Activation of the Cardiac Renin–Angiotensin System in High Oxygen-Exposed Newborn Rats

Angiotensin Receptor Blockade Prevents the Developmental Programming of Cardiac Dysfunction

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Abstract—Newborn rats exposed to high oxygen (O₂), mimicking preterm birth-related neonatal stress, develop later in life cardiac hypertrophy, dysfunction, fibrosis, and activation of the renin–angiotensin system. Cardiac renin–angiotensin system activation in O₂-exposed adult rats is characterized by an imbalance in angiotensin (Ang) receptors type 1/2 (AT1/2), with prevailing AT1 expression. To study the role of renin–angiotensin system in the developmental programming of cardiac dysfunction, we assessed Ang receptor expression during neonatal high O₂ exposure and whether AT1 receptor blockade prevents cardiac alterations in early adulthood. Sprague–Dawley newborn rats were kept with their mother in 80% O₂ or room air (control) from days 3 to 10 (P3–P10) of life. Losartan or water was administered by gavage from P8 to P10 (n=9/group). Rats were studied at P3 (before O₂ exposure), P5, P10 (end of O₂), and P28. Losartan treatment had no impact on growth or kidney development. AT1 and Ang type 2 receptors were upregulated in the left ventricle by high O₂ exposure (P5 and P10), which was prevented by Losartan treatment at P10. Losartan prevented the cardiac AT1/2 imbalance at P28. Losartan decreased cardiac hypertrophy and fibrosis and improved left ventricle fraction of shortening in P28 O₂-exposed rats, which was associated with decreased oxidation of calcium/calmodulin-dependent protein kinase II, inhibition of the transforming growth factor-β (TGFβ)/SMAD3 pathway, and upregulation of cardiac angiotensin-converting enzyme 2. In conclusion, short-term Ang II blockade during neonatal high O₂ prevents the development of cardiac alterations later in life in rats. These findings highlight the key role of neonatal renin–angiotensin system activation in the developmental programming of cardiac dysfunction induced by deleterious neonatal conditions. (Hypertension. 2016;67:00-00. DOI: 10.1161/HYPTENSIONAHA.115.06745.)

Key Words: angiotensin receptors ■ animal model of human disease ■ cardiac remodeling ■ heart development ■ neonatal oxygen stress

Renin–angiotensin system (RAS) is a key component of cardiovascular and renal system homeostasis. RAS activation during development is well described and contributes to organogenesis and growth, especially in the cardiovascular and renal systems. Among RAS components, angiotensin (Ang) II is the major peptide acting during fetal and neonatal life. Both subtypes of Ang receptors are expressed during fetal development but have different patterns of expression during the fetal–neonatal transition. In rats, Ang type 2 (AT2) receptors are upregulated in the heart, large vessels, lungs, and kidneys during fetal development and progressively decline after birth, suggesting their contribution to fetal organogenesis. AT1 and Ang type 1 (AT1) receptors, on the other hand, are detected late in fetal development, increase soon after birth, and are considered to contribute mainly to tissue maturation, growth, and postnatal adaptation. This switching pattern of Ang receptors suggests that modifications of AT1/AT2 balance play a key role in different developmental stages. A disruption of this balance, depending on the stage of development, may negatively affect cardiovascular and renal homeostasis and contribute to the establishment of cardiovascular diseases.

Deleterious perinatal conditions, including preeclampsia, intrauterine growth restriction, and preterm birth can lead to developmental programming of cardiovascular risk factors and diseases. Preterm-born individuals in particular, whose numbers are growing in the population because of recent numbers are growing in the population because of recent...
advances in neonatal intensive care, develop higher blood pressure, cardiac shape alterations, and cardiac dysfunction in adulthood, indicating that this population is at risk to develop cardiovascular diseases later in life.

Because ex utero life is relatively hyperoxic compared with fetal development, we have used a rodent model of increased oxygen (O₂ 80%) exposure during the developmental time equivalent in humans to the last trimester of gestation to replicate premature ex utero marked rise in PO₂ (blood partial pressure in oxygen) occurring at preterm delivery. We have shown that this model develops hypertension and vascular dysfunction in adults, as well as cardiac fibrosis, hypertrophy, and left ventricular (LV) dysfunction. RAS activation also occurs in the heart of adult rats exposed to high O₂ as newborns and is characterized by an AT1/AT2 receptor imbalance, with prevailing AT1 receptor expression. Interestingly, cardiac alterations and RAS activation induced by neonatal high O₂ occur before the rise in blood pressure, suggesting a direct effect of high O₂ on heart development and on cardiac adaptation throughout development to adult life.

In addition, Ang II–mediated cardiac hypertrophy and fibrosis occurs predominantly through AT1 receptor stimulation and subsequent reactive oxygen species (ROS) generation, which triggers already known profibrotic signaling pathways, such as the transforming growth factor-beta (TGF-β) superfamily and downstream SMAD3 (mothers against decapentaplegic homolog 3). Ang II stimulation of ROS production in the heart also promotes calcium/calmodulin-dependent protein kinase II (CaMKII) oxidation and disruption of calcium (Ca²⁺) homeostasis, a mechanism directly involved in the development of impaired myocardium contractility. Further, alterations in the cardiac levels of angiotensin converting enzyme 2 (ACE2), a counter-regulator of RAS activation in the heart, may also be involved in the development of cardiac alterations induced by neonatal high O₂ exposure.

Therefore, to better understand the participation of RAS on the developmental programming of cardiac dysfunction, the 2 aims in this study were (1) to determine whether RAS activation occurs during neonatal O₂ exposure and ≤28 days (P28) in rats and parallels the development of cardiac dysfunction and remodeling; and (2) to determine whether neonatal short-term treatment with an AT1 receptor blocker, losartan, in the last 3 days of neonatal O₂ exposure (from days 8 to day 10 of life) prevents cardiac alterations in young rats at P28.

## Materials and Methods

The materials and methods are described in detail in the online-only Data Supplement.

### Animal Model

All experimental procedures were approved by the Animal Ethics Committee of the Sainte-Justine University Hospital (CHU Sainte-Justine) Research Center and followed the guidelines of the Canadian Council on Animal Care and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Litters with Sprague–Dawley pups (culled to n=12, 6/sex) were kept with their mother in 80% O₂ using an oxyccyler (ProOx P110, Biosherix, Lacona, NY) from P3 to 10 of life (group O₂) as previously described. To avoid maternal morbidity associated with O₂ toxicity, the dams were interchanged every 12 hours with a surrogate mother of a litter maintained at room air. Pups from the control group were maintained at room air with their mother (without interchange); we have previously shown that mother interchange does not affect pup growth and blood pressure. Only male offspring were studied.

Two experiments were performed in this study. In the first, to assess the gene expression of Ang receptors during O₂ exposure, hearts were extracted from neonatal control (room air) and O₂-exposed rats at P3 (before O₂), P5 (during O₂), and P10 (immediately after O₂) of life. No more than 3 animals per litter were used for each time point (2 litters/group, establishing a total n=6 rats per group/age). Hearts were rapidly extracted after decapitation under isoflurane anesthesia (2:1 O₂), LV and right ventricles (RV) were separated, frozen in liquid nitrogen, and kept at −80°C until RNA extraction and evaluation of AT1a, AT1b, and AT2 receptors mRNA expression by reverse transcription quantitative real-time polymerase chain reaction (see complementary information in the online-only Data Supplement).

In a second experiment, neonatal rats from control and O₂ litters were administered tap water (H₂O, 50 μL) or losartan (20 mg/kg/day; total volume of 50 μL) by gavage from P8 to P10 (last 3 days of O₂ exposure); this therapeutic time frame was chosen to avoid impact on nephrogenesis, which proceeds ≤7 days postnatal in rats. Pups were killed at P10 or P28. No more than 3 rats were used per treatment in each litter (3 litters/group, establishing a total n=9 rats/group/age). At P28, cardiac LV function and structure and pulmonary artery resistance were determined by 2-dimensional guided M-mode echography of LV short-axis and 2-dimensional guided pulse wave Doppler echography of the mitral valve and pulmonary artery flow. Hearts from the second experiment were extracted at P10 and P28 to evaluate AT1a, AT1b, and AT2 receptors, as well as ACE2, TGF-β1, SMAD3, and oxidized/total CaMKII were assessed by Western blot. The base of the heart was fixed in 4% paraformaldehyde for paraffin embedding and histomorphometry analysis (see complementary information in the online-only Data Supplement).

Researchers were blinded to the treatment group of the animals during all analyses.

### Statistical Analysis

Data are presented as mean±SEM in the table and figures. Data were analyzed by 2-way analysis of variance followed by Bonferroni post hoc test, considering oxygen exposure (O₂-exposed versus control) and age (for RAS assessment) or treatment (losartan experiment) as factors. Repeated measures analysis of variance was used to analyze growth curves. The software GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA) was used for all tests performed. The significance level was established at p<0.05.

### Results

#### Ang II Receptor Gene Expression During Neonatal High O₂ Exposure

Different patterns of AT1a, AT1b, and AT2 gene expression in the LV were observed during the first 10 days of life of room air controls versus high O₂-exposed rats. In controls, AT1a mRNA expression progressively increases from P5 to P10 (Figure 1A), whereas AT1b and AT2 mRNA expression were significantly decreased at P5 and P10 compared with P3 (Figure 1B and 1C). Neonatal high O₂ exposure led to an overall upregulation of all Ang receptor subtypes studied compared with controls at P5 and P10. Although AT1a mRNA showed a greater increase in O₂-exposed versus controls from P3 to P10, AT1b and AT2 remained upregulated in O₂-exposed compared with controls at P5 and P10 but at levels similar to P3 (Figure 1A–1C). Our results indicate that neonatal high O₂ exposure causes upregulation of Ang receptors.
during postnatal cardiac development and abrogates the natural downregulation of AT1b and AT2 in the LV.

**Neonatal AT1 Receptor Blockade**

**Body Growth and Kidney Development**

No significant changes in the mothers' body weight and food intake were observed between groups. Body weight of pups was assessed from P3 to P28, and renal histomorphometric analysis was performed at P10 (Figure S1 in the online-only Data Supplement). Neither O2 exposure nor losartan treatment impacted growth of the rats (Figure S1A). At P10, losartan treatment did not modify renal glomerulus area and renal cortex width (Figure S1B and S1C) in control and O2-exposed rats.

**Ang II Receptor and ACE2 Expressions After Blockade**

Ang II receptors AT1a, AT1b, and AT2 mRNA and protein expression were assessed in LV tissue at 2 different ages (Figure 2A experimental design): at P10 (immediately after losartan treatment/O2 exposure) and P28 (later impact of losartan treatment). In controls, losartan did not significantly impact AT1 receptor (mRNA and protein) expression at P10 and P28 (Figure 2B). At P10, losartan treatment blunted AT1 receptor protein and AT1b mRNA upregulation observed in O2-exposed pups. At P28, losartan prevented upregulation of AT1b in O2-exposed rats versus controls without significant impact on AT1 protein expression, whereas treatment upregulated AT1a mRNA expression in O2-exposed rats.

Figure 2C shows AT2 receptor gene and protein expression in the LV at P10 and P28. In controls, losartan treatment upregulated AT2 protein (but not mRNA) expression at P10 and had no impact at P28. In O2-exposed rats, losartan prevented upregulation of AT2 protein and mRNA expression at P10. At P28, O2-exposed rats had decreased AT2 receptor protein and mRNA expression in the LV, and although losartan increased AT2 protein expression in rats previously exposed to high O2, AT2 mRNA was not different between treated and nontreated O2-exposed groups.

ACE2 mRNA expression was further evaluated in the LV of control and O2-exposed rats by quantitative polymerase chain reaction (Figure S2). ACE2 mRNA expression was significantly decreased in the O2 group immediately after exposure (P10) and at P28. Losartan prevented this downregulation in the O2-exposed group at P10 and more markedly at P28. Losartan also decreased ACE2 mRNA at P10 in controls, although this effect did not persist at 28 days.

**Effect of Losartan Treatment on Cardiac Function and Hypertrophy**

Losartan treatment prevented the increased heart to body weight ratio, an index of cardiac hypertrophy, and the increased cardiomyocyte surface area in LV of P28 high O2-exposed rats (Figure 3A and 3B). Cardiac LV function and remodeling were assessed by echocardiography in rats at P28, 18 days after end of treatment and O2 exposure (Table). O2-exposed rats exhibited a 14% decrease in fractional shortening and a 12% increase in LV posterior wall thickness in diastole compared with controls, which were prevented by losartan treatment. LV mass index was also decreased by 11% in losartan-treated O2-exposed rats compared with nontreated rats exposed to high O2. No significant modification was observed in control rats treated with losartan.

Findings from pulse wave Doppler echocardiography of pulmonary artery flow were indicative of increased pulmonary artery resistance in O2-exposed rats. Pulmonary acceleration time (PAT) and the ratio between PAT and pulmonary ejection time were significantly decreased in O2-exposed rats. Losartan did not prevent the decrease of PAT/pulmonary ejection time ratio in O2-exposed rats, whereas in controls, losartan treatment decreased PAT with no effect on PAT/pulmonary ejection time ratio. Histomorphometric analysis of the RV wall at P28 showed increased cardiomyocyte surface area in O2-exposed rats; this RV hypertrophy was prevented by losartan (Figure 3B).

Interstitial fibrosis (Masson’s trichrome) was increased in the RV and LV (Figure 3C) of P28 O2-exposed versus control rats. Neonatal losartan treatment reduced interstitial fibrosis in the LV of O2-exposed rats, but not significantly in the RV fibrosis. In both LV and RV of control animals, losartan did not significantly impact interstitial fibrosis.

**CaMKII Oxidation and TGF-β as Potential AT1 Downstream Pathways Activated by Neonatal O2 Exposure**

Total and oxidized CaMKII expression was assessed in LV homogenates at P10 and P28 (Figure 4A and 4B). At P10, no difference was observed in total and oxidized CaMKII expression in O2-exposed compared with control rats. Losartan,
however, decreased oxidized/total CaMKII ratio in treated O₂-exposed versus nontreated (Figure 4A) rats. At P28, LV total CAMKII was decreased in O₂-exposed rats, whereas oxidized CaMKII was significantly increased, suggesting a late effect of neonatal O₂ exposure on CaMKII expression and Ca²⁺ homeostasis. Losartan blunted the increased oxidized/total CaMKII ratio but did not impact total CaMKII (Figure 4B).

TGF-β family mRNA and protein expression were assessed in the LV at P10 and P28. At P10, the mRNA expression of TGF-β subunits 1, 2, and 3 was not different between groups; however, losartan significantly decreased mRNA expression of subunits 1 and 2 in treated versus nontreated O₂-exposed, as well as TGF-β receptor 1 mRNA in treated O₂-exposed compared with control rats (Figure S3A). No difference was observed in TGF-β family and receptor mRNA expression at P28 (Figure S3B). The expression of TGF-β1 protein, however, was higher in O₂-exposed LV compared with controls at P10 but not different at P28; losartan treatment decreased TGF-β1 expression at both ages P10 and P28 (Figure 4C and 4D). No difference in SMAD3 protein expression was observed between groups at P10 (Figure 4E). At P28, although SMAD3 protein expression increased in control rats treated with losartan, in the O₂-exposed animals, losartan treatment decreased its expression (Figure 4F).

**Discussion**

The present study shows that neonatal high O₂ exposure is associated with activation of the RAS in the newborn heart and that treatment with the AT1 receptor antagonist losartan prevents the persistent changes in Ang receptor expression, cardiomyocyte hypertrophy, and LV fibrosis associated with neonatal O₂ exposure. These results indicate that the RAS, primarily AT1 receptor, is a key mediator of developmental...
cardiac remodeling triggered by deleterious neonatal hyperoxic conditions.

We had previously shown the determinant role of RAS, characterized by AT1/AT2 receptor imbalance with prevailing AT1 expression, in programmed hypertension and vascular and cardiac dysfunction of adult rats exposed to neonatal high O2.12,13 Importantly, cardiac AT1/AT2 imbalance was present in young rats (4 weeks), before the elevation of blood pressure. The current study further confirms a determinant role for early RAS activation (here in the neonatal period) on persistent changes in Ang receptor expression and in cardiac structure and function triggered by neonatal high O2 exposure.

Ang receptor ontogeny in rodents has been described by Shanmugam et al4,19 who showed that AT1 and AT2 receptors are differentially regulated and distributed in organs and tissues during neonatal development. The timing of the switch between AT2 and AT1 receptor predominance in fetal/neonatal heart may indicate a key stage of cardiac development susceptible to perturbations by unfavorable neonatal conditions. The latter is corroborated by studies showing that prenatal and early postnatal treatment with AT1 blockers and ACE inhibitors is associated with cardiac22 and kidney23,24 abnormal development. Cardiac hypertrophy and fibrosis and changes in Ang receptors have been reported in late fetal or young offspring subjected to deleterious fetal conditions25–27 To our knowledge, no study beside our previous one13 has reported cardiac expression of Ang receptors after neonatal hyperoxic exposure or other conditions associated with preterm infant environment. In adults, upregulation of AT1 receptors later in life is associated with structural and functional changes in the myocardium, as well as development of cardiovascular disease.28

Figure 3. Effect of losartan on cardiac mass, myocardial hypertrophy, and fibrosis. A, Heart to body weight index of cardiac hypertrophy. B, Right (RV) and left ventricular (LV) cardiomyocyte cross-sectional surface area and representative microphotographs of LV sections (subendocardium) stained with hematoxylin/eosin. C, Interstitial fibrosis in the RV and LV with representative microphotographs of LV sections (subendocardium) stained with Masson’s trichrome from control and O2-exposed P28 rats, treated with H2O or losartan. Scale bars, 50 μm. Data presented as the mean±SEM. *P<0.05 and **P<0.01 vs group indicated, n=6 to 9/group.
Hypertension

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blunts AT1b and AT2 postnatal regulation, the persistence of these receptors at the time of the losartan treatment may be related, if not directly contributing, to the observed regulatory effects. Losartan-mediated AT1 and AT2 downregulation was also previously shown in adult rats subjected to aortic coarctation.32 The authors postulate that losartan leads to the internalization of AT1 by stimulating the increase of circulating Ang II. Furthermore, the binding of Ang II could promote the internatalization of the ligand–AT1 receptor complex, protecting the ligand from degradation, decreasing the number of functional receptors.32 Our study corroborates this postulate by showing that losartan decreased the protein expression of both AT1 and AT2 receptors, what could contribute to downregulate RAS during the last days of O2 exposure and re-establish the Ang receptor regulation during this critical stage of neonatal cardiac development.

Losartan also inhibited key mechanisms related to cardiac remodeling and fibrosis, such as the TGF-β pathway, which we had previously described to be activated in the heart of adult rats exposed to neonatal high O2. The canonical TGF-β pathway contributes to cardiac fibrosis. In the heart, TGF-β is mainly derived from myofibroblasts; however, under Ang II stimulation, cardiomyocytes, endothelial cells, and immune cells present in the heart, or infiltrating cardiac tissue in response to Ang II, can also produce TGF-β, exacerabating extracellular matrix production and remodeling.13,34 Indeed, we showed that neonatal O2-exposed rats have increased cardiac interstitial fibrosis at P28, and losartan treatment decreased cardiac fibrosis, more markedly so in the LV versus RV.

Short-term treatment with losartan also prevented cardiac hypertrophy and dysfunction in P28 rats exposed to high O2 as newborns, similarly so in the LV and RV. In the O2-exposed RV, losartan treatment prevented cardiomyocyte hypertrophy without changes in pulmonary artery resistance, suggesting a direct effect of AT1 receptor in modulating cardiomyocyte hypertrophy independently of workload; high pulmonary artery resistance and pressure is a hallmark of chronic lung damage associated with this experimental model.35

We studied CaMKII as a potential mechanism regulated by the RAS and related to myocardial hypertrophy and contractility through the maintenance of Ca2+ homeostasis. Autophosphorylation or oxidation of CaMKII (such as in conditions with increased ROS) can maintain its activation, despite reduced cytosolic Ca2+ concentration, altering cardiac contractility.17 Ang II can disrupt Ca2+ homeostasis in cardiac cells by promoting CaMKII oxidation through increased ROS production.18,19 In vitro, treatment of cardiac ventricular and atrial cells with losartan prevented Ang II-induced increment of cytosolic Ca2+.36 In vivo, postmyocardial infarction treatment with losartan ameliorated LV function and improved myocyte Ca2+ homeostasis in rats without changing myofilament function.37 In the present study, losartan treatment prevented the increased oxidized/total CaMKII ratio in O2-exposed rats. Although we did not measure cardiac ROS in

Table. Echocardiographic Imaging Data Obtained in 28-Day-Old Male Rats Exposed to Neonatal High Oxygen (O2) and Controls (Room Air) Treated With Water (H2O) or Losartan From Days 8 to 10 (n=9/Group)

<table>
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<th>Parameters</th>
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<th>Losartan</th>
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<th>Losartan</th>
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<tr>
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<td>HR, bpm</td>
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<td>IVSd, mm</td>
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<tr>
<td>LVIDd, mm</td>
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<td>LVPWd, mm</td>
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<td>44±3</td>
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<td>LV mass, mg</td>
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<td>LV mass index, mg/g</td>
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<td>2.76±0.05†</td>
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<td>LV mitral valve</td>
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<td>E velocity, cm/s</td>
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<td>Pulmonary artery flow</td>
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<td>PAT, ms</td>
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Data presented as the mean±SEM. A indicates mitral A wave; E, mitral E wave; FS, fractional shortening; HR, heart rate; IVSd interventricular septal thickness in diastole; LV, left ventricle; LVIDd, LV internal diameter in diastole; LVPWd, LV posterior wall thickness in diastole; PAT, pulmonary acceleration time; and PET, pulmonary ejection time.

*P<0.05 vs control (H2O) group.
†P<0.05 vs O2-exposed treated with H2O, 2-way analysis of variance, and Bonferroni post hoc test.
current study, we can postulate that decreased CaMKII oxidation may be linked to lower ROS production resulting from Ang receptor blockade. These results also suggest that the positive impact of losartan treatment on cardiac function in O2-exposed rats could be linked to improved Ca2+ homeostasis through lower CaMKII oxidation. Intriguingly, at P28, both O2-exposed groups (treated and nontreated) had a significant decrease in total CaMKII, suggesting a prolonged effect of neonatal O2 exposure on the LV expression of this kinase.

Although our results suggest that CaMKII and TGF-β mechanisms are interconnected with the RAS activation in neonatal O2 exposure because of their changes in losartan-treated groups, whether they, instead, occur in parallel or are key drivers of the cardiac phenotype in this model would be confirmed by inhibiting these mechanisms individually in future experiments.

Losartan also prevented cardiac ACE2 downregulation in rats exposed to high O2 as newborns. ACE2 is a carboxypeptidase that metabolizes Ang II to Ang-(1–7), counter-regulating the Ang II/AT1 receptor. Interestingly, a study from Patel et al, treating ACE2 knocked out mice with Ang receptor blocker irbesartan, reversed cardiac dysfunction and was associated with decreased NADPH oxidase activity and superoxide production. Losartan may also prevent Ang II/AT1-mediated ACE2 shredding from the heart in O2-exposed rats. This mechanism was previously described in rats under Ang II infusion, in which Ang receptor blocker treatment has also preserved cardiac ACE2.

In summary, the current work confirms that neonatal high O2 exposure (from days 3–10 of life) leads to upregulation of AT1a, AT1b, and AT2 in the heart compared with age-matched rats maintained at room air. We have also shown

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**Figure 4.** Early and late effects of losartan treatment on calcium/calmodulin-dependent protein kinase II (CaMKII) expression, transforming growth factor-beta (TGF-β), and SMAD3 pathway. Representative images and quantitative analysis of total and oxidized CaMKII protein (A and B), TGF-β1 (C and D), and SMAD3 proteins expression (E and F) assessed by Western blotting in the LV of O2-exposed vs control rats at P10 and P28, respectively, treated with H2O or losartan (P8–P10). Data presented as the means±SEM. *P<0.05 and **P<0.01 vs group indicated, n=4 to 6/group.
that high O₂ exposure abrogates natural AT1b and AT2 downregulation, modifying the overall Ang signaling during neonatal development. Furthermore, this study reveals that the neonatal AT1 receptor is a major RAS component mediating cardiac remodeling and dysfunction observed later in life, mainly through activation of mechanisms related to myocardial hypertrophy, fibrosis, and Ca²⁺ homeostasis through activation of TGF-β/SMAD3 pathway and CaMKII oxidation, as well as ACE2 downregulation. Short-term treatment with losartan (administered after the completion of kidney development) prevented cardiac alterations and activation of AT1 receptor downstream mechanisms, with beneficial responses sustained into early adulthood in rats exposed to neonatal high O₂. These findings suggest that a therapeutic window exists during perinatal development that could allow preventing the developmental programming of cardiac alterations induced by neonatal high O₂ exposure in immature subjects.

**Perspectives**

We had previously shown the role of RAS in the adult cardiovascular disease induced by neonatal exposure to high O₂. The current study further shows that high O₂ exposure leads to neonatal activation of RAS, at an important heart development stage, and have a significant impact on later-life cardiac structure and function. Our study shows beneficial effects of early short-term RAS blockade. Our findings generate new concepts in the investigation of ideal strategies and time windows to treat and prevent developmental diseases caused by perinatal deleterious conditions. Short-term treatment with losartan, an AT1 receptor blocker, and the prevention of cardiac alterations into early adulthood suggest a modified RAS activation induced by early-life exposures, such as neonatal high O₂, and its perpetuation into later life, potentially contributing to maladaptive cardiac responses into adulthood. Further investigation into the mechanisms underlying alterations in the cardiac RAS activation and the cause of its persistence in rats exposed to neonatal high O₂ are needed and may contribute to a better understanding of the key perinatal factors mediating the developmental programming of cardiovascular disease in preterm-born individuals, a population susceptible to O₂-mediated stress.

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**Disclosures**

None.

**References**

Cardiac RAS Activation in Neonatal Developmental Programming

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What Is New?
- Neonatal high O₂ exposure upregulates Angiotensin receptors during cardiac development.
- Short-term treatment with losartan during neonatal high O₂ exposure is effective in preventing late cardiac remodeling and dysfunction.
- Angiotensin type 1 receptors, the downstream transforming growth factor-β/SMAD3 pathway, and calcium/calmodulin-dependent protein kinase II are key mediators of the developmental programming of cardiac alterations induced by neonatal high O₂ exposure.

What Is Relevant?
- Describing renin–angiotensin system as a major mechanism activated during neonatal high O₂ exposure, and a key player in the programming of cardiac dysfunction, supports treatments modulating renin–angiotensin system components to prevent developmental cardiovascular diseases in preterm-born individuals susceptible to premature high O₂ stress.

Summary
Neonatal high O₂ exposure in rats leads to early and late alterations in cardiac renin–angiotensin system imprint, with prevailing angiotensin type 1 receptor expression as a key mediator of developmental cardiac remodeling and dysfunction into adulthood. This study is relevant in that it addresses important mechanisms activated during neonatal life and their impact on cardiac development and identifies potentially effective strategies to prevent developmental cardiac alterations in individuals exposed to premature high O₂ and at risk to develop cardiovascular disease later in life.
Activation of the Cardiac Renin–Angiotensin System in High Oxygen-Exposed Newborn Rats: Angiotensin Receptor Blockade Prevents the Developmental Programming of Cardiac Dysfunction

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ACTIVATION OF THE CARDIAC RENIN-ANGIOTENSIN SYSTEM IN HIGH OXYGEN-EXPOSED NEWBORN RATS: ANGIOTENSIN RECEPTOR BLOCKADE PREVENTS THE DEVELOPMENTAL PROGRAMMING OF CARDIAC DYSFUNCTION

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SUPPLEMENTAL MATERIALS

Supplemental methods
References
Supplemental Figure S1
Supplemental Figure S2
Supplemental Figure S3
Supplemental methods

All experimental procedures were approved by the Animal Ethics Committee of the Sainte-Justine University Hospital (CHU Sainte-Justine) Research Centre and followed the guidelines of the Canadian Council on Animal Care and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Echocardiography

Left ventricle (LV) cardiac function and remodeling were determined by echocardiography in rats at 28-days of life under isoflurane anesthesia (2:1 O₂) as previously described¹⁻⁴ using an ACUSON CV70 ultrasound imaging system (Siemens Medical Solutions, Burlington, ON) equipped with a 12 MHz scan head. In brief, rats were weighed, anesthetized with 2.5% isoflurane mixed with O₂ at 1 L/min, the chest shaved and two-dimensional guided M-mode images were obtained from a short axis LV view at the papillary muscle level to assess the LV systolic function and remodeling. The LV internal diameter in diastole (LVIDd) and the interventricular septum and LV posterior wall thickness in diastole (IVSd and LVPWd) were measured. LV mass, LV mass index and fractional shortening (FS) were calculated as follows. LV mass (mg) = [(LVIDd + IVSd + LVPWd)³ - LVIDd³] x 1.055 x 0.8, where 1.055 is the density of the rat myocardium (in mg/mm³)⁵ and 0.8 a correcting factor to compensate for the overestimation of LV mass. LV mass index (mg/g) = LV mass/body weight. FS (%) = [(LVIDd + LVIDd/LVIDd) x 100.

Diastolic function was derived from analysis of the mitral valve flow pulse wave (PW) Doppler signal, obtained from the long axis LV view, by determining the peak E and A, the E/A ratio, the deceleration time and rate (DT and DR, respectively) of the E wave from the mitral valve PW Doppler spectrum, and the isovolumic relaxation time (IVRT).

Pulmonary artery (PA) resistance was estimated from analysis of the PA flow pulse wave (PW) Doppler signal, obtained from the long axis LV view, by determining PA acceleration time (PAT) and ejection time (PET), as well as their ratio as described by Thibault et al⁶.

Tissue Processing and Histological Analysis

Immediately after echocardiography imaging, rats were killed under isoflurane anesthesia (3:1 O₂). Hearts were rapidly removed, washed in potassium chloride (100 mM KCl in saline) to induce diastolic arrest, and weighed. Hearts (atria removed) from P10 rats were immediately frozen in liquid nitrogen (for molecular analysis) or immersion-fixed in 4% paraformaldehyde (PFA) for paraffin embedding and histomorphometry analysis. P28 hearts (atria removed) were transversely (short axis) cut; the LV and RV were separated and fixed for 24-48 hours in 4% paraformaldehyde. The apex of the separated LV was immediately frozen in liquid nitrogen.

Transverse cross-sections of LV and RV were paraffin-embedded and 5 µm sections stained with hematoxylin and eosin for the measurement of cardiomyocyte surface area. In addition, ventricular sections (5 µm) were stained with Masson’s Trichrome to evaluate cardiac fibrosis. For all histological analyses, three pictures were obtained randomly each from the sub-endocardium, the sub-epicardium and the mid-myocardium of the LV and RV. Cardiomyocyte size was evaluated in the sub-endocardium and sub-epicardium by measuring the perimeter and surface area of cells with a visible nucleus. Cardiac fibrosis was assessed by quantifying the blue staining pixels (corrected as % of total pixels) obtained from the Masson’s trichrome staining.
The software Image J 1.36b (http://rsbweb.nih.gov/ij/) was used for stereological analysis and pixel quantification as previously described\textsuperscript{7,8}.

The right kidneys from rats at P10 (after the completion of nephrogenesis) were fixed in 4% paraformaldehyde, cut in half (long axis sagittal cut), and embedded in paraffin. Five µm sections from the central region of the kidney (across the full coronal plane) were stained with hematoxylin and eosin. To assess renal cortex width, four images of the cortex and medulla were taken with a 10X objective for each section. Using image analysis software (Image J), the width of the renal cortex (from the cortico-medullary junction to the superficial edge of the outer renal cortex) was measured twice in each of the four images, and the average calculated for each kidney. To determine renal glomerulus size, a complete section from each kidney was systematically sampled (with a 40X lens) at a step length of 300 µm. At each field of view, Image J software was used to trace the perimeter of the Bowman’s capsule of every glomerulus (> 50 glomeruli were sampled for each kidney). The average cross-sectional area of the renal glomerulus was then calculated for each kidney.

**Western Blotting**

Hearts were homogenized in RIPA (Radioimmuno-precipitation assay buffer) buffer containing protease inhibitors (Na-deoxycholate 10%, EDTA 100 mM, SDS 10%, Complete mini (Roche) 0.05X, phenylmethanesulfonylfluoride (PMSF) 100mM, Igepal 10%). Antibodies against angiotensin type 1 (AT1) receptors (ab9391, 1/1,000 dilution, Abcam, Cambridge, MA; sc1173, 1/1,000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), angiotensin type 2 (AT2) receptors (sc-9040 and sc-7420, 1/1,000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), transforming growth factor (TGF)-β1 (ab64715, 1/1,000 dilution, Abcam, Cambridge, MA), SMAD3 (Small Mothers Against Dpp or Decapentaplegic homolog 3) (ab28379, 1/1,000 dilution, Abcam, Cambridge, MA), calcium/calmodulin-dependent protein kinase II (CaMKII) total and oxidized (GTX61641 and GTX36254, 1/1,000 dilution, GeneTex Inc, Irvine, CA) were used in this study. Antibody against β-tubulin (T0198, 1/2,500 dilution, Sigma-Aldrich Canada Co., Oakville, ON) was used as control. Protein bands were developed with an enhanced chemiluminescence substrate (PerkinElmer Inc, Waltham, MA) and quantified using Image J.

**Reverse transcription - quantitative PCR**

The mRNA expression levels of angiotensin receptors were determined by reverse transcription (RT) of total RNA followed by quantitative PCR (qPCR). Total RNA was extracted from LV using RNeasy Mini Kit (Qiagen Inc, Toronto, ON). One µg of total RNA was reversed transcribed using Omniscript RT Kit (Qiagen Inc, Toronto, ON) and qPCR was performed using SYBER Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) with a MX3000P Stratagene real-time PCR cycler (Agilent Technologies, Mississauga, ON, Canada). The following PCR conditions were used: DNA was denatured for 10 min at 95°C followed by 45 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 1 min. The following cDNAs were amplified with the primers indicated in parentheses. *Agtr1a* (AT1a) (forward 5’-CCAAGTCCCACTCAAGCCT-3’ and reverse 5’-TTGCCAGTGTGCTTTGAACC-3’), *Agtr1b* (AT1b) (forward 5’-GCACCTTTCTCTACCCGCCC-3’ and reverse 5’-CCTTCTCTGTCTCAACCTC-3’), *Agtr2* (AT2) (forward 5’-TGTGGCATTGACATCTTTG-3’ and reverse 5’-AGAAGTGGTCACCCGAG-3’), and ACE2 (forward 5’-CAGTCTGAGGACCATTAG-3’ and reverse 5’-TCCTGATGTAACTTCACT-3’). The 40S ribosomal protein S16 (*Rps16*) was used as
internal control (forward 5’-TCTGGGCAAGGAGAGATTTG-3’and reverse 5’-CCGCCAAACTTCTTGATTC-3’). Primers were designed to have a melting temperature (Tm) of 60°C and a 3’ GC clamp using Primer3.9

**TGF-β family mRNA expression**

The mRNA expression levels of TGF-β subunits 1, 2 and 3, as well as TGF-β receptor 1 were assessed using rat angiogenesis RT² Profiler PCR Array (PARN-024Z, Qiagen Inc, Toronto, ON). Total RNA was extracted from LV tissues of controls+H2O, O2+H2O and O2+losartan (n=4/group) using RNeasy Mini Kit (Qiagen Inc, Toronto, ON). cDNA templates were obtained through RT² First Strand Kit using 0.8 µg of RNA/sample followed by RT² SYBR Green qPCR Mastermixes (provided in the kit, Qiagen Inc, Toronto, ON) prepared according to manufacturer instructions. Data analysis was performed using RT² Profiler PCR Array Data Analysis version 3.5 (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php), are presented as fold change ± 95% confidence interval (CI), and differences between groups (calculated by the Profiler software) were considered statistically significant when \( P<0.05. \)

**ONLINE SUPPLEMENT REFERENCES**


Online Supplemental Figures

Supplemental Figure S1 – Growth curves and kidney development. A, growth curves showing body weight variations of rats exposed to neonatal high O₂ (O₂) or kept at room air (Ctrl) from P3 (before O₂ exposure) to P10 (immediately after O₂ exposure), treated with water (H₂O) or losartan (Los) from P8-10, and up to their maturation at 28 days. B, renal glomerulus area and C, renal cortex width measurements from kidney sections of high O₂-exposed rats or controls at P10, treated with H₂O or losartan (n=7-9 rats/group). Data presented as the mean ± SEM.
Supplemental Figure S2 – Early and late effects of losartan treatment on angiotensin-converting enzyme 2 (ACE2). ACE2 mRNA expression assessed in the LV of (A) P10 and (B) P28 controls and O₂-exposed rats, treated with H₂O or losartan from P8-10 by quantitative-PCR. Data presented as mean ± SEM. *P<0.05 and **P<0.01 vs. group indicated, n=4-6/group.
Supplemental Figure S3 - Early and late effects of losartan treatment on transforming growth factor-beta (TGF-β). TGF-β superfamily types 1, 2, 3 and receptor-1 expression in the LV of (A) P10 and (B) P28 controls and O₂-exposed rats, treated with H₂O or losartan from P8-10. Data presented as average fold change ± 95% CI. *P<0.05 vs. group indicated, n = 4/group.