Long-Term Angiotensin II Receptor Blockade Limits Hypertension, Aortic Dysfunction and Structural Remodelling in a Rat Model of Chronic Kidney Disease

Supplementary Methods

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Supplementary Methods

In vitro organ bath experimental protocols

The following protocols were performed consecutively on individual aortic rings, with at least 20-min washout period allowed between different drug conditions:

Protocol I: Aortic ring contractility was assessed in response to cumulative concentration-responses to final bath concentrations of 1×10^{-10} – 3×10^{-4}M NA and then to 5 – 100 mM KCl.

Protocol II: Aortic rings were first precontracted with 1μM of the α_1-adrenergic receptor agonist PE. Upon achieving the maximum plateau response, endothelium-dependent relaxation was assessed using cumulative concentrations (1×10^{-10} – 1×10^{-4}M) of ACh. Following washout and return to baseline levels, the rings were again precontracted with PE and endothelium-independent relaxation evaluated using 1×10^{-11} – 1×10^{-5}M cumulative concentrations of SNP.

Protocol III: Cumulative concentration-responses to ACh were recorded in aortic rings precontracted with PE (1μM) preincubated for 20 min with either 10 mM L-Arg, 10 μM L-NAME or in the presence of both.

Protocol IV: **A**ortic rings were preincubated for 20 min with L-NAME (10 μM) and the non-selective cyclooxygenase inhibitor indomethacin (10 μM), and precontracted with PE (1μM) as previously described [1]. Following this, responses to cumulative additions of ACh were assessed.

Calculation of organ bath aortic responses

Contractile force responses to NA and KCl were normalized to the wet weight of the individual ring measured after completion of the experiment, as previously described [2] to give a measure in N/g. Concentration-response curves were fitted to a sigmoidal curve: Y=Lower plateau + (R_{max}- Lower plateau)/(1+$10^{(LogEC50-X)}$) where Y is the force response and X the drug concentration. The 50% effective concentration (EC_{50}) calculated from the sigmoidal curve fitting was used to evaluate sensitivity to vasoactive substances. The largest response induced by the agonists (NA, KCl, ACh and SNP) was considered the $R_{max\ and}$ % responses were calculated relative to it. The total vascular reactivity was represented by the area under the cumulative concentration-response curve (AUC) of the respective agonist [2, 3].

Time control experiments showed that the aortic rings retained comparable vascular responses over the course of the procedure (before and after experiment protocols: Lewis untreated NA R_{max} values; % 93.2±1.9 vs. 89.1±3.2 and N/g 2.4±0.4 vs. 2.2±0.5 N/g; P>0.05).

Biochemical assays and metabolic data

Urine samples collected over a 24-hour period were used for determination of urinary protein, creatinine and urinary protein: creatinine (UPC) ratio. All biochemical analyses were determined using an IDEXX VetTest® Chemistry Analyser (IDEXX, NSW, Australia). UPC was not calculated for those animals where urinary protein was <0.05 g/L, being below the detection limit of the analyzer. Urine flow rate (UFR) was determined [4] as: UFR (mL/min) = urine volume (L) / time (24 hours) × rat body weight (grams), and glomerular filtration rate (GFR) was estimated using the following equation: GFR (mL/min) = urinary creatinine (g/L) × urine volume (L) / plasma creatinine (μmol/L) [4].

Histomorphometry and immunohistochemistry

Internal and external diameters, media thickness and cross-sectional area (CSA) were measured. Wall thickness measurements were averaged from at least 8 locations evenly distributed around the

vessel circumference. Total elastin, collagen, and calcium were quantified by applying a threshold to construct a binary image from the appropriate colour (red, blue, black respectively) and measuring the percentage of red (elastin), blue (collagen) or black (calcium) occupied in the region of interest. A built-in software algorithm automatically counted nuclei. Density of elastin, collagen, calcium and nuclei were expressed as a percentage of total CSA. The elastin to collagen ratio was also calculated. The medial elastin network was characterised by the relative area occupied by elastin lamellae and interlamellae elastin, the mean thickness of each elastin lamella, and the mean interlamellae distance. Elastin lamellae fracture points were quantified by manual counting of lamellae elastin discontinuation around the aortic section, which was normalized to the number of elastin lamellae and the averaged inner and outer circumferences to control for the increased lamellae population, as per the following equation:

$$\frac{\text{Number of fracture points}}{\text{Average of inner and outer circumference} \times \text{number of lamellae}}$$

Quality control in the application of a threshold to obtain a binary image was ensured by visual inspection and comparison with the original image to evaluate the integrity of the customised automated software.

Elastin and collagen was also assessed using Weigert's method for elastic fibres and Sirius red-picric acid stain for collagen on paraffin-embedded cross-sections of abdominal aorta (5μm). Sections were analyzed by means of computer-aided histomorphometry by using of a ×40 objective (×660 on monitor) (BZ-II Analyser Biorevo BZ 9000 Keyence Japan). Aortic cysts, identified in the media layer, were additionally quantified in Sirius Red-stained sections by means of computer-aided histomorphometry. The total area of media in the microscopic field at x 40 magnifications was determined at 4 points around the aorta (12, 3, 6, 9 clock) in each animal. The cystic areas in these regions (detected as white stained area) were expressed as percent area of cystic degeneration/ area for each image and then averaged for each animal.

Immunohistochemical staining was assessed as the proportional area of media in the microscopic field at x 40 magnification from each group by applying a threshold (moments) to each image after first using a colour deconvolution plug-in within Image J for 3-amino-9-ethylcarbazole (AEC). The aortic medial wall was selected manually excluding the adventitia and endothelium.

Supplementary References

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