Fetal Programming

Neonatal Oxygen Exposure in Rats Leads to Cardiovascular and Renal Alterations in Adulthood

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Abstract—Long-term vascular and renal consequences of neonatal oxidative injury are unknown. Using a rat model, we sought to investigate whether vascular function and blood pressure are altered in adult rats exposed to hyperoxic conditions as neonates. We also questioned whether neonatal O2 injury causes long-term renal damage, important in the pathogenesis of hypertension. Sprague-Dawley pups were kept with their mother in 80% O2 or room air from days 3 to 10 postnatal, and blood pressure was measured (tail cuff) from weeks 7 to 15. Rats were euthanized, and vascular reactivity (ex vivo carotid rings), oxidative stress (lucigenin chemiluminescence and dihydroethidium fluorescence), microvascular density (tibialis anterior muscle), and nephron count were studied. In male and female rats exposed to O2 as newborns, systolic and diastolic blood pressures were increased (by an average of 15 mm Hg); ex vivo, maximal vasoconstriction (both genders) and sensitivity (males only) specific to angiotensin II were increased; endothelium-dependent vasodilatation to carbachol but not to NO-donor sodium nitroprussiate was impaired; superoxide dismutase analogue prevented vascular dysfunction to angiotensin II and carbachol; vascular superoxide production was higher; and capillary density (by 30%) and number of nephrons per kidney (by 25%) were decreased. These data suggest that neonatal hyperoxia leads in the adult rat to increased blood pressure, vascular dysfunction, microvascular rarefaction, and reduced nephron number in both genders. Our findings support the hypothesis of developmental programming of adult cardiovascular and renal diseases and provide new insights into the potential role of oxidative stress in this process. (Hypertension. 2008;52:889-895.)

Key Words: hypertension ■ vascular dysfunction ■ developmental origin of adult onset disease ■ oxygen ■ angiotensin ■ microvascular rarefaction ■ nephron number

Premature babies, representing ≈8% of all births, have decreased antioxidant defenses and are exposed on birth to high oxygen (O2) concentration relative to the intrauterine milieu.1-2 This results in high O2-derived free radicals. Evidence in humans and animal studies indicate that premature newborns are more susceptible to oxidative tissue damage, leading to pathologies such as retinopathy of prematurity and bronchopulmonary dysplasia.3-4 However, the long-term vascular and blood pressure consequences of neonatal hyperoxic injury are unknown.

It is becoming increasingly evident that conditions early in life can influence adult diseases; however, the mechanisms are incompletely understood.5-6 Recent data suggest that perinatal oxidative stress may be the initiating trigger in long-term programming of cardiovascular function. In a previous study, we found that cellular antioxidant glutathione is decreased in the fetus of dams fed a low-protein (LP) diet during gestation. In that model, administration of the peroxidation inhibitor lazaroid to the pregnant dam prevented elevated blood pressure, vascular dysfunction, and microvascular rarefaction of the adult offspring.7 In spontaneously hypertensive rats, although considered a model of genetic hypertension, supplementation with antioxidants during gestation and early postnatal weeks resulted in persistent reduction of adult blood pressure.8

The current studies were undertaken to test the hypothesis that neonatal O2 injury causes long-term vascular damage and hypertension. Moreover, considering that the kidney plays a role in the development of hypertension and that altered renal development (resulting in lower nephron count) can be triggered by perinatal events, which contribute to adult blood pressure elevation, we also hypothesized that neonatal O2
injury causes long-term renal damage. Supporting these postulates, recent studies demonstrate higher blood pressure and smaller kidneys in former premature infants.9,10

Materials and Methods

Animals

Animals were used according to a protocol of the animal care committee of the Centre Hospitalier Universitaire Sainte-Justine in accordance with the principles of the Canadian Council on Animal Care Guide for the Care and Use of Experimental Animals.

Sprague-Dawley rat pups (Charles River, St-Constant, Québec, Canada) were maintained in 80% O2 (mixture of medical-grade 100% O2 and room air with an oxycycler A82OCV, Biosherix) from days 3 to 10 of life (O2-exposed group [H]; n=12 litters). To avoid maternal mortality associated with high O2 exposure, the dam was alternated every 12 hours with a surrogate mother of a litter maintained in room air (control group NH: pups in normoxia/mother partly in hyperoxia; n=12 litters). To control for the fact that an NH litter is fed by a dam subjected 12 hours per day to hyperoxia, additional litters were maintained in room air, and dams were interchanged every 12 hours (control group NN: pups in normoxia/mother in normoxia; n=12 litters). To control for the stress of interchanging the dams, a fourth series of litters was kept in room air (control group NNI; normoxia not interchanged; n=10 litters). Pups were weaned at 4 weeks of age to regular chow. Both male and female offspring were studied. No more than 2 animals per litter were used for each series of studies.

Experimental Procedures

Please see Figure S1 (available online at http://hyper.ahajournals.org) for the time line.

After habituation for 1 week, systolic and diastolic blood pressures and heart rate (HR) of the offspring were determined at 4 weeks and from 7 to 14 weeks (males) and to 15 weeks (males) of age by the tail-cuff method (50-001 Rat Tail Blood Pressure System, Harvard Apparatus). Rats were then euthanized for ex vivo vascular reactivity studies, and the aorta was also sampled in a subgroup of animals for evaluation of superoxide anion production (see below). Freshly excised carotid arteries rings from 16 to 20 weeks (males) and 20 to 24 weeks (females) of age were studied as described (using Dasylab 5.6 software, Data Acquisition System Laboratory).11 Four to 8 rings from 1 rat were used for 1 experiment. Cumulative concentration-response curves were generated with angiotensin II (Ang II; 1 pM to 1000 pM), or Krebs solution, carbachol, phenylephrine hydrochloride, U46619, and apocynin (Sigma Chemical), as well as Tempol (Fluka Chemika).

Statistical Analysis

Values are expressed as means±SEMs. Ex vivo concentration-response curves to Ang II, phenylephrine, and U46619 were analyzed by a computer fitting to a 4-parameter sigmoid curve using the Prism 3 program (GraphPad) to evaluate the EC50 and Emax, the maximum asymptote of the curve. Analysis of differences within and between groups was performed using 2-way repeated-measure ANOVA followed by post-ANOVA comparison among means using the Bonferroni test, as well as by Student t test for paired or unpaired observations. Statistical significance was set at P<0.05.

Results

The average number of pups per litter was 14.4±0.4. No significant difference in weight gain during O2 exposure was observed between groups (expressed in grams per rat per 7 days of exposure): H, 12.8±0.7; NH, 13.0±0.8; NN, 12.8±0.6; and NNI, 13.0±1.0. Thereafter, weight of H females was slightly but significantly decreased compared with NH, NN, and NNI (at 14 weeks, in grams: H, 300±10; NN, 318±6; NNI, 316±11; and NNI, 319±9). No difference in weight between male groups was observed (at 15 weeks, in grams: H, 553±21; NH, 543±20; NNI, 537±10; and NNI, 562±9). On average, 1 pup per litter was found dead from days 3 to 10 for all of the study groups. Survival was unchanged between groups throughout the entire study period.

Blood Pressure and HR

For both genders, blood pressures and HRs were similar between groups at 4 weeks. In males, systolic blood pressure was also similar at 7 weeks but was significantly higher by 9 weeks and persistently elevated throughout the remainder of the study period in the H compared with the NH, NN, and NNI groups; for diastolic blood pressure, values were significantly increased by 7 weeks of age in the H group versus NH, NN and NNI and remained elevated for the remainder of the study period (Figure 1A). In H females, systolic and cence,11 after 30-minute preincubation with Krebs solution, Krebs+Ang II (1 μmol/L), or Krebs+Ang II+apocynin (1 mmol/L; inhibitor of the assembly of the NADPH oxidase complex).

Evaluation of Vascular Production of Superoxide Anion by Hydroethidine

Aortic superoxide levels were evaluated in H and NH male adults by the oxidative fluorescent dye hydroethidine, as described.11

Nephron Count in the Kidney

At 25 to 35 weeks of age, a group of rats was anesthetized with pentobarbital. The left kidney was isolated and weighed to determine the number of nephrons per kidney, as described previously.14 Briefly, whole kidneys were incubated in 50% hydrochloric acid for 45 minutes at 57°C. Kidneys were rinsed with tap water and stored overnight at 4°C in a gauged flask. After mechanical dissociation, tubules and glomeruli were suspended in water. Three 0.5-mL aliquots were taken and placed in a hemocytometer-like chamber, and the glomeruli were counted microscopically by 3 investigators who were unaware of the specimen origin. The 3 results were averaged, and then this value was used to determine the total number of glomeruli in the sample and, therefore, in the kidney.

Chemicals

The following agents were purchased: Ang II, sodium nitroprussiate, carbachol, phenylephrine hydrochloride, U46619, and apocynin (Sigma Chemical), as well as Tempol (Fluka Chemika).
Diastolic blood pressures were significantly increased by 7 weeks compared with all of the control groups and for the remainder of the study period (Figure 1B). Blood pressures did not differ among the control groups within each gender. Overall systolic and diastolic blood pressures were higher \( (P < 0.01) \) in males compared with females within each study groups.

HR did not significantly differ between groups up to 12 weeks in males and 11 weeks in females. HR then gradually increased and was significantly higher in H rats versus NH, NN, and NNI for the remainder of the study period (Figure 1C and 1D).

**Ex Vivo Vasomotor Function in Adults**

For males and females, the maximal constriction of carotid rings generated by Ang II (Emax) was significantly increased in H rats compared with all of the control groups (Figure 2A and 2B). NH and NN dose-response curves were similar, whereas sensitivity of the vasoconstriction to Ang II was slightly but significantly decreased in NNI males only (logEC50 ± SEM: H, −7.90 ± 0.12; NH, −7.76 ± 0.11; NN, −8.01 ± 0.13; NNI, −7.12 ± 0.10; \( P < 0.05 \) NNI versus all of the other groups). Cumulative dose-response curves to thromboxane A2 analogue U46619 and to phenylephrine were similar for all of the groups in males and for H, NH, and NN in females (NNI females not tested; please see Figure S3). For males and females, superoxide dismutase analogue Tempol abrogated the increased vasoconstriction to Ang II in H animals to values not different from control groups (Figure 2A and 2B). Tempol also reduced the sensitivity of Ang II dose-response curve of H males to values not different to NNI males. Tempol had no effect in the NN and NH groups (data not shown; NNI Ang II + Tempol not tested).

For both genders, vasodilatation generated by the acetylcholine analogue carbachol was significantly impaired in H animals, and this was totally prevented by Tempol (Figure 2C and 2D); Tempol had no effect in NH and NN animals (data not shown; NNI carbachol + Tempol not tested). Responses to cumulative concentrations of sodium nitroprussiate, an NO donor, were similar for all of the groups for males, as well as for H, NH, and NN for females (NNI females not tested; please see Figure S4).

**Effect of Neonatal Oxygen Exposure on Vascular Production of Reactive Oxygen Species in Adults**

For both genders, a nonsignificant trend of higher baseline levels of superoxide measured using lucigenin-enhanced chemiluminescience was noted in H compared with NH animals (please see Figure S5A and S5B). Ang II increased superoxide generation to significantly higher levels in the H versus NH animals; coincubation of the aortic segments with apocynin prevented the increase in superoxide generation observed in the presence of Ang II. In the NH group, levels of superoxide production did not significantly differ between the different experimental conditions. Superoxide production evaluated by the oxidative fluorescent dye hydroethidine was markedly increased for both genders in aorta from H versus NH and NN groups (NNI not tested) and localized mainly in vascular smooth muscle cells (please see Figure S5C and S5D); no difference was observed between the NH and NN groups.
Effects of Neonatal Oxygen Exposure on Microvascular Density

Capillary density was significantly reduced in the tibialis anterior muscle from 4-week–old H (963 ± 46 g/mm²) versus NH (1405 ± 93 g/mm²) and NNI rats (1386 ± 110 g/mm²; n = 5 per group; P < 0.05 H versus NH and NNI; males only studied; NN not tested; Figure 3).

Effects of Neonatal Oxygen Exposure on the Final Nephron Number

Kidney (and total body) weight were significantly lower in females (versus males), but the ratio kidney/total body weight did not differ between gender and between experimental groups (Figure 4). For male and female H animals, the number of nephrons per kidney was significantly decreased compared with all of the control groups (by 25% versus NN and NNI). Nephron number was also significantly reduced but to a lesser extent (10% to 12%) in NH compared with NN and NNI animals.

Discussion

Findings from our study demonstrate that hyperoxic exposure of newborns has long-term vascular and renal consequences.
leading in adulthood to elevated blood pressure, vascular dysfunction associated with enhanced vascular production of reactive oxygen species, decreased microvascular density, and decreased nephron number. In addition, our data reveal a significant reduction in nephron number in adults that had remained in room air during the neonatal period but were fed by a dam exposed to high O$_2$.

In humans, fetal/neonatal programming of cardiovascular dysfunction is described mainly in low birth weight individuals and has been attributed primarily to intrauterine growth restriction but also to prematurity. Antioxidant defenses of premature and intrauterine growth restriction infants are deficient and less inducible, rendering them particularly vulnerable to oxidative damage. Reactive oxygen and nitrogen species are considered to be central factors in major complications of prematurity, including bronchopulmonary dysplasia, periventricular leukomalacia, and retinopathy of prematurity. More recent studies indicate that former prematurely born individuals also display cardiovascular dysfunction (elevated blood pressure and abnormal retinal vasculature) as adults. Mechanisms linking prematurity and cardiovascular dysfunctions associated with enhanced vascular production of reactive oxygen species, decreased microvascular density, and impaired renal function in former preterm children, this model is particularly relevant to examine long-term vascular consequences of neonatal oxidative injury of immature subjects.

One could hypothesize that H animals are more susceptible to stress-induced (by the tail-cuff method) elevation of blood pressure; however, odds that this accounts for all of the differences observed are small, because the difference was not present initially, and the same person took all of the measures, 2 to 3 times per week for each animal, and no “habituation” decrease in blood pressure was observed in either group.

The HR increased significantly in H animals of both genders compared with all of the control groups after the rise in blood pressure, which could suggest that increased HR is secondary to hypertension associated with afferent baroreceptor desensitization. Alternatively, HR can increase secondary to central activation of the efferent sympathetic nervous system input to the heart, mediated by blunted central regulation of the arterial baroreflex or primary increased activity. Both mechanisms can be mediated by Ang II and are plausible in the current experiments. In humans, Phillips and Barker also reported increased resting HR in adults with low birth weight.

Elevated blood pressure may result from vascular dysfunction, vascular structural changes, or perturbation in regulating systems, such as the kidneys and the brain. Carotid arteries, as studied in the current experiments, are not resistance vessels and cannot be assumed to participate in resulting blood pressure. Nevertheless, one can postulate that the vascular dysfunction observed in carotids of H adult rats is also present in resistance vessels. The enhanced maximal vasoconstriction, as well as sensitivity (for males), to Ang II in H animals seems mediated by exaggerated production of superoxide because, in the presence of Tempol, the vasmotor response to Ang II was nearly identical to that of the NNI group. Whether enhanced superoxide production by NN and NH vessels also prevails and mediates their increased sensitivity to Ang II compared with NNI is plausible but could not be evidenced by the current experiments.

Increased vasmotor response specifically to Ang II and enhanced NADPH oxidase-mediated superoxide production is present in other models of developmental programming of hypertension, such as in offspring of dams fed an LP diet or food restricted during gestation. The pattern distribution of endothelial nitric oxide synthase in the media of the aorta, as reported by others and previous studies by us. Whether vascular expression of Ang II type 1 receptors is increased in H adults, as in the LP model, and whether this could explain the enhanced vasoconstriction specifically to Ang II is unclear. Also, whether NADPH oxidase expression or activity is increased or whether superoxide anion scavenging systems are decreased is to be determined.

We did not examine arterial remodeling of the carotid arteries, but the similar response to KCl under different tension between groups indicates that tensional force capacity is unaltered by the current experimental procedure and, therefore, that arterial remodeling, if present, does not play a role in the observed vascular dysfunction. Furthermore, the enhanced vasoconstriction observed specifically to Ang II and not to Phe and U-46619 also supports this.

Impaired endothelium-mediated vasodilatation in H animals seems mediated by impaired NO synthesis or NO scavenging by superoxide (supported by attenuation of the impaired vasodilatation to carbachol of H animals in the presence of Tempol), because responses to NO-donor sodium nitroprussate were similar to controls. Alternatively, we cannot exclude a role for other endothelium-mediated vasodilators, such as prostacyclin and endothelium-dependent hyperpolarizing factor, in modulating the impaired vasodilatation. Whether elevated blood pressure could precede and be a causal element of the vascular dysfunction and superoxide production remains to be determined.

Vascular dysfunction was present in both male and female H rats; however, enhanced sensitivity to Ang II in H, NH, and NN versus NNI animals was observed only in males. Overall, blood pressures were also higher in males than females for all of the study groups. The mechanistic pathways underlying these gender differences are unknown. Similar differing patterns in blood pressures are reported in aging humans and animals, as well as in animal models of hypertension, and are attributed to sex steroids, their differing effect on vascular superoxide production, and expression of the renin-angiotensin-aldosterone system components. Interestingly, gender differences prevail in the antioxidant capacity of low birth weight babies and could contribute to the differential
incidence of morbidities in newborns and adults, as well as the differing mechanisms of hypertension programming between genders.\textsuperscript{30}

The vascular impact of early hyperoxic exposure has been studied in the small lung arteries of 2-week–old pups exposed to \textit{O}_2 since birth, where it leads to enhanced vasocontractile properties and decreased NO release and NO-mediated vasodilatation.\textsuperscript{31} However, whether these changes prevail in the systemic vasculature and persist into adulthood was so far unknown.

Microvascular rarefaction is a vascular structural anomaly reported in association with hypertension.\textsuperscript{32} We found a very similar degree of capillary rarefaction in 7- and 28-day–old, but not in fetal, LP rats,\textsuperscript{13} which was prevented by coadministration of the lipid peroxidation inhibitor lazaxorid to the LP-fed dams,\textsuperscript{7} suggesting early postnatal disruption in normal microvesSEL development. These, along with current data, also suggest that microvascular rarefaction can be a primary event in the development of hypertension associated with neonatal oxidative stress. In humans, microvesSEL rarefaction can be present at very early stages in (and even before) the development of hypertension in high-risk individuals.\textsuperscript{33,34} In individuals who suffered adverse conditions in perinatal life, reduced retinal vascularization and lower peripheral skin blood flow have been reported in young adults born at term but intrauterine growth restricted\textsuperscript{35} or born preterm (these individuals had not been diagnosed with formal retinopathy of prematurity during their neonatal period).\textsuperscript{17,36}

Intrauterine growth–restricted infants, as well as rats exposed to an LP diet during gestation or to partial uterine artery ligation, have fewer nephrons.\textsuperscript{37,38} Nephron number is decreased in adult patients with primary hypertension.\textsuperscript{39} These observations led to the suggestion that impairment of renal growth with resulting fewer nephron counts could play a role in the development (programming) of hypertension.\textsuperscript{40} In the current studies, the kidneys used for nephron count were from older animals than those studied for blood pressure and vascular reactivity. However, considering that nephrogenesis is completed by 5 to 8 days after birth in rats and that nephron count is not modified afterward, we do not believe that the older age of the animals studied has impacted on the results.\textsuperscript{19} Lower glomerular number in the H group suggests disruption of nephron formation by the hyperoxic insult. LP offspring exhibit increase in kidney nitrotyrosine staining early in life; treatment of the young rats with Tempol for 3 weeks prevents the increased renal nitrotyrosine staining and the elevated blood pressure.\textsuperscript{41} Oxygen tension can have a regulatory role in nephrogenesis through hypoxia-inducible factor,\textsuperscript{42} which regulates expression of genes involved in angiogenesis. In rat metanephric organ culture, DNA content, vasculogenesis, and tubulogenesis are enhanced when tissues are kept under low (1% to 3%) rather than standard (20%) oxygen concentration.\textsuperscript{43} Furthermore, reduced NO bioavailability interferes with hypoxic signaling and hypoxia-inducible factor expression.\textsuperscript{44}

In addition to the above-discussed findings, the current experimental design allowed other important observations. The significant reduction in nephron number of the NH group suggests that \textit{O}_2 breathing by the lactating mother can impact organ development in the pups through mechanisms that remain to be determined. We also observed that, among the 3 control groups, there were no differences in blood pressures; however, Ang II sensitivity of the NN and NH male groups was significantly enhanced compared with the NNI group. This suggests that perturbation of the care-providing mother in the neonatal period can impact on long-term vascular function of the male pups; whether this is mediated by neonatal and/or maternal stress and how is currently not known.

**Perspectives**

These experiments show that neonatal hyperoxic exposure results in decreased vascular tree development and nephron numbers that could participate in the observed hypertension in adults. Whether vascular dysfunction and renal changes are primary or secondary events to elevated blood pressure remains to be determined. Other elements could contribute to elevated blood pressure in this model: enhanced activity of brain renin-angiotensin system and effenter sympathetic activity, resistance arteries remodeling, and the role of other vasoactive systems, such as endothelin and endotheliumb-dependent hyperpolarizing factor. The results of the current study support the hypothesis of developmental programming of adult vascular and renal diseases by neonatal/perinatal oxidative stress and are of importance for the growing population of former premature born individuals.

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**Disclosures**

None.

**References**


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NEONATAL OXYGEN EXPOSURE IN RATS LEADS TO CARDIOVASCULAR AND RENAL ALTERATIONS IN ADULTHOOD

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**Figure S1:** Schematic time line of the presented studies.
Figure S2: Vasoconstrictor response of carotid artery rings from H and NH 16-20 week old male rats (n = 4 each group) to KCl (80 mM) under increasing tension. Data are mean ± SEM.
**Figure S3:** Vasoconstrictor response to thromboxane A2 analogue U46619 (A and B) and to phenylephrine (PHE) (C and D) of carotid artery rings from H, NH, NN and NNI 16-20 (male) and 20-24 (female) week old rats. Symbols legend as in Figure 1. Constriction is expressed relative (percent) to the response elicited by KCl (80 mM). Data are mean ± SEM of n = 5-6 rats per group.
Figure S4: Vasodilatation response to SNP of carotid artery rings from 16-20 week old H, NH, NN and NNI male (left) rats, and 20-24 week old H, NH and NN female (right) rats. Vasodilatation is expressed as percent reversal of U46619 (0.3 μM)-induced vasoconstriction. Data are mean ± SEM of n = 5 rats per group.
Figure S5: Superoxide levels measured by lucigenin-enhanced chemiluminescence in aortas from 16-20 (male; A) and 20-24 (female; B) week old H (■) vs. NH (□) rats in baseline conditions, after 5 minutes preincubation with angiotensin II (AngII, 1 µM) or with AngII plus apocynin (1 mM). RLU: relative light units. Data are mean ± SEM of n = 6 male and 5 female rats per group. * p < 0.05 compared with NH in same conditions; # p = 0.07 compared to H AngII plus apocynin. C and D: Representative sections of aorta from male NH (C) and H (D) rats, after treatment with hydroethidine (2 µM) (see Methods). Images were obtained with a laser scanning confocal microscope (LSM 510 laser scanning microscope, Zeiss) equipped with an argon laser. Fluorescence was detected with a 514-nm long-pass filter. Bar scale = 10 µm.