Sympathetic Nervous System

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


Abstract—Genetically hypertensive mice (BPH/2J) are hypertensive because of an exaggerated contribution of the sympathetic nervous system to blood pressure. We hypothesize that an additional contribution to elevated blood pressure is via sympathetically mediated activation of the intrarenal renin–angiotensin system. Our aim was to determine the contribution of the renin–angiotensin system and sympathetic nervous system to hypertension in BPH/2J mice. BPH/2J and normotensive BPN/3J mice were preimplanted with radiotelemetry devices to measure blood pressure. Depressor responses to ganglion blocker pentolinium (5 mg/kg IP) in mice pretreated with the angiotensin-converting enzyme inhibitor enalaprilat (1.5 mg/kg IP) revealed a 2-fold greater sympathetic contribution to blood pressure in BPH/2J mice during the active and inactive period. However, the depressor response to enalaprilat was 4-fold greater in BPH/2J compared with BPN/3J mice, but only during the active period ($P=0.01$). This was associated with 1.6-fold higher renal renin messenger RNA (mRNA; $P=0.02$) and 0.8-fold lower abundance of micro-RNA-181a ($P=0.03$), identified previously as regulating human renin mRNA. Renin mRNA levels correlated positively with depressor responses to pentolinium ($r=0.99; P=0.001$), and BPH/2J mice had greater renal sympathetic innervation density as identified by tyrosine hydroxylase staining of cortical tubules. Although there is a major sympathetic contribution to hypertension in BPH/2J mice, the renin–angiotensin system also contributes, doing so to a greater extent during the active period and less during the inactive period. This is the opposite of the normal renin–angiotensin system circadian pattern. We suggest that renal hyperinnervation and enhanced sympathetically induced renin synthesis mediated by lower micro-RNA-181a contributes to hypertension in BPH/2J mice. (Hypertension. 2013;62:775-781.)

Key Words: hypertension ■ kidney ■ microRNAs ■ renin–angiotensin system ■ sympathetic nervous system

BPH/2J mice are a genetic model of hypertension developed by Schlager1 by crossing 8 normotensive strains and selecting for elevated blood pressure (BP). Normotensive BPN/3J control mice were bred concurrently by crossing randomly selected mice from the same base population. Recently, the mechanism of the hypertension has been recognized as neurogenic because ganglion blockade abolished the hypertension in BPH/2J mice.2 Furthermore, spectral analysis of BP revealed greater power in the autonomic frequency band, suggesting overactivity of the sympathetic nervous system (SNS), most prominently during the nocturnal active period.2 BPH/2J mice also display exaggerated day–night differences in BP, which are associated with greater neuronal activity in regions of the hypothalamus and amygdala known to be important for cardiovascular regulation.3

Given the recent success of renal sympathetic nerve ablation for the treatment of resistant hypertension,4 the importance of renal influence on the expression of neurogenic hypertension has been highlighted. Importantly, the peripheral renin–angiotensin system (RAS) is closely linked to renal sympathetic nerve activity (RSNA) via its ability to stimulate renin secretion,4 and also through angiotensin II–mediated facilitation of SNA.5 However, the interaction of the kidney and renal RAS with SNS-mediated hypertension in BPH/2J mice has not been investigated thoroughly. The role of the RAS has been examined in a variety of ways in BPH/2J mice including by measurement of messenger RNA (mRNA) in tissues and various pharmacological assessments.6–10 Iwao et al10 reported normal renin activity in plasma, kidney, and submandibular gland of BPH/2J mice, although others found greater renin activity...
in the submandibular gland of BPH/2J mice and 1.3-fold higher renal expression of angiotensin-converting enzyme (ACE) mRNA compared with BPN/3J mice. Furthermore, chronic angiotensin II type 1 (AT1) receptor blockade led to comparable BP reductions in both BPH/2J and BPN/3J mice, suggesting that the hypertension is independent of the RAS. However, chronic ACE inhibition caused an 8% greater hypertensive response in BPH/2J compared with BPN/3J mice. Thus, on the basis of these contrasting findings, it is unclear whether the RAS contributes to hypertension in BPH/2J mice or not. High-dose chronic losartan and chronic ACE inhibition with captopril are capable of inhibiting both the peripheral and the central RAS. Thus, a distinct contribution from the peripheral RAS to BPH/2J hypertension is unclear.

The aim of the present study was to determine whether the peripheral RAS contributes to the elevation in BP in hypertensive BPH/2J mice, either independently or through interactions with the SNS. We addressed this using radiotelemetry to determine the relative BP effect of pharmacological inhibition of the RAS and SNS or both. To delineate the contribution of the RAS and SNS to hypertension in BPH/2J mice as opposed to the contribution to normal BP maintenance, direct comparisons were made with normotensive BPN/3J mice. Although there may be some physiological differences between these 2 strains that are independent of BP, the advantage of directly assessing the effect on BP of inhibiting each system in both strains is that the contribution to hypertension can be examined. The ACE inhibitor enalaprilat was used to determine the contribution of the peripheral RAS because enalaprilat does not readily cross the blood brain barrier in the acute setting. Furthermore, because renin is rate limiting in the RAS, Ren1 mRNA concentration was assessed as a measure of renal RAS activation. Renin mRNA was used to reflect the state of renin production and hence dynamic contribution to BP within the 12-hour periods rather than measurement of renal renin protein or its surrogate, renin enzyme activity, which more closely reflect renin storage levels. We also measured the micro-RNA (miRNA) miR-181a because its human homolog has been shown to negatively regulate human renin mRNA and is reduced in the kidney in human hypertension. Tyrosine hydroxylase (TH) staining, a marker of sympathetic innervation, was used to quantify renal innervation.

**Methods**

**Animals**

Cardiovascular experiments were performed with age-matched normotensive BPN/3J (n=7–13) and hypertensive BPH/2J (n=7–11) male adult mice with an average age of 17 weeks. Experiments were approved by the Alfred Medical Research Education Precinct Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for Scientific Use of Animals, in line with international standards.

**Cardiovascular Measurements**

BP telemetry transmitters (model TA11PA-C10; Data Sciences International, St Paul, MN) were implanted as detailed in the online-only Data Supplement. Ten days after surgery, recordings of systolic arterial pressure, diastolic arterial pressure, calculated mean arterial pressure (MAP), heart rate, and locomotor activity were obtained in freely moving mice in their home cage. During the light (inactive) period and during the dark (active) period, cardiovascular parameters were measured for 30 minutes before and for 30 minutes after intraperitoneal injections of pentolinium (5 mg/kg; Sigma-Aldrich), and enalaprilat (1 mg/kg; Merck & Co). In addition, pentolinium (5 mg/kg) was administered 30 minutes after pretreatment with enalaprilat (1 mg/kg). The response to pharmacological treatment was represented by the difference between the control periods compared with 20 to 30 minutes after treatment.

**Measurement of Renin mRNA and miR-181a Levels in the Kidney**

Ren1 mRNA and miRNA-181a abundance were measured in BPH/2J and BPN/3J mouse kidneys collected during the dark (active) period (n=6 per group) and light (inactive) period (n=3–4 per group; for further details see Methods in the online-only Data Supplement).

**Kidney TH Staining**

TH staining was performed on kidney sections from BPH/2J (n=4) and BPN/3J (n=4) mice and the percentage of TH staining in the cortical tubules was semiquantitatively assessed (for further details see Methods in the online-only Data Supplement).

**Statistical Analysis**

Data were expressed as mean or mean change±SEM and analyzed by ANOVA (further details see Methods in the online-only Data Supplement). A P value of <0.05 was considered significant.

**Results**

**Baseline Cardiovascular Measurements**

Average 24-hour MAP and heart rate were higher in BPH/2J mice (n=10), compared with BPN/3J mice (n=11; Pstrain<0.001; Figure S1 in the online-only Data Supplement). During the dark (active) period BPH/2J mice had 27% higher MAP (Pstrain<0.001). During the light (inactive) period, MAP in BPH/2J mice was 17% higher than in BPN/3J mice (Pstrain=0.001).

**Effect of Pentolinium During the Dark Period**

Treatment with pentolinium alone reduced MAP in both BPH/2J (n=7; –39±4 mmHg; P<0.001) and BPN/3J mice (n=7; –28±3 mmHg; P<0.001). The BP reduction was 39% greater in the BPH/2J strain (Pstrain=0.04; Figure 1A).

**Effect of Pentolinium During the Light Period**

Pentolinium treatment induced depressor responses in BPN/3J (–11±1 mmHg; P<0.001; n=10) and BPH/2J mice (–11±4 mmHg; P=0.001; n=8), which were comparable between strains (Pstrain=0.9; Figure 1B).

**Effect of Pentolinium After Enalaprilat Pretreatment During the Dark Period**

Administration of pentolinium after enalaprilat treatment produced 56% greater depressor responses in BPH/2J (–58±4 mmHg; n=8) than in BPN/3J mice (–37±4 mmHg; n=9; P strain<0.001; Figure 1A). Enalaprilat pretreatment augmented the response to pentolinium by 33% in BPH/2J mice and 49% in BPN/3J mice (P treatment<0.001), and although there was an effect of strain (Pstrain<0.001), there was no strain-by-treatment interaction (Pinteraction=0.3).

**Effect of Pentolinium After Enalaprilat Pretreatment During the Light Period**

The depressor response induced by pentolinium after enalaprilat was 53% greater in BPH/2J (–50±3 mmHg;
n=7) compared with BPN/3J mice (–33±3 mm Hg; n=9; \(P_{\text{strain}}<0.001\); Figure 1B). Enalaprilat pretreatment augmented the depressor response to pentolinium by 3-fold in BPN/3J and 4.7-fold in BPH/2J mice (\(P_{\text{treatment}}<0.001\)), and although there was an effect of strain (\(P_{\text{strain}}=0.009\)), there was no strain-by-treatment interaction (\(P_{\text{interaction}}=0.1\)).

**Effect of Enalaprilat During the Dark Period**

After treatment with enalaprilat, MAP decreased in BPH/2J (–11±2 mm Hg; \(P<0.001\); n=9) but not in BPN/3J mice (–3±2 mm Hg; \(P=0.1\); n=8; \(P_{\text{strain}}=0.004\); Figure 1C).

**Effect of Enalaprilat During the Light Period**

Enalaprilat treatment elevated MAP in BPH/2J (+10±4 mm Hg; \(P=0.004\); n=7) but not BPN/3J mice (1±3 mm Hg; \(P_{\text{strain}}=0.05\); n=9; Figure 1D). Compared with the response to vehicle, there were marked effects of enalaprilat treatment (\(P_{\text{treatment}}<0.001\)), but no effect of strain (\(P_{\text{strain}}=0.2\)), and there was a strain-by-treatment interaction (\(P_{\text{interaction}}=0.04\)).

**Effect of Vehicle During the Dark Period**

Administration of vehicle elevated MAP in BPH/2J (5±2 mm Hg; \(P=0.02\); n=7) but not in BPN/3J mice (0±1 mm Hg; \(P=0.9\); n=9; Figure 1C). However, changes in MAP were similar between strains (\(P_{\text{strain}}=0.1\)).

**Effect of Vehicle During the Light Period**

After vehicle treatment, MAP was elevated in BPN/3J (18±2 mm Hg; \(P<0.001\); n=11) and BPH/2J mice (17±4 mm Hg; \(P<0.001\); n=8) to a similar extent in each strain (\(P_{\text{strain}}=0.8\); Figure 1D).

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Mean arterial pressure (MAP) response to administration of agents in BPN/3J (gray) and BPH/2J (black) mice. A, Response induced by pentolinium (left) and pentolinium after enalaprilat pretreatment (right) during the dark period and during the light period (B). C, Response induced by saline (left) and enalaprilat (right) during the dark period and during the light period (D). Each point represents the mean value averaged across a 5-minute period. The dashed vertical reference line represents the time-point of administration of treatment. Shaded area represents the period analyzed for comparison of the effect of treatment. Bar graphs represent average changes in MAP in response to agents in 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). Bar graphs values are mean±SEM. Significance refers to between-strain difference in response is shown as *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\).
Contribution of SNS and RAS to BP in BPH/2J and BPN/3J Mice

The relative contributions of the RAS and SNS to BP during the inactive and active periods were calculated using the differences among the pentolium, enalaprilat, and combination treatments as follows. The basal, that is, RAS and SNS independent, level of BP was taken as the BP reached after pentolium and enalaprilat. To take into consideration that injections involved disturbing the conscious mice, which itself induced mild increases in BP, the responses to drugs were compared relative with the effect of vehicle injection. The contribution of the RAS was taken as the difference between the BP achieved after enalaprilat relative to the BP reached after vehicle. The SNS contribution was taken as the remaining difference between this basal value and the level of BP observed after enalaprilat (Figure 2). The calculated contribution of the SNS to BP was 1.7-fold greater in BPH/2J mice compared with BPN/3J mice during both the inactive period (45 versus 26 mm Hg) and the active period (55 versus 33 mm Hg). The contribution of the RAS was calculated to be 2-fold greater in BPH/2J than BPN/3J mice during the active period (19 versus 9 mm Hg) and 0.6-fold during the inactive period (8 versus 14 mm Hg; Figure 2).

Ren1 mRNA and miR-181a Levels in the Kidney

Renal Ren1 mRNA in BPH/2J mice was 55% higher than in BPN/3J mice during the active period (P=0.01) and was 42% higher when compared with BPH/2J kidneys collected during the inactive period (P=0.07). In contrast, in BPN/3J mice, renal Ren1 mRNA levels were not significantly lower in the inactive period (P=0.6) and there was no difference between strains during the inactive period (P=0.4; Figure 3A). Renal miR-181a was 33% lower during the active period in BPH/2J compared with BPN/3J mice (P=0.04). Furthermore, renal miR-181a in BPH/2J mice during the active period was 53% lower than during the inactive period (P=0.005), although miR-181a in BPN/3J mice was comparable during the active and inactive periods (P=0.2). Moreover, miR-181a levels were comparable between strains during the inactive period (P=0.9; Figure 3B).

Correlations

There was a negative correlation between Ren1 and miR-181a values for each animal (r=−0.52; P=0.04; Figure 4A). The mean inactive and active depressor response to pentolium in each strain exhibited a strong positive correlation with Ren1 (r=0.99; P<0.001; Figure 4B) and a trend toward a negative correlation with miR-181a (r=−0.85; P=0.07; Figure 4C). The level of renal miR-181a showed a negative correlation with resting MAP (r=−0.92; P<0.04; Figure 4D).

TH Staining

The percentage of TH staining in kidney tubules was greater in BPH/2J (26±2%; n=4) compared with BPN/3J mice (19±1%; n=4; P=0.03; Figure 5).

Discussion

Our study found that during the active period, when the hypertension is greatest in BPH/2J mice, acute ganglion blockade and ACE inhibition each produced greater falls in BP. This indicated greater contributions to BP in BPH/2J mice from both the SNS and the peripheral RAS. In contrast, during the inactive period when hypertension in BPH/2J mice is least evident, there remains a greater contribution from the SNS, as determined by ganglionic blockade, but only a minimal contribution from the RAS. Importantly, the greater contribution of the RAS during the active period was associated with greater renal Ren1 mRNA expression and lower levels of renal miR-181a, a negative regulator of renin mRNA. We also observed a 1.4-fold greater abundance of renal sympathetic nerve fibers in BPH/2J mice, as identified by greater TH staining. Taken together, these findings suggest that although the SNS is a major contributor to hypertension in BPH/2J mice, the RAS also contributes to the BP elevation, doing so more during the dark (predominantly awake) period and less during the light (predominantly asleep) period. This is the opposite of the circadian
pattern of normotensive rodents and humans, where the contribution of the RAS to BP is lower during the awake period and greater during sleep.\textsuperscript{17,18} The reversed renin pattern is a likely additional factor besides the overall greater SNS activity in contributing in part to the hypertension in BPH/2J mice. The mechanism could possibly involve renal hyperinnervation, as well as enhanced sympathetically induced renin synthesis mediated by a diminution in miR-181a, a negative posttranscriptional regulator of Ren1 mRNA.

**Contribution of SNS to Hypertension in BPH/2J Mice**

The present study confirms our previous finding that the SNS drives the BP elevation in BPH/2J mice during the active period.\textsuperscript{2} During the inactive period, the effect of ganglion blockade alone suggests comparable contributions of the SNS to BP maintenance between strains. However, ganglion blockade with prior ACE inhibition revealed compensatory effects mediated by the peripheral RAS which mask the full contribution of the SNS to BP maintenance. Importantly, the unmasked contribution of the SNS to BP maintenance is consistently 1.7× greater in BPH/2J compared with BPN/3J mice independent of the circadian period (dark/light), suggesting an elevated tonic sympathetic drive. In support, prior analysis of BP variability has indicated that vasomotor SNA is elevated in BPH/2J mice.\textsuperscript{2} The present study is also the first to demonstrate that renal sympathetic innervation is greater in BPH/2J compared with BPN/3J mice, as indicated by TH staining in kidneys.

**Contribution of RAS to Hypertension in BPH/2J Mice**

Our finding that the peripheral RAS contributed to 44% of the BP elevation in BPH/2J mice during the dark (predominantly awake) period, but made little contribution during the light (predominantly asleep) period, is opposite to other findings which show peripheral RAS activity peaks during the sleep period and decreases during the awake period.\textsuperscript{17,18} This overactivity of the RAS in BPH/2J mice during the active period suggests that there is not an inherent tonic overactivity of the peripheral RAS, but more likely an abnormal regulation of the RAS during the active period. The greater depressor response to enalaprilat is unlikely to merely reflect a greater sensitivity of BPH/2J mice to RAS inhibition because AT\textsubscript{1} receptor inhibition has previously showed no discernable difference in sensitivity of BPN/3J and BPH/2J mice when the AT\textsubscript{1} receptor inhibitor is administered at threshold and maximal doses.\textsuperscript{9} Although ACE inhibition can influence BP through bradykinin accumulation, this is unlikely to be contributing to the greater depressor response to enalaprilat in BPH/2J mice because the vasodilatory response to bradykinin is reportedly reduced in BPH/2J compared with BPN/3J mice.\textsuperscript{19} The greater depressor response to enalaprilat was not the only indication of an elevated

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**Figure 4.** Correlations between renal miR-181a and renal Ren1 mRNA (A), depressor response to pentolinium (Δ mm Hg) and renal Ren1 mRNA (B), depressor response to pentolinium (Δ mm Hg) and renal miR-181a (C), baseline MAP (mm Hg) and renal miR-181a (D). Data displayed from the inactive period (○) and active period (●) in BPN/3J (gray) and BPH/2J mice (black).
contribution of the peripheral RAS to BP in the active period. The pattern was accompanied by a greater abundance of Ren1 mRNA in the kidneys of BPH/2J mice during the active period, consistent with a role for kidney-derived renin production in driving the elevated RAS activity. Plasma renin activity is normally elevated during sleep and reduced during the awake period.20 Although renal Ren1 mRNA abundance was comparable between strains during the inactive period, levels were elevated during the active period in BPH/2J mice. This finding provides an explanation for the lack of differential expression of Ren1 mRNA in a prior transcriptome-wide array study6 and also for the similar renin expression demonstrated previously in the kidney of BPH/2J and BPN/3J mice.8 The greater contribution of the RAS in BPH/2J mice is consistent with the slightly greater hypotensive effect of chronic ACE inhibition with captopril in BPH/2J mice.10 However, the present findings contrast our previous finding of a similar contribution of the RAS to 24-hour BP in BPH/2J and BPN/3J mice.9 The discrepancy could be explained by the exclusion of a contribution from the central angiotensin system in the present study, although this might suggest that the contribution of the central angiotensin system to BP could also be aberrant in BPH/2J mice.

What Is the Association Between Overactivity of RAS and SNS?

We noticed a remarkably strong relationship between renal Ren1 and SNS activity as determined by depressor responses to ganglion blockade. Although this correlation does not necessarily indicate a direct or causal association, it does suggest some relationship. Furthermore in light of the elevated TH staining and greater RAS contribution in BPH/2J mice, it is possible that this relationship could reflect RSNA-driven overactivity of the RAS or even potentiation of SNA by the RAS. Although the peripheral RAS is capable of facilitating SNS activity,5 this is unlikely to be the case in BPH/2J mice because SNS overactivity, as measured by the depressor response to ganglion blockade, was apparent with and without ACE inhibition. Thus, SNS overactivity seems to be independent of RAS activity. The association could also reflect greater RSNA-mediated renin release and subsequent natriuretic and vasoconstrictor effects. Although there is a disparity between the apparently tonic overactivity of the SNS and the state-dependent RAS overactivity in BPH/2J mice, this may reflect circadian changes in regional SNA. Indeed RSNA is greater during activities, such as exercise and grooming, compared with sleep,20,21 whereas vasomotor sympathetic activity as measured by analysis of BP variability is greater during activity and lower during sleep.22 Renal denervation would be useful to help validate the contribution of the RSNA to hypertension. One would expect that it would cause a substantial reduction in the RAS-mediated contribution to hypertension during the dark (awake) period in BPH/2J mice. However, given the lack of influence of the RAS on hypertension during the light period (predominantly asleep), the influence of renal denervation would be expected to be less profound at this time.

Is Reduced miR-181a Mediating the Ren1 Overexpression and Hypertension?

The importance of miR-181a as a potential novel mediator of the BP elevation in hypertension has been largely unrecognized until recently.15 That miR-181a is likely to be a negative regulator of Ren1 mRNA expression in mice is supported by its strong negative correlation with Ren1 mRNA level and by the ability of human miR-181a to downregulate human renin mRNA in transfection experiments.15 Marques et al15 showed that hypertensive individuals have marked underexpression of miR-181a in the kidney, accompanied by marked overexpression of renin mRNA. Such a pattern was seen in BPH/2J mice in our study. Expression of miR-181a in monocytes has since been reported to be negatively correlated with systolic BP in obese subjects.23 Indeed our present finding of a negative correlation between renal miR-181a and BP supports a role for this micro-RNA in BP regulation in mice. Production of miR-181a during the inactive period is comparable in BPH/2J and BPN/3J mice, but its underproduction in the active period in BPH/2J mice indicates that a negative control mechanism is switched on at this time. Downregulation of miR-181a in BPH/2J, but not in BPN/3J mice, during the active period suggests factor(s) unique to the BPH/2J strain during the active period. Given the strong positive association between SNA and Ren1, a possible negative regulator of miR-181a might be SNA, either directly or indirectly. Although there was a negative correlation between miR-181a and response to pentolinium, this did not quite reach statistical significance. Nonetheless, regardless of how miR-181a is regulated in BPH/2J mice, given its ability to regulate Ren1 mRNA, synthetic miR-181a mimics could represent novel therapeutics in the treatment of hypertension.

Limitations

The present study shows strain differences in Ren1 mRNA, TH staining, and miR-181a and clear correlations among miR181a, Ren1, and BP. Although these strain differences and correlations do not necessarily indicate causal relationships with BP or hypertension, in the context of the pharmacological findings, these factors add support to the hypothesis that sympathetically mediated activation of the intrarenal RAS is contributing to hypertension in BPH/2J mice. Importantly though, further assessment to verify a contribution to hypertension is necessary. As such these findings are likely to motivate further interventional studies, such as renal denervation and administration of miR-181a mimetics to confirm an influence on hypertension in vivo. Another point to consider is that the present findings represent the contribution of the SNS and RAS to establish hypertension because tail-cuff measurements indicate hypertension is present in BPH/2J mice from as young as 7 weeks of age.24 Similar pharmacological assessment in very young BPH/2J mice would be useful to determine the influence of the RAS and SNS during hypertension development, but obtaining radiotelemetric BP measurements in such young mice would be technically challenging.

Conclusion and Perspectives

Hypertension in BPH/2J mice involves tonic overactivity of the SNS and elevated renal sympathetic innervation. During the sleep and awake states, the elevated sympathetic drive is likely manifested differently by circadian-related changes in regional sympathetic activity. During the active state, the elevation in sympathetic activity likely drives RSNA-induced renin production, which is reflected by the elevated contribution of the peripheral RAS to hypertension in BPH/2J mice at this time. However, underexpression of the negative regulator of Ren1 mRNA, miR-181a, may also be responsible for elevation of Ren1 expression and hypertension.
There are significant implications for our present findings in a mouse model of hypertension that has renal hyperinnervation and enhanced sympathetically induced renin synthesis. Hypertensive BPH/2J mice may prove to be an excellent animal model to investigate the mechanism mediating the hypotensive effect of renal sympathetic nerve ablation observed in treatment-resistant patients with hypertension. Furthermore, given the present study, the first to identify a mouse model of hypertension with aberrant renal miR-181a, akin to that seen in patients with essential hypertension, BPH/2J mice could serve as a unique animal model for the investigation of miR-181a-mediated BP regulation which could lead to novel therapeutic targets for hypertension.

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**Disclosures**

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**References**


**Novelty and Significance**

**What Is New?**

- This is the first study to demonstrate that the BPH/2J mouse strain is a mouse model of hypertension that has renal hyperinnervation and enhanced sympathetically induced renin synthesis, which seems to contribute to the hypertension, and that this is likely to be mediated by lower levels of the microRNA, miR-181a.

**What Is Relevant?**

- Importantly, this is the first study to describe aberrant renal expression of miR-181a in a hypertensive mouse model which is akin with that observed in patients with essential hypertension.

**Summary**

Our findings suggest that renal hyperinnervation and enhanced sympathetically induced renin synthesis, mediated by lower levels of the microRNA, miR-181a, are together responsible for the hypertension in BPH/2J mice.
A Novel Interaction Between Sympathetic Overactivity and Aberrant Regulation of Renin by miR-181a in BPH/2J Genetically Hypertensive Mice


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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:
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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.

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. 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. The beat-to-beat arterial pressure and heart rate (HR) were detected on-line and analyzed later using a program written in Labview.

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. 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). A combined residual was used that pooled the between- and within-animal variance as described previously. 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 \( \Delta \Delta C_T \) 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\(_2\)O\(_2\)/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\(_2\)O\(_2\) (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'-GGCCUCUGCCACCCAGCCAGCCUGCCUCAGGGCCAGCCUGCCACCCAGCUGGGAACACUCUCUGGAGAUGCCCUUGCCUGGCUUUGCCUCAGAUGGGAGUGGUCCUGCCACCCGUGACACAG-3'

Mouse renin 1 (Ren1) 3'UTR

3'UTR 5'-GGCCUCUGCCACCCAGCCAGCCUGCCUCAGGGCCAGCCUGCCACCCAGCUGGGAACACUCUCUGGAGAUGCCCUUGCCUGGCUUUGCCUCAGAUGGGAGUGGUCCUGCCACCCGUGACACAG-3'

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