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

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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)² - LVIDd²] 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 + LVIDs)/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\(^7,\)\(^8\).

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 \(\mu m\) 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 \(\mu m\). 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 glomeruli 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)-\(\beta\)1 (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 \(\beta\)-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 \(\mu g\) 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’-GCACTCTTTTCTACCGCCCT-3’ and reverse 5’-CACTTTCTCTGCTCAACCT-3’), \(Agtr2\) (AT2) (forward 5’-TGTGGCTTTGATCATTTG-3’ and reverse 5’-AGAAGTGGTTTTTGGCAAG-3’), and ACE2 (forward 5’-CAGCTGAGGAGGCGATATG-3’ and reverse 5’-TCCTGTGGGGGTATTTCT-3’). The 40S ribosomal protein S16 (\(Rps16\)) was used as
internal control (forward 5'-TCTGGGCAAGGAGAGATTG-3'and reverse 5'-
CCGCCAAACTTCTGGATTC-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

Supplemental Figure S1 – Growth curves and kidney development. **A**, growth curves showing body weight variations of rats exposed to neonatal high O\(_2\) (O\(_2\)) or kept at room air (Ctrl) from P3 (before O\(_2\) exposure) to P10 (immediately after O\(_2\) exposure), treated with water (H\(_2\)O) 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 O\(_2\)-exposed rats or controls at P10, treated with H\(_2\)O 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.