Online Supplement

A Novel Interaction Between Sympathetic Overactivity and Aberrant Regulation of Renin by miR-181a in BPH/2J Genetically Hypertensive Mice


Short title: Neural and Renin Contribution to Hypertension

Footnote on title page:
From the Baker IDI Heart and Diabetes Research Institute, Melbourne (K.L.J., A.M.D.W., K.P.R., T.-P. N.-H., P.J.D., G.A.H.); Department of Pharmacology, Monash University Melbourne (K.L.J., G.A.H.); School of Health Sciences, University of Ballarat, Ballarat, (F.Z.M., F.J.C.), Victoria; Basic & Clinical Genomics Laboratory, School of Medical Sciences and Bosch Institute, University of Sydney, Sydney, New South Wales (B.J.M), Australia

P.J.D. and G.A.H. are co-senior authors.

Correspondence to Geoffrey A. Head, Neuropharmacology Laboratory, Baker IDI Heart and Diabetes Research Institute, P.O. Box 6492, St Kilda Road Central, Melbourne, Victoria 8008, Australia. E-mail: geoff.head@baker.edu.au

Tel: +61-3-8532-1330 Fax: +61-3-8532-1100
Supplement Materials and Methods

Animals

The genetically hypertensive BPH/2J (n=24) and normotensive BPN/3J (n=27) male mice used in the present study came from inbred colonies bred at the Alfred Medical Research and Education Precinct Animal Centre (Generation 15-20) from breeders purchased at generation 20-36 from Jackson laboratories. The original breeding selection program, took place in the 1970’s for at least 23 generations and then brother sister mating followed to create these inbred strains ¹.

Animals in the present study were housed in individual cages in a room with a 12:12 hour light-dark cycle (1am–1pm light/day) with ad libitum access to water and mouse chow (Specialty Feeds, Glen Forrest, Western Australia; 19% protein, 5% fat, 5% fibre, 0.2% sodium).

Telemetry transmitter implantation

Blood pressure (BP) telemetry transmitters (model TA11PA-C10; Data Sciences International, St Paul, MN) were implanted under isoflurane open circuit anesthesia (5% induction and 1.5-2% maintenance) (Forthane, Abbott, Botany, NSW, Australia) delivered via oxygen. Carprofen (5mg/kg)(Rimadyl, Pfizer Australia Pty Ltd, West Ryde, NSW, Australia) was administered subcutaneously just prior to surgery and 24 hours post-surgery for analgesia. A lateral incision and blunt dissection were used to expose the left carotid artery which was temporarily occluded using a non-absorbable silk tie (Dysilk 1-0, Dynek Pty Ltd, SA, Australia). The catheter of the telemetry device was inserted into the carotid artery and secured using silk ties and the body of the probe was positioned subcutaneously along the right flank.² A subcutaneous continuous stitch using an absorbable suture (Polysorb, Covidien, Mansfield, MA) was used to close the incision. Mice were allowed at least ten days recovery prior to BP measurement. Experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific purposes.

Cardiovascular measurements

Cardiovascular and locomotor recordings were sampled at 1000 Hz using an analog-to-digital data acquisition card (National Instruments 6024E) as described previously.³ The beat-to-beat arterial pressure and heart rate (HR) were detected on-line and analyzed later using a program written in Labview.⁴

The cardiovascular effect of each drug or drug combination was assessed on separate days. Baseline cardiovascular parameters were determined during the light (inactive) period or during the dark (active) period at least 2 hours before or after lights off. The doses of drug administered in the present study were based on those reported to elicit depressor effects.⁵-⁷ Drugs were dissolved in isotonic 0.9% saline (vehicle) and freshly prepared each day.

Statistical Analysis

All cardiovascular data were analysed by a multi-factor, split-plot analysis of variance (ANOVA).⁸ A combined residual was used that pooled the between- and within-animal variance as described previously.⁹ For cardiovascular responses to pharmacological treatments the between-groups sums of squares was partitioned into main effects of treatment, strain (BPH/2J and BPN/3J), and their interaction (treatment x strain). Renal TH staining and RNA data were presented as mean ± SEM and Student’s t-test was used to compare between-strains differences. A P value of <0.05 was considered significant.
**Measurement of renin mRNA and miR-181a levels in the kidney**

Collection of the tissues at different times was as described previously. Briefly, age matched adult BPH/2J and BPN/3J mice (n=6/group) were killed with an overdose (100 mg/kg) of pentobarbitone (Lethobarb, Virbac Animal Health, NSW, Australia) during the dark (active) period at the peak of the circadian variation in BP, 2 hours after lights out, when average MAP difference between the strains is maximal (30 mmHg). Hypertensive BPH/2J (n=3) and age-matched BPN/2J mice (n=4) were killed in the same way during the light (inactive) period (2 hours after lights on) when the MAP levels of the BPH/2J and BPN/3J mice differ by only 16 mmHg. TRIzol® reagent (Life Technologies, Mulgrave, Australia) was used for RNA extraction according to the manufacturer’s recommendations. Complementary DNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit for total cDNA and the TaqMan® MicroRNA Reverse Transcription Kit for miRNA cDNA (Life Technologies). Amplification reactions used the TaqMan® Fast Advanced Master Mix (Life Technologies). TaqMan probes were used for gene expression to assess *Ren1* mRNA (assay Mm02342887, Life Technologies) and mature miRNA miR-181a levels (assay 000480, Life Technologies), together with reference genes. Samples were run in duplicates. A quantitative real-time PCR (qPCR) system (model ViiA™ 7 qPCR, Life Technologies) and the ∆∆C_T method were used to determine the levels of *Ren1* mRNA and miR-181a.

**Kidney tyrosine hydroxylase (TH) staining**

Hypertensive BPH/2J (n=4) and normotensive BPN/3J mice (n=4), were anesthetized deeply with an i.p. injection of 100 mg/kg pentobarbitone (Lethobarb, Virbac Animal Health) in the active period, 2 hours after lights out. Mice were perfused with 20 ml of 0.9% saline then 60 ml of 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO) dissolved in 0.1 mol/L phosphate buffer (PB), pH 7.2. Kidneys (n=4/group) were cryopreserved in 20% sucrose overnight before embedding in paraffin. Four micrometer sections were processed as described previously; briefly endogenous peroxidase was blocked (10 minutes, 3% H_2O_2/TRIS-buffered saline (TBS)), sections were blocked in 10% normal horse serum/TBS, followed by endogenous avidin-biotin blocking (Avidin-Biotin blocking kit, Vector Laboratories, Burlingame, CA). Sections were incubated in rabbit anti-TH (Millipore Australia, North Ryde, NSW, Australia) overnight, then incubated with biotinylated anti-rabbit (Vector) and visualized with 3,3′-diaminobenzidine tetrahydrochloride/H_2O_2 (DAB; Sigma-Aldrich, St Louis, MO). The percentage of TH staining in cortical tubules was semi-quantitatively assessed with 10 images per animal captured under identical light/exposure (Olympus BX-50, Olympus Optical; Q-imaging MicroPublisher 3.3 RTV camera, Surrey, BC, Canada). The percentage area of the image that stained positively was assessed in a blinded manner as described previously (Image Pro-Plus 6.0 software; Media Cybernetics, Silver Spring, MD) based on red, green and blue channels.
References
S1. Hourly averaged data showing the circadian variation of MAP (mmHg), HR (beats/min) and activity (units) during the active (night) (outer panels) and inactive (day) (middle panel) phases in BPN/3J (●; n=10) and BPH/2J mice (○; n=11). Bar graphs on right represent average MAP, HR, and locomotor activity during the inactive (Day) and Active (Night) periods in BPN/3J (N) and BPH/2J (H) mice. Values are mean±SEM. For comparisons between strains across the entire 24 hours, *P<0.05; **P<0.01 and ***P<0.001.
S2. Line graph represents 5-minute averages of MAP, HR, and locomotor activity before and after administration of pentolinium (left) and pentolinium after enalaprilat pre-treatment (right) between BPN/3J (gray) and BPH/2J (black) mice during A, the active period, and B, the inactive period. Dashed vertical line represents time-point of administration. Shaded area represents the response period analyzed. Bar graphs are mean response ± SEM of MAP, HR, and locomotor activity to pentolinium (center) or pentolinium following enalaprilat pre-treatment (right) for BPN/3J (N) and BPH/2J mice (H). Squares on the far right indicate effect of treatment (T), strain (S) and treatment by strain interaction (T×S). Significance refers to between-strain difference in response and is shown as *P<0.05; **P<0.01; ***P<0.001.
S3. Line graph represents 5-minute averages of MAP, HR, and locomotor activity before and after administration of vehicle (left) and enalaprilat (right) between BPN/3J (gray) and BPH/2J (black) mice during A, the active period, and B, the inactive period. Dashed vertical line represents time-point of administration. Shaded area represents the response period analyzed. Bar graphs are mean response ± SEM of MAP, HR, and locomotor activity to vehicle (center) or enalaprilat (right) for BPN/3J (N) and BPH/2J mice (H). Squares on the far right indicate effect of treatment (T), strain (S) and treatment by strain interaction (T×S). Significance refers to between-strain difference in response and is shown as *P<0.05; **P<0.01; ***P<0.001
Human renin (REN) 3'UTR

3'UTR 5'-GGCCUCUCGCACCAGGCGGUGAGCCUGCUACAGGAGGAACACUACUAGAGAUGGCCCUU
CUGCCUGGCUUAGGCCCUACAGCAUGUUGGAUGGCUUGCUUGGCUUGCCUACAGGACUGGAGGGCUUG

[Diagram showing the sequences with positions for hsa-miR-181a-5p]

CCCUCUACUACUACUACUACUACU

Mouse renin 1 (Ren1) 3'UTR

3'UTR 5'-GGCCUCUCGCACCAGGCGGUGAGCCUGCUACAGGAGGAACACUACUAGAGAUGGCCCUU
GUGGCUCUUAGGCCCUACAGGACUGGAGGGCUUGCUUGGCUUGCCUACAGGACUGGAGGGCUUG
CACAGAGACCAGGACCUAACGAGUGGGCCUCUACUAACGAGUGUGGACACUUGGAGGG

[Diagram showing the sequences with positions for mmu-miR-181a-5p]

AAAUGUACCGUGCAUGAGAAGGAAACG

S4: Target sequence for annealing of the microRNA miR-181a with the 3'untranslated region of human and mouse renin mRNA.