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Cardiac sympathetic dysfunction in the prehypertensive spontaneously hypertensive rat

Julia Shanks, Sotiria Manou-Stathopoulou, Chieh-Ju Lu, Dan Li, David J. Paterson, and Neil Herring

Burdon Sanderson Cardiac Science Centre, Department of Physiology, Anatomy and Genetics, Sherrington Building, University of Oxford, Oxford, United Kingdom

Submitted 27 March 2013; accepted in final form 30 July 2013

Shanks J, Manou-Stathopoulou S, Lu CJ, Li D, Paterson DJ, Herring N. Cardiac sympathetic dysfunction in the prehypertensive spontaneously hypertensive rat. Am J Physiol Heart Circ Physiol 305: H980–H986, 2013. First published August 2, 2013; doi:10.1152/ajpheart.00255.2013.—Recent studies in prehypertensive spontaneously hypertensive rats (SHR) have shown larger calcium transients and reduced norepinephrine transporter (NET) activity in cultured stellate neurons compared with Wistar-Kyoto (WKY) controls, although the functional significance of these results is unknown. We hypothesized that peripheral sympathetic responsiveness in the SHR at 4 wk of age would be exaggerated compared with the WKY. In vivo arterial pressure (under 2% isoflurane) was similar in SHRs (88 ± 2/50 ± 3 mmHg, n = 18) compared with WKYs (88 ± 3/49 ± 4 mmHg, n = 20). However, a small but significant (P < 0.05) tachycardia was observed in the young SHR despite the heart rate response to vagus stimulation (3 and 5 Hz) in vivo being similar (SHR: n = 12, WKY: n = 10). In isolated atrial preparations there was a significantly greater tachycardia during right stellate stimulation (5 and 7 Hz) in SHRs (n = 19) compared with WKYs (n = 16) but not in response to exogenous NE (0.025–5 μM; SHR: n = 10, WKY: n = 10). There was also a significantly greater release of [3H]NE to field stimulation (5 Hz) of atria in the SHR (SHR: n = 17, WKY: n = 16). Additionally, plasma levels of neuropeptide Y sampled from the right atria in vivo were also higher in the SHR (ELISA, n = 12 for both groups). The difference in [3H]NE release between SHR and WKY could be normalized by the NET inhibitor desipramine (1 μM; SHR: n = 10, WKY: n = 8) but not the α2-receptor antagonist yohimbine (1 μM; SHR: n = 7, WKY: n = 8). Increased cardiac sympathetic neurotransmission driven by larger neuronal calcium transients and reduced NE reuptake translates into enhanced cardiac sympathetic responsiveness at the end organ in prehypertensive SHRs.

At 16 wk of age, there is evidence of left ventricular hypertrophy and both mean arterial pressure and heart rate are significantly elevated in the SHR (13). In addition, an increase in cardiac norepinephrine (NE) release (measured by 3H-radiolabeling; Ref. 21) and a larger tachycardia on stimulation of the right stellate ganglia (13) is also observed. Cultured neurons from the stellate ganglion of adult SHRs also have larger depolarization-induced calcium transients compared with WKYS (20), and this may drive a greater calcium dependent exocytic release of NE. Increased NE release appears to be further exacerbated by a reduction in the autoinhibitory action of the presynaptic α2-adrenoceptor (36), as well as a reduction in NE reuptake by the presynaptic NE uptake transporter (NET; Ref. 31). Conversely, the heart rate response to stimulation of the cervical vagus nerve is diminished in the adult SHR compared with age-matched WKYS (11).

A number of recent studies provide evidence that dysregulation of cardiac sympathetic neurotransmission may arise even before the onset of hypertension within the SHR. At ~4 wk of age when the SHR is still normotensive, an increased intracellular calcium transient in response to neuronal depolarization has been described in stellate and superior cervical ganglia neurons (20). A reduction in the activity of NET has also recently been reported in cultured stellate (but not superior cervical ganglia or renal ganglia) neurons of young prehypertensive SHRs compared with age-matched WKYS (31). However, the functional significance of these observations in terms of NE release and peripheral cardiac sympathetic control of heart rate is unknown. Whether a reduction in α2-receptor autoinhibition also augments NE release at this developmental stage is also unclear.

We therefore hypothesized that stimulation of the right stellate ganglion in young prehypertensive SHRs would produce a significantly larger increase in heart rate and release of NE compared with age-matched WKY controls due to a reduction in NET or α2-receptor autoinhibition. We also investigated whether the ability of the vagus nerve to reduce heart rate in the prehypertensive SHR was impaired.

MATERIALS AND METHODS

Animals. Age- and weight-matched male SHR and WKY rats (4 wk of age, SHR: n = 53, WKY: n = 48) were purchased from Harlan (Bicester, UK) and housed under standard laboratory conditions. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and the Animals Scientific
Procedures (ASP) Act 1986 (UK). Procedures were performed under British Home Office license requirements (PPL 30/2366).

**Hemodynamic measurements and heart rate responses to vagus nerve stimulation in-vivo.** Rats were ventilated under anesthesia (2% isoflurane and 100% oxygen) as a terminal procedure. The left carotid artery was cannulated with a 3-F portex cannula, based on a method previously described (19). The cannula was connected to a calibrated pressure transducer, and data were acquired using a biopac M150 system connected to a Macbook Pro computer running AcqKnowledge 3.9.2 software. Heart rate and blood pressure recordings were taken as an average over 30 s after a 5-min stabilization period, recorded at 200 data points/s. In a subset of experiments, the right vagus was isolated, crushed distal to the heart, and stimulated at 3 and/or 5 Hz (20-V, 1-ms pulse duration for 30 s) and the heart rate response was recorded.

**Measurement of heart rate response to right stellate stimulation and NE in vitro.** Spontaneously beating atria with intact sympathetic innervations and right stellate ganglion were isolated, and responses to sympathetic nerve stimulation were measured based on a method previously described (13, 25). In brief, the double atrial preparation was transferred to a preheated (37 °C), water jacketed, carbogen-aerated water bath containing 100 ml Tyrode solution. A suture through the left atria was attached to a stainless steel hook at the bottom of the water bath, whilst the right atrial suture was connected with an average pretension of 5 mN to an isometric force transducer (model: 60-2997; Harvard Apparatus) linked to a signal amplifier. The atria were field stimulated at 5 Hz (15-V, 1-ms pulse duration for 30 s), or increasing cumulative rate stabilized (5 beats/min). The stellate was stimulated at 1, 3, 5, or mean arterial blood pressure compared with WKY rats (*n* = 20), when measured while animals were under general anesthesia in vivo. However, the SHR had a small but significant resting tachycardia under these conditions. After equilibration in the organ bath in vitro, there was no difference in baseline atrial rate between SHR (80°C. Neuropeptide Y (NPY) concentration was measured using a commercially available ELISA (S 1346; Peninsula Laboratories, Bachem) against serially diluted protein standards.

**Solutions and drugs.** Rat Tyrode solution for the isolated atria preparation contained the following: (in mmol/l): 120 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 2 CaCl2, and 11 glucose, constantly aerated with carbogen (5% CO2-95% O2) to maintain pH 7.4. Experiments using NE, [3H]NE, and ascorbic acid were carried out in the dark due to the light sensitivity of the drug. Desipramine (1 μM; Sigma) was used at a concentration previously shown to completely inhibit NET (31), and yohimbine (1 μM; Sigma) was used at a concentration to completely block the α2-adrenoceptor in a similar preparation (3). Drugs were made up and stored as stock solutions (1 mM) and diluted to the desired concentration on the day. All drugs under went no more than one freeze thaw cycle.

**Statistics.** All statistical analysis was carried out using GraphPad Prism (GraphPad Software, San Diego, CA). Data are presented as means ± SE of the mean, and all data passed a normality test. Comparisons between SHR and WKY groups were done using an unpaired student’s t test assuming unequal variance, whereas within group analysis was performed using a one-way ANOVA with a Newman-Keuls post hoc analysis. Statistical significance was accepted at *P < 0.05.

**RESULTS**

**Baseline hemodynamic characteristics.** At 4 wk of age, the SHR (*n* = 18) did not show any change in systolic, diastolic, or mean arterial blood pressure compared with WKY rats (*n* = 20), when measured while animals were under general anesthesia in vivo. However, the SHR had a small but significant resting tachycardia under these conditions. After equilibration in the organ bath in vitro, there was no difference in baseline atrial rate between SHR (n = 19) and WKYs (n = 16). Animals were age matched and were of a similar weight as shown in Table 1.

**Heart rate response to parasympathetic and sympathetic stimulation.** There was no difference in the heart rate (beats/min) response to right vagal stimulation between the SHR and WKY at either 3 or 5 Hz (3 Hz: SHR, *n* = 9, WKY, *n* = 6; 5 Hz: SHR, *n* = 12, WKY, *n* = 10) in vivo (see Fig. 1, *D* and *E*). However, the heart rate response to stimulation of the right stellate ganglia in the isolated atrial preparation was significantly higher in the prehypertensive SHR (n = 19) compared with age-matched WKYs (n = 16) at 5 and 7 Hz (5 Hz: SHR, 91.1 ± 4.2 beats/min, WKY, 77.1 ± 3.8 beats/min; 7 Hz: SHR, 74.2 ± 3.9 beats/min, WKY, 61.9 ± 3.9 beats/min).

**Table 1. Measurements in 4-wk-old SHR and WKYs**

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>SHR</th>
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<tr>
<td>In vivo systolic blood pressure, mmHg</td>
<td>88 ± 3 (<em>n</em> = 20)</td>
<td>89 ± 2 (<em>n</em> = 18)</td>
</tr>
<tr>
<td>In vivo diastolic blood pressure, mmHg</td>
<td>49 ± 4 (<em>n</em> = 20)</td>
<td>51 ± 3 (<em>n</em> = 18)</td>
</tr>
<tr>
<td>In vivo mean arterial blood pressure, mmHg</td>
<td>66 ± 3 (<em>n</em> = 20)</td>
<td>69 ± 3 (<em>n</em> = 18)</td>
</tr>
<tr>
<td>In vivo heart rate, beats/min</td>
<td>298 ± 11 (<em>n</em> = 20)</td>
<td>334 ± 9 (<em>n</em> = 19)</td>
</tr>
<tr>
<td>In vitro heart rate, beats/min</td>
<td>286 ± 9 (<em>n</em> = 16)</td>
<td>298 ± 7 (<em>n</em> = 19)</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>97 ± 4 (<em>n</em> = 48)</td>
<td>96 ± 3 (<em>n</em> = 53)</td>
</tr>
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Values are means ± SE. No statistical difference in in vivo systolic, diastolic, or mean arterial blood pressure or in vitro atrial rate is observed between 4-wk-old spontaneously hypertensive rats (SHRs) and Wistar-Kyoto rats (WKYs), which are also of a similar body weight. However, in vivo heart rate was significantly increased in the SHR (*P < 0.05, unpaired t-test).
Despite this the heart rate responses to cumulative doses of NE (0.025–5 μM) in the isolated atria were similar in the SHR (n = 10) and WKY (n = 10) as shown in Fig. 1C. In vivo the heart rate response to vagal nerve stimulation was also similar in the prehypertensive SHR (3 Hz: n = 9; 5 Hz: n = 12) compared with WKY (3 Hz: SHR, n = 6; 5 Hz: n = 10) as shown by the representative raw data trace (D) and group mean data (E).

**Sympathetic neurotransmitter release.** The level of [3H]NE release in response to field stimulation of double atrial preparations was significantly higher in the prehypertensive SHR compared with age-matched WKYs (SHR: 82.14 ± 10.46%, n = 17 vs. WKY: 56.05 ± 6.98%, n = 16; Fig. 2B). Similarly, plasma levels of the sympathetic cotransmitter NPY sampled from the right atria in vivo, were also significantly elevated in the SHR (SHR: 15.70 ± 3.10 nM, n = 12; WKY: 8.461 ± 1.351 nM, n = 12) as shown in Fig. 2C.

**Reuptake and autoinhibition of NE release.** To determine whether the rate of NE reuptake contributed to the increased NE release observed in the SHR, desipramine was used as an inhibitor of NET. Desipramine normalized the difference in release of [3H]NE to field stimulation between the SHR and WKY (SHR: 97.19 ± 7.752%, n = 10; WKY: 106.0 ± 11.53%, n = 8) as shown in Fig. 3E. To determine if reduced levels of α2-adrenoceptor autoinhibition may contribute to the increased NE release observed in the SHR, yohimbine was
used a potent and selective \( \alpha_2 \)-antagonist. Yohimbine did not normalize the difference in \( [3H] \)NE release between prehypertensive SHRs \( (n = 7) \) and WKYs \( (n = 8) \). In fact the difference in \( [3H] \)NE release between the SHR and WKY was significantly increased. Yohimbine also significantly increased the heart rate response to stimulation of the right stellate ganglia in vitro in the SHR at 7 Hz \( (99.9 \pm 7.0 \) to \( 119.2 \pm 8 \) beats/min, \( n = 9 \)\) with a strong trend to increasing the response at 5 Hz \( (85.9 \pm 7.2 \) to \( 100.9 \pm 7.2 \) beats/min, \( P = 0.09 \)) but this was not observed in the WKY \( (7 \) Hz: \( 86.6 \pm 4.9 \) to \( 96 \pm 8.4 \) beats/min; \( 5 \) Hz: \( 73.4 \pm 5.2 \) to \( 76.4 \pm 7.4 \) beats/min, \( n = 9 \)\).

Yohimbine did not significantly alter baseline heart rate in either group.

**DISCUSSION**

This present study reports three novel findings concerning peripheral cardiac autonomic control in the prehypertensive SHR. First, resting heart rate was significantly higher in vivo when measured while the animals were under general anesthesia in the SHR compared with age- and weight-matched WKYs, despite the intrinsic heart rate of the isolated atria and...
the ability of the vagus nerve to lower heart rate remaining unchanged. Secondly, the tachycardia to stimulation of the right stellate ganglia was significantly increased in the SHR although the response to exogenous NE was not different to that observed in the WKY. Finally, the release of NE and the sympathetic cotransmitter NPY was also significantly elevated in the SHR. This may be in part due to impaired reuptake of NE through NET rather than through reduced α₂-adrenoceptor autoinhibition. When these results are taken together with other reports (20, 31), they indicate that impaired reuptake of NE and enhanced neuronal calcium transients in the SHR translate to an increase in NE release and tachycardia before hypertension develops (Fig. 4).

Disrupted autonomic balance in the prehypertensive SHR. Hypertension is associated with well-characterized physiological changes, including cardiac remodeling due to stresses in the myocardium resulting in left ventricular hypertrophy, and an increase in sympathetic nervous system activity (27). Within the 4-wk-old SHR, arterial blood pressure was not different from age- and weight-matched WKY controls when measured while the animals were under general anesthesia. In addition, we have previously reported that ventricular weight-to-body weight ratios were also not different (20), suggesting no evidence of left ventricular hypertrophy. However, there was a small but significant increase in resting heart rate in vivo in the prehypertensive SHR. Previous studies have also demonstrated no difference in arterial blood pressure between the SHR and WKY at 4–5 wk of age using the noninvasive tail-cuff method (15, 33) and also following surgical implantation of a radiotelemetry device (16). While the telemetry study reports a significant tachycardia in the SHR, reports from tail-cuff data vary from a significant tachycardia being observed in the WKY (15) to a tachycardia being observed in the SHR (33). One common feature of these studies is the degree of variability in the measurements made. The aim of our invasive blood pressure recordings while the animals were under general anesthesia was to minimize the noise and variation in the data due to recovery surgery from implantation of radiotelemeters and variations in animal activity (the SHR is often used as a model of attention deficit hyperactivity disorder), which may complicate these measurements. While the mean arterial pressures we record are lower than those observed by these other methods (≈70 vs. 90–120 mmHg), they are still within a physiological resting range. Despite our carefully controlled conditions, recordings in 38 animals (SHR: n = 20, WKY: n = 18) were required to demonstrate statistical significance in terms of a tachycardia in the SHR group, suggesting that the resting phenotype is subtle. We have previously observed this resting tachycardia to be more pronounced in the adult animal once hypertension has set in (20).

The fact that there was no difference in the beating rate of isolated atria from both the prehypertensive SHR and WKY despite the in vivo resting tachycardia suggests that the difference observed was due to a change in autonomic balance. The magnitude of vagally mediated bradycardia is small in both the SHR and WKY compared with the tachycardia mediated by sympathetic stimulation as has been previously reported in adult animals (11, 13). Unlike the adult SHR, which has a smaller heart rate response to vagus nerve stimulation than the WKY, we observe no difference in the degree of bradycardia at 4-wk of age, suggesting there is no functional cardiac parasympathetic impairment at this developmental stage. Others have reported a slight increase in the heart rate response to vagal nerve stimulation at high stimulation frequencies at ~6 wk of age, which quickly normalizes as the animal ages (24). It is therefore unlikely that a change in vagal tone is responsible for the difference in in vivo heart rate we observe.

Conversely, we demonstrated that direct stimulation of the right stellate ganglion at 5 and 7 Hz produced a larger tachycardia in the SHR than the WKY. This was not as marked as in the adult animal where stimulation frequencies between 1 and 7 Hz produce significantly larger responses (13). However, at this age (16 wk) both [³H]NE release and β-adrenergic receptor responsiveness are also greatly increased (13). We report a greater release of NE during stimulation at 5 Hz in the 4-wk-old SHR compared with the WKY although the heart rate response to exogenous NE was no different, suggesting that it is increased neurotransmitter release that is responsible for the larger tachycardia observed both on stimulation of the right stellate in vitro and at rest in vivo.

Plasma levels of the sympathetic cotransmitter NPY are also elevated in the prehypertensive SHR compared with the WKY. Plasma catecholamine levels fluctuate dramatically making it difficult to draw accurate conclusions regarding sympathetic activity when taking measures at one time point. NPY by comparison is a slowly diffusing cotransmitter with a long half-life. Plasma levels have little diurnal variation, and so it is a more accurate indicator of sustained sympathetic hyperactivity (4, 17, 22). For these reasons we chose to measure its plasma levels sampled directly from the right atria in vivo. NPY is also able to cross talk and inhibit vagal acetylcholine release following high frequency sympathetic stimulation (12, 14). We observed no difference in the size of the vagal responses in the young SHR despite higher circulating NPY levels. This sug-

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**Fig. 4.** Schematic diagram of a cardiac peripheral sympathetic nerve terminal within the 4-wk hypertensive SHR. Increased depolarization induced intracellular calcium transients (20) and reduced NE reuptake via the NET (31) facilitate greater release of NE and the cotransmitter NPY at the neuromuscular junction. The increased intracellular calcium is due to reduced mitochondrial buffering of intracellular calcium rather than changes in endoplasmic reticulum ryanodine receptor (RyR) function, despite a more rapid uptake into the endoplasmic reticulum by the smooth endoplasmic reticulum ATPase (SERCA; Ref. 15). An increase in the action of α₂-adrenoceptor autoinhibition may act as a compensatory mechanism within the prehypertensive SHR to limit NE release, although this is lost in the adult animal. The increase in NE release to stimulation of the right stellate ganglion leads to a greater increase in the beating rate of the sinoatrial node. Green lines represent stimulatory pathways and red lines inhibitory pathways impacting on neurotransmission.
gests the NPY levels were not high enough to impair vagal neurotransmission although both these measurements were taken in the resting state under general anesthesia, rather than following high-level sympathetic stimulation where NPY release may increase further. Nevertheless, it is conceivable that elevated cardiac NPY levels may contribute to resting vagal impairment in the adult animal when very high levels of sympathetic hyperactivity have become established (11).

A previous study has attempted to examine the changes in peripheral autonomic control of heart rate in the prehypertensive SHRs by examining the heart rate responses to field stimulation (6). Field stimulation of the entire atria will depolarize all sympathetic, parasympathetic, efferent, and afferent neurons making the subsequent atrial heart rate response to the many local neurotransmitters and cotransmitters that would be released extremely difficult to interpret. We believe that selective stimulation of the right stellate ganglion is more physiological and reliable, and we are not aware of any other previous work that has achieved this in this age group. We also demonstrate no difference in the chronotropic response to the physiological agonist NE and directly measure NE release to support our physiological heart rate data and add to their novelty.

Ideally, we would have liked to evaluate vagal responses in vitro in the SHR and WKY at this age but despite considerable effort have not been able to overcome this technical challenge. We have previously been able to perform these dissections in guinea pigs of a similar size and mice, but the mediastinal anatomy and narrow neck of the young rat makes it extremely difficult to produce a viable preparation. We therefore chose to stimulate the right vagus by exposing it at the cervical level in vivo. Conversely, it is difficult to reach the right stellate ganglion in vivo without causing a pneumothorax (given its position posteriorly below the first rib). Measuring NPY release in vitro is also very difficult in such a small preparation when release concentrations are very low. In guinea pigs three to four times this size, measurements of NPY in atrial perfusate are on the limit of detection of ELISA assays. We have therefore chosen what we feel to be the most appropriate and accurate methodologies currently available.

Regulation of cardiac sympathetic neurotransmission in the prehypertensive SHR. The adult SHR has evidence of larger calcium transients on depolarization of cultured stellate neurons (20), reduced NET activity (31), and impaired α2-adrenoceptor autoinhibition of NE release (36). The stroke-prone SHR also has increased sympathetic innervation of the left ventricle with higher tyrosine hydroxylase positive nerve fiber density (18). We observed that inhibition of NET with desipramine normalizes the difference in NE release between prehypertensive SHRs and WKYs. However, the difference in NE release between the young SHR and WKY became significantly larger following administration of the α2-adrenoceptor antagonist yohimbine suggesting that increased α2-adrenoceptor autoinhibition may be a compensatory mechanism limiting increased sympathetic neurotransmission at this developmental stage. There is strong cellular basis underpinning the enhanced functional sympathetic responses reported in this study. Specifically, NET activity is impaired in stellate neurons from 4-wk-old SHRs (31), and those neurons also have larger calcium transients on neuronal depolarization, which may drive more exocytotic release of NE (20). We have also recently reported that there appears to be heterogeneity in NET activity between neurons from the stellate ganglion and the superior cervical ganglion (which mainly innervates the vasculature of the head and neck), and the celiac/superior mesenteric ganglia (which innervate the kidneys and other abdominal organs). Only stellate neurons demonstrated impaired NET activity in the SHR at 4 wk of age compared with the WKY. We therefore do not feel that our results regarding cardiac neurotransmission can necessarily be extrapolated to other areas of the peripheral sympathetic nervous system.

Implications and future perspectives. We present evidence that dysregulation in cardiac adrenergic signaling in the SHR develops before the onset of hypertension and functionally translates to a small but significant resting tachycardia and larger increases in heart rate on stimulation of the right stellate ganglion. Sympathetic dysfunction may therefore be an early marker of the disease and contribute to its pathogenesis rather than being an epiphenomenon that develops once hypertension has set in. The causes of increased blood pressure in the SHR are clearly multifactorial and involve changes in vascular, renal, and autonomic function at many different anatomical sites. The increase in peripheral cardiac, sympathetic control is unlikely to be the sole cause of the increased blood pressure but may be an early marker of the disease and help drive left ventricular hypertrophy (27). Increased sympathetic nerve activity within borderline hypertensive patients and normotensive individuals with first degree relatives who are hypertensive has also previously been observed (5, 10). With further research, sympathetic cardiac hyperactivity may act as an early marker for those individuals predisposed to develop hypertension, potentially providing novel therapeutic targets for treatment. Currently used treatments for hypertension target the sympatho-adrenal axis and renin-angiotensin pathways treating the end organ after manifestations of the disease have already developed. Greater identification of early hallmarks of the disease may help develop therapies that prevent the progression of hypertension if sympathetic hyperactivity can be targeted.

Whether the increase in peripheral cardiac sympathetic function we observe occurs in isolation or in response to changes in central drive or even changes in afferent brainstem input also remains unclear. For example, greater respiratory sympathetic coupling has been observed in a working heart brainstem preparation from SHRs at 3 to 5 wk of age compared with the WKY, which may reflect an increase in central sympathetic drive (32). Changes in peripheral chemoreceptor sensitivity have also been observed in the SHR compared with the WKY before the onset of hypertension (34), and carotid sinus nerve denervation in the SHR at 4 wk of age has recently been shown to reduce the development of hypertension in this model (1). How these phenomena interact and whether one component helps drive changes in the other will be an interesting area of further study.

GRANTS

N. Herring, J. Shanks, and D. J. Paterson acknowledge support from the British Heart Foundation (BHF) Centre of Research Excellence (CRE; RE/08/ 004). This work was supported by a BHF project grant (PG/11/6/28660). N. Herring is a BHF CRE Intermediate Fellow at the University of Oxford and Senior Registrar in Cardiology at Oxford University Hospital National Health Service Trust.

AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00255.2013 • www.ajpheart.org
DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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