the enzyme expression. The lung ACE mRNA expression was reduced significantly in fostered animals at 7 but not 14 days of age when compared with control pups (Fig. 12).

DISCUSSION

In the present study, we showed that cross-fostering results in a significant reduction in maternal-pup contact without affecting the animals’ breast-milk intake and weight gain during the first 2 wk of life. Fostered animals exhibited PV changes and RV hypertrophy at 2 wk of age that persisted into adulthood. The factors responsible for the fostering-induced PV changes involve RAAS and are age dependent. Fourteen-day-old fostered pups showed increased ROS generation and impaired eNOS-dependent pulmonary vasodilatation secondary to reduced eNOS expression and BH4 deficiency-induced enzyme uncoupling. These changes were associated with a transient decrease in lung ACE expression at 1 wk of age and AT1 receptor expression upregulation from 14 days until adulthood. Functionally, an increased pulmonary vasomotor response to U46619 and ANG II was documented later in life in fostered animals, and ROS scavenging normalized the U46619-induced enhanced vasoconstriction. Lastly, PV remodeling changes were present in fostered animals at 14 days of age and during adulthood.

There is evidence that adverse intrauterine conditions increase the risk of cardiovascular disease in humans (46, 47). Although the mechanism responsible for these changes is poorly understood, RAAS has been implicated in the fetal programming of late-onset systemic hypertension (8, 46). In newborn rodents, maternal separation-induced stress results in attenuation of the hypercapnia response (21), alterations in the hypothalamic-pituitary-adrenocortical axis (7), behavioral changes (30), and ANG II-mediated, adult-onset systemic hypertension (39). Limited studies, however, addressed the effect of neonatal stressors on the pulmonary vasculature.

Maternal pup-rearing behavior is known to modulate behavioral and hemodynamic regulation later in life in rodents (15, 43). In the present study, we showed that cross-fostering in the immediate postpartum period resulted in reduced maternal-pup interaction when compared with the animals reared by their biological mothers. We here propose that the neglect-like behavior exhibited by fostered mothers induced a significant neonatal stress that resulted in PV changes within the first 14 days of life that persisted until adulthood.

The RAAS pathway is developmentally regulated and thus susceptible to early-life stressors. The newborn ANG II plasma levels are highest immediately after birth and gradually decrease afterwards in humans (44) and other mammals (20, 50,

![Fig. 6. Pulmonary vasomotor relaxation. Endothelium-dependent [acetylcholine (ACH); A and C] and \(-\)independent [S-nitroso-N-acetyl-penicilamine (SNAP); B and D] vasorelaxation dose response in U46619 precontracted near-resistance pulmonary arteries from control and fostered 14-day-old (n = 14 and 18, respectively) and adult (n = 12 for both groups) animals. Data expressed as means ± SE. **P < 0.001 compared with control animals by 2-way ANOVA and Tukey-Kramer multiple comparison testing.](http://ajplung.physiology.org/)

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Log [SNAP] (M) vs. Log [ACH] (M) for 14 days and adult animals. Figure 6A shows the relaxation response to SNAP at 14 days and Figure 6B shows the relaxation response to ACh at 14 days. Figure 6C shows the relaxation response to SNAP at adulthood and Figure 6D shows the relaxation response to ACh at adulthood.
AT1 receptors modulate the ANG II-dependent pulmonary vasomotor tone. Inhibition of the AT1 receptor in newborn pigs blunts the hypoxic pulmonary vasoconstriction response (12). In rats, the lung tissue AT1 mRNA expression was reported by Morrell et al. (45) to be low early in life, whereas Gao et al. (28) showed that AT1 receptor protein expression in the same tissue is highest during the fetal and neonatal period. This apparent discrepancy likely reflects the well-known lack of specificity of commercially available AT1, AT2, and Mas receptor antibodies (6). As such, in the present study, we chose instead to use RT-PCR to evaluate mRNA expression changes of the distinct RAAS pathway components.

ACE expression in the rat lung is developmentally regulated and lower in the newborn when compared with adult animals (64). Lung ACE activity has been developmentally studied in rodents and found to be low at birth and progressively increased with maturation (61). RAAS has been widely recognized as an important player in the pathobiology of adult-onset pulmonary arterial hypertension (2, 42, 55). Chronic hypoxia (36)- and monocrotaline (35)-induced pulmonary hypertension in adult rats is associated with a reduction in ACE activity. In the fetal period, the ACE activity changes associated with pulmonary hypertension have been studied. In the nitrofen-induced prenatal rat model of diaphragmatic hernia and pulmonary hypertension, the lung ACE activity increases when compared with control animals (10). A reduction in lung ACE protein expression, however, was documented in fetal mice subjected to hypoxemia (31), a noxious stimulus capable of inducing pulmonary hypertension (41). Together, these studies suggest that ACE expression and/or activity are susceptible to change during the fetal-neonatal period and may play a role in the pathogenesis of pulmonary hypertension. In the present study, the fostered pups’ total lung tissue ACE mRNA expression was reduced at 1 wk but not 14 days of life. This finding suggests that the cross-fostering-induced decrease in ACE mRNA expression led to the upregulation of AT1.

Fig. 7. Lung endothelial nitric oxide synthase (eNOS) expression. eNOS protein in lung endothelial cells of control and fostered 2-wk-old (A; n = 3 and 4, respectively) and adult (B; n = 3 for both groups) animals, normalized to tubulin expression. Representative Western blots are shown. All samples from control and cross-fostered animals were run on the same gel, and nonessential lanes were removed during image processing. **P < 0.01 compared with samples of age-matched control animals by unpaired Student’s t-test.

Fig. 8. Lung reactive oxygen species (ROS) generation. Control and fostered 2-wk-old and adult lung tissue ROS measured as polyethylene glycol-superoxide dismutase-inhibitable H2O2 content (A) and total and Nω-nitro-L-arginine methyl ester (L-NAME)-inhibitable lucigenin-dependent chemiluminescence (B). Both assays’ data were normalized to tissue protein content. Means ± SE. A: 2-wk-old (control, n = 4; fostered, n = 3). Adult (control, n = 4; fostered, n = 4). **P < 0.01, compared with samples of age-matched control animals by unpaired Student’s t-test. B: n = 3 for both ages and groups. *P < 0.05 by 1-way ANOVA and multiple comparison testing.
receptor expression at 14 days of age via reduction in lung ANG II content during the 1st wk of life.

It is interesting to note that although AT1 receptor mRNA expression is increased in fostered animals at both 14 days of age and during adulthood when compared with controls, the pulmonary arteries’ ANG II-induced force response is only enhanced later in life. This likely reflects the reduced lung AT1 receptor expression documented by others during the neonatal period (45). Similarly, when compared with same-age control rats, the adult-fostered animals’ enhanced pulmonary vasocontraction to U46619 is likely mediated via NOX-derived ROS generation. This is so, since adult-fostered animals exhibited upregulated expression of NOX isoforms, and incubation with the ROS scavenger Tempol abolished the enhanced, U46619-induced vasocontraction in fostered animals in a similar manner as reported by others (16).

It is known that ANG II induces endothelial dysfunction via a reduction in endothelial cell BH4 content, thus facilitating eNOS uncoupling and ROS generation (13, 29, 48, 53). BH4 is an essential cofactor for eNOS activity that is crucial for eNOS stabilization in its coupled form. BH4 is formed de novo via a GTP cyclohydrolase I pathway or via a DHFR enzyme-dependent alternative salvage pathway. Previous studies in rodents showed that in the presence of DHFR dysregulation, the resulting BH4 deficiency and BH2 accumulation lead to eNOS uncoupling and are associated with systemic hypertension (13, 17, 33). Thus the present-study data suggest that the pulmonary arterial eNOS uncoupling and ROS production in 14-day-old fostered animals were caused by a transient DHFR dysregulation.

In the present study, we documented that fostering-induced PV remodeling was present at 14 days of age and continued into adulthood (Fig. 4). Such changes have been reported in association with RAAS upregulation in adult animal models of pulmonary hypertension and ameliorated by AT1 blockade (18). Following monocrotaline-induced pulmonary hypertension in adult rats, for instance, overexpression of Ang-(1–7) or DHFR / TubulinDHFR / Tubulin

Control Fostered

0 2

4 6

Control Fostered

Fig. 9. Lung eNOS uncoupling and biopterin content. Control and fostered 2-wk old rat lung tissue eNOS dimer/monomer ratio (A; n = 4 for both groups) and tetrahydrobiopterin (BH4) and dihydrobiopterin (BH2) ratio (B; n = 3 for both groups). A, inset: Western blot is shown. All samples were run on the same gel, and nonessential lanes were removed during image processing. *P < 0.05 and **P < 0.01 compared with control samples by unpaired Student’s t-test.

L1194 NEONATAL STRESS AND PULMONARY VASCULAR CHANGES

Fig. 10. Lung dihydrofolate reductase (DHFR) expression. Control and fostered 2-wk-old (A; n = 4 for both groups) and adult (B; n = 3 for both groups) lung endothelial cell DHFR protein normalized to tubulin expression. Representative Western blots are shown. All samples were run on the same gel and nonessential lanes removed during image processing. **P < 0.01 compared with control samples by unpaired Student’s t-test.
ACE2 attenuates the PV remodeling process and reduces RV hypertrophy (54).

The extent to which the PV endothelial, mechanical, and histological changes documented in fostered pups are indicative of the presence of pulmonary hypertension in these animals merits further discussion. On one hand, the higher Fulton index values in experimental animals, when compared with control rats, suggest a fostering-induced PVR increase. Yet, we failed to confirm this finding by echocardiography, suggesting that if a group difference in PVR at either age is present, then the magnitude of PVR increase is small.

Nevertheless, the echocardiographic assessment of PVR relies on the PAAT and RVET measurements that are dependent on myocardial systolic performance. In fostered adult rats, TAPSE, an echocardiographic parameter of RV function (37), was abnormally decreased when compared with control animals (Fig. 3).

Reduced TAPSE heralds the presence of RV dysfunction in fostered adult rats. Others have shown that RV remodeling, associated with increased afterload, occurs not only in response to PVR rise but also following enhanced pulmonary arterial stiffness (56). Thus the echocardiographic evidence of reduced TAPSE in fostered adult animals, in the absence of a group difference in PVR index, can be explained as follows. Firstly, fostered animals exhibited PV remodeling that was likely associated with increased pulmonary arterial stiffness, thus possibly accounting for the reduced TAPSE values. Secondly, in the swine pulmonary hypertension model, a decrease in TAPSE values was shown to be present in the early-adaptive RV-remodeling phase before the onset of heart failure (1).

Fig. 11. ANG II receptor expression in the lung. Two-week-old (n = 3/group) and adult (n = 6/group) control and fostered lung tissue ANG II receptor types 1 (AT1; A and B) and 2 (AT2; C and D), and Mas (E and F) receptor mRNA, normalized to their respective mRNA GAPDH expression. Means ± SE. **P < 0.01 compared with samples of age-matched control animals by unpaired Student’s t-test.

Fig. 12. Lung angiotensin-converting enzyme (ACE) expression. Control and fostered lung ACE mRNA normalized to their respective mRNA GAPDH expression at 7 and 14 days of life. **P < 0.01 compared with samples of age-matched control animals by unpaired Student’s t-test.
Lastly, in adult subjects with progressive pulmonary hypertension undergoing echocardiographic assessment, minimal increases in pulmonary arterial systolic pressure result in a significant decrease in TAPSE measurements (38). Taken together, the reduced TAPSE values documented in fostered adult rats, when compared with the control animals, are suggestive of a progressive increase in RV afterload without a significant rise in pulmonary arterial pressure at the time the measurements were obtained.

In summary, we demonstrated that cross-fostering interferes with the maternal-pup interaction and results in neonatal programming of PV changes. The mechanism involved in this process includes RAAS-mediated changes in the PV AT₁ receptor that promote endothelium dysfunction, ROS generation, and PV remodeling. Figure 14 outlines the pathways involved in the cross-fostering-induced PV changes.

The present findings have experimental and translational significance. Cross-fostering is commonly used to ensure adequate maternal milk supply when chronically subjecting newborn rodents to hypoxia (66) or hyperoxia (3) to induce pulmonary hypertension experimentally. The cross-fostering effects on the pulmonary vasculature ought to be distinguished from the chronic hypoxia/hyperoxia-induced pulmonary hypertension. Translationally, disturbance of the parental-newborn interaction is inevitable in infants requiring intensive care. The impact of parental-newborn disruption on the transition from fetal to neonatal life and the pathogenesis of PPHN merit further investigation.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


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**Fig. 13. Lung NADPH oxidase (NOX) expression.** Adult control animals (n = 54) and fostered rats (n = 3) NOX isoforms 1 (A), 2 (B), and 4 (C) mRNA expression, normalized to their respective mRNA GAPDH expression. **P < 0.01 compared with control data.

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**Fig. 14. Pathogenesis of fostering-induced PV changes.** The diagram outlines the mechanism by which cross-fostering induces an increase in PVR during the neonatal period and adulthood. TXA₂ (TP) R, thromboxane A₂ receptor.
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