Supplement
[Category: Original Research article]

Vascular Cell-Specific Roles of Mineralocorticoid Receptors in Pulmonary Hypertension

Supplemental Methods

Animals: MR floxed, Cre negative littermates of the SMC-MR$^{+/+}$ mice treated with tamoxifen were used as controls for tamoxifen-induced SMC-MR$^{-/-}$ mice; MR floxed, Cre negative littermates (EC-MR$^{+/+}$) were used for comparisons with EC-MR$^{-/-}$ mice; and WT mice treated with placebo pellets were controls for the spironolactone treated mice (Supplemental Table S1). All control groups had similar hemodynamic measurements, the proportion of muscularized vessels and inflammation in the lungs, as well as the degree of collagen deposition in the RVs (six normoxic groups and 3 hypoxic controls groups [Control/Placebo, SMC-MR$^{+/+}$, and EC-MR$^{+/+}$], respectively). Therefore, data from these groups have been combined. To confirm the pooled analysis of RV fibrosis, a more specific comparison was performed, with each experimental group being compared with their respective controls (Supplemental Table 3). For analysis of gene expression in the RV, normoxic and sugen/hypoxia-exposed EC-MR$^{+/+}$ and EC-MR$^{-/-}$ littermates were compared.

Experimental model of pulmonary hypertension, Sugen/hypoxia model: Mice were injected once a week for 4 weeks with the VEGF receptor 2 antagonist Sugen 5416 (80 mg/kg) or diluent (DMSO) on day 1. Beginning on day 2, the Sugen-treated animals were exposed to 4 weeks of normobaric hypoxia 11.5% O_2 while the DMSO-injected mice were

exposed to control normoxia. This model was shown previously to produce significant pulmonary hypertension.[1]

Hemodynamic Measurements: At the end of the hypoxia exposure period, animals were anesthetized with pentobarbital (20 mg/kg ip) and ketamine (60 mg/kg im). The trachea was cannulated and lungs were ventilated with a rodent ventilator using room air. A 25-gauge needle attached to a Statham P23-G pressure transducer by a short segment of P-50 tubing was inserted directly into the RV using a transthoracic approach. A similar technique was used to record left ventricular (LV) pressures. At the end of the experiment, animals were euthanized with a pentobarbital injection (120 mg/kg ip). The thorax was opened immediately. Lungs were inflated for histological preparation, as previously described.[2] Heart was excised and chambers weighed and the Fulton index [RV/LV+Septum (S)] was determined as a measure of RV hypertrophy.[3]

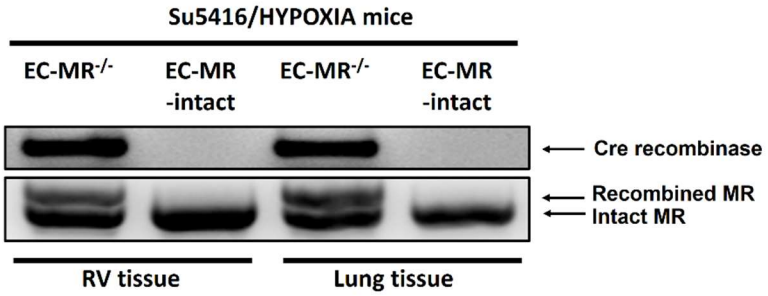
Assessment of RV cardiomyocyte cross-sectional area and collagen deposition: All analyses were performed by investigators blinded to genotype or treatment. 12-15 regions of photomicrographs covering the whole section of the RV were obtained and interrogated for myocytes cut in cross section and exposing the nucleus centrally. Cross-sectional area was measured using an Olympus CH2 microscope with a DP25 camera and DP2-BSW software (Tokyo, Japan). Perivascular and interstitial fibrosis were evaluated separately. For collagen fraction quantification, stained areas and myocyte areas from each section were determined using color-based thresholding.[4] The total fibrosis area was calculated as a percentage of total surface area, using image analysis

software (Image-Pro Plus 7.0) as the summed stained areas divided by total ventricular area.

RNA extraction and real-time Polymerase Chain Reaction (PCR) analysis: Total RNA of RV tissue was isolated by TRI reagent, and real-time PCR was performed as previously described,[5] using primers listed in Supplemental Table S2.

PCR analysis of Nr3c2 genomic DNA: EC-specific and SMC-specific MR gene recombination was previously characterized as described. Here we confirm Cre-specific recombination in tissues from the Sugen/hypoxia mouse model. DNA was extracted from hypoxic lung and RV tissues with the DNeasy kit (Qiagen) and PCR for recombined MR (454 base pair band) and intact floxed MR (364 base pair band) was performed as previously described,[6-8] using a combination of 3 primers (Supplemental Table S2).

Supplemental Data:



Supplemental Figure S1: MR gene recombination in Sugen/hypoxia-exposed mice with selective deletion of the mineralocorticoid receptor in endothelial cells.

RV and lung tissue were collected from EC-MR$^{-/-}$ and EC-MR-intact mice after exposure to Su5416/hypoxia and genomic DNA was isolated to determine the degree of MR recombination. Genomic DNA PCR was performed as described. Representative image of DNA gel electrophoresis showing that: 1) Cre band is present only in Cre positive, EC-MR$^{-/-}$ mice; 2) The intact floxed MR band is 364 bp and the recombined band is 454 bp; 3) MR gene recombination occurs only in the Cre positive animals. 4) There is intact MR in both tissues, consistent with the presence of non-endothelial cells in the heart (e.g., cardiomyocytes) and lung (e.g., pneumocytes). 5) The proportion of recombined MR is greater in the lung, consistent with the larger fraction of endothelial cells in lung compared to heart tissue.

Supplemental Table S1. Comparative groups in sugen/hypoxia conditions and their respective normoxic controls

Normoxia control	Sugen/Hypoxia
1.Control/Placebo	7.Control/Placebo
2.SMC-MR$^{+/+}$ tamoxifen	8.SMC-MR$^{+/+}$ tamoxifen
3.EC-MR$^{+/+}$	9.EC-MR$^{+/+}$
4.Control/SP	10.Control/SP
5.SMC-MR$^{-/-}$ tamoxifen	11.SMC-MR$^{-/-}$ tamoxifen
6.EC-MR$^{-/-}$	12.EC-MR$^{-/-}$

Normoxic control groups had similar hemodynamic measurements, proportion of muscularized vessels and inflammation in the lungs, as well as the degree of collagen deposition in the RVs. Therefore, the data from these six normoxic/control groups have been combined into a single control group for the figures and statistical analyses. Among the sugen/hypoxia groups, similar findings were noted in groups 7, 8, and 9 and these results were pulled. Abbreviations: Control/SP: Control/Spironolactone treated mice; EC-MR$^{+/+}$: Endothelial cell – Mineralocorticoid receptor intact mice; EC-MR$^{-/-}$: Endothelial cell – Mineralocorticoid receptor knockout mice; SMC-MR$^{+/+}$ tamoxifen: Smooth muscle cell - Mineralocorticoid receptor intact, Cre negative and tamoxifen treated mice; SMC-MR$^{-/-}$ tamoxifen: tamoxifen-

induced smooth muscle cell - Mineralocorticoid receptor knockout mice.

Supplemental Table S2. Primers used for Real time Polymerase Chain Reaction (RT-PCR) analysis of MR, inflammatory and fibrotic mediator gene expression analysis.

MR Gene Recombination Primers	
MR-4	CCA CTT GTA TCG GCA ATA CAG TTT AGT GTC
MR-5	CAC ATT GCA TGG GGA CAA CTG ACT TC
MR-3	CTG TGA TGC GCT CGG AAA CGG

Gene	**Forward Primer**	**Reverse Primer**
β-actin	TCTACGAGGGCTATGCTCTCC	TTTGATGTCACGCACGATTTCC
Collagen I A1	TACTCGAACGGGAATCCATC	GAGCGGAGAGTACTGGATCG
Collagen III A1	CTGTAACATGGAAACTGGGGAAA	CCATAGCTGAACTGAAAACCACC
E-selectin	TGACCACTGCAGGATGCAT	ATCCAACGAACCAAAGACTCG
Galectin-3	CAGTGCTCCTGGAGGCTATC	ATTGAAGCGGGGGTTAAAGT
ICAM-1	TGTTTCCTGCCTCTGAAGC	CTTCGTTTGTGATCCTCCG
TNFα	CATGAGCACAGAAAGCATGATCCG	AAGCAGGAATGAGAAGAGGCTGAG

Abbreviations: ICAM-1: Intercellular Adhesion Molecule 1; TNFα: Tumor Necrosis Factor α.

Supplemental Table S3. Analysis of RV perivascular fibrosis of each experimental Sugen/hypoxic group as compared to their respective normoxic controls

Group	**Normoxia control**	**Sugen/Hypoxia**
WT mice /Placebo	0.5 (0.2-3.1)	8.3 (7.7-13)‡‡‡
WT mice /Spironolactone	1.7 (1.2-2.2)	3.9 (2.7-6.85)‡,**
EC-MR$^{+/+}$	1.95 (0.6-3.3)	8.95 (6.4-10.6)‡‡‡
EC-MR$^{-/-}$	0.9 (0.6-2.4)	4.5 (3.3-7.7)‡,†
SMC-MR$^{+/+}$ tamoxifen	1.15 (0.9-1.4)	12 (4.9-13.6)‡‡‡
SMC-MR$^{-/-}$ tamoxifen	0.6 (0.5-3.0)	9.9 (7.8-13)‡‡‡

Abbreviations: WT: wild type; MR: Mineralocorticoid Receptor; EC: Endothelial Cell; SMC: Smooth Muscle Cell; EC-MR$^{+/+}$: EC-MR-intact mice; EC-MR$^{-/-}$: EC-MR knockout mice; SMC-MR$^{+/+}$ tamoxifen: SMC-MR-intact, Cre negative, tamoxifen treated mice; SMC-MR$^{-/-}$ tamoxifen: tamoxifen-induced SMC-MR knockout mice. Data are presented as mean ± SEM. All hypoxic groups exhibited increased collagen deposition compared with their respective normoxic controls (‡$p < 0.05$; ‡‡$p < 0.01$; ‡‡‡$p < 0.001$). When sugen/hypoxia groups were compared, both spironolactone and selective MR deletion in ECs attenuated the increase in collagen deposition (**$p = 0.01$ sugen/hypoxia/Spironolactone vs. sugen/hypoxia placebo; †$p = 0.03$ sugen/hypoxia EC-MR$^{-/-}$ vs. hypoxia EC-MR$^{+/+}$).

Supplemental References:

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