Abnormal Intracellular Calcium Homeostasis in Sympathetic Neurons From Young Prehypertensive Rats

Dan Li, Chee-wan Lee, Keith Buckler, Anant Parekh, Neil Herring, David J. Paterson

Abstract—Hypertension is associated with cardiac noradrenergic hyperactivity, although it is not clear whether this precedes or follows the development of hypertension itself. We hypothesized that Ca\(^{2+}\) homeostasis in postganglionic sympathetic neurons is impaired in spontaneously hypertensive rats (SHRs) and may occur before the development of hypertension. The depolarization-induced rise in intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\); measured using fura-2-acetoxymethyl ester) was significantly larger in cultured sympathetic neurons from prehypertensive SHRs than in age matched normotensive Wistar-Kyoto rats. The decay of the [Ca\(^{2+}\)]\(_i\) transient was also faster in SHRs. The endoplasmic reticulum Ca\(^{2+}\) content and caffeine-induced [Ca\(^{2+}\)]\(_i\) amplitude were significantly greater in the young SHRs. Lower protein levels of phospholamban and more copies of ryanodine receptor mRNA were also observed in the young SHRs. Depleting the endoplasmic reticulum Ca\(^{2+}\) store did not alter the difference of the evoked [Ca\(^{2+}\)]\(_i\) transient and decay time between young SHRs and Wistar-Kyoto rats. However, removing mitochondrial Ca\(^{2+}\) buffering abolished these differences. A lower mitochondrial membrane potential was also observed in young SHR sympathetic neurons. This resulted in impaired mitochondrial Ca\(^{2+}\) uptake and release, which might partly be responsible for the increased [Ca\(^{2+}\)]\(_i\), transient and faster decay in SHR sympathetic neurons. This Ca\(^{2+}\) phenotype seen in early development in cardiac stellate and superior cervical ganglion neurons may contribute to the sympathetic hyperresponsiveness that precedes the onset of hypertension. (Hypertension. 2012;59:00-00.) ● Online Data Supplement

Key Words: hypertension ■ sympathetic neuron ■ calcium homeostasis ■ endoplasmic reticulum ■ mitochondria

Hypertension is a multiorgan disease involving the kidney, vasculature, and autonomic nervous system. In particular, abnormal neurohumoral activation is a hallmark of hypertension and is a negative prognostic indicator for sudden cardiac death and a strong independent predictor of mortality.\(^1,2\) Much evidence supports the observation that sympathetic hyperresponsiveness is involved in the pathophysiology of human and animal primary hypertension.\(^3-6\) Increases in muscle sympathetic nerve activity in response to mental stress have been documented in normotensive offspring linked to a family history of hypertension.\(^6\) This suggests that dysregulated sympatho humoral activation may be an early marker of hypertension in those who are genetically predisposed to the disease.

Intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) plays a pivotal role in triggering neurotransmitter release from sympathetic neurons.\(^7\) In superior cervical ganglion (SCG) neurons, Ca\(^{2+}\) signals govern the release of norepinephrine (NE).\(^8,9\) In turn, NE release plays a critical role in the regulation of blood pressure and cerebral blood flow distribution.\(^10-11\) We have shown in the spontaneously hypertensive rat (SHR), enhanced heart rate responses and evoked NE release from the postganglionic neurons innervating the heart when compared with normotensive Wistar-Kyoto (WKY) rats.\(^11\) Postsynaptically, basal and NE-stimulated L-type calcium current in single sinoatrial node cells was also enhanced in the SHR, as was the heart rate response to bath-applied NE.\(^14\) Taken together, these results suggest that a significant component of the sympathetic hyperresponsiveness in the SHR occurs at the end-organ level.

We tested the hypothesis that intracellular Ca\(^{2+}\) homeostasis is dysregulated in the SHR and that this is genetically programmed and precedes the subsequent development of hypertension. To investigate the mechanisms involved in the potential differences between the SHR and WKY rat, we targeted calcium handling by the endoplasmic reticulum (ER) and mitochondria in an attempt to delineate whether disruption of Ca\(^{2+}\) handling proteins in intracellular stores might explain the enhanced exocytotic response in the SHR.\(^12,15\)

Methods

Animals

Neonatal (4–7 days), prehypertensive young (4–6 weeks), and hypertensive adult (15–17 weeks) male SHRs and WKY rats were...
used in this study. Cells were isolated from the SCG and cardiac stellate ganglion for phenotyping. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication No. 85–23, revised 1996) and the Animals (Scientific Procedures) Act 1986 (United Kingdom).

An expanded Materials and Methods section is available in the online-only Data Supplement.

Results

Animal Characteristics

The phenotypic characteristics of both young and adult SHRs and WKY rats are summarized in Table S1 (please see the online-only Data Supplement). No differences in ventricular weight:body weight ratio (an indication of left ventricular hypertrophy), mean arterial blood pressure, or heart rate measured in vivo were observed in the young SHRs, whereas these measures were significantly increased in the adult SHRs when compared with the age-matched WKY rats.

Intracellular Free Calcium Transients in Sympathetic Neurons

Sympathetic neurons were confirmed by the catecholamine neuronal marker antityrosine hydroxylase stain (Figure 1A). Intracellular calcium concentration was performed by ratiometric recordings (using fura-2-acetoxymethyl ester, fura-2/AM) in single sympathetic neurons (Figure 1B). A typical calcium intensity profile of a sympathetic neuron responding to 100 mmol/L of KCl was shown in Figure 1C. In the SHR, high K⁺ evoked [Ca²⁺], was significantly enhanced in both stellate ganlion (Figure 2) and SCG neurons (Figure 3A and 3B) when compared with age-matched WKY rats. In-depth profiling of the neural phenotype was performed on SCG cells because of the higher yield of neurons and ease of dissection.

Baseline [Ca²⁺], was significantly higher in SCG neurons from the SHRs when compared with age-matched WKY rats in all 3 of the age groups (Table S2). The baseline [Ca²⁺], in the SHR decreased with age, whereas this was not seen in the WKY rat. During depolarization with high K⁺, SCG neurons from the neonatal SHR had a greater increase in [Ca²⁺], when compared with those from the WKY rat (Figure 3A and 3B). This effect was present in animals of all age groups, from neonatal to adult rats. There was a significant increase in evoked [Ca²⁺], between the young and adult SHRs (Figure 3B). SCG neurons from the WKY rats also showed an increase in depolarization evoked [Ca²⁺], with age, but the response remained smaller than the SHRs (eg, at adult, SHR: +32.10%; WKY: +13.92% compared with young rats; Figure 3B). We also calculated the area under the curve of the Ca²⁺ response to high K⁺ (Figure S1 in the online-only Data Supplement), and observed that this was significantly greater in the SHRs from neonatal to the fully developed hypertensive rat when compared with the age-matched WKY rats. Analyzing the responses as a percentage of change from baseline also resulted in greater [Ca²⁺], responses in the young SHRs, although this was not observed in the neonatal or adult rats (Table S3 in the online-only Data Supplement).

Increased Rate and Decay Time of the Calcium Transient in SCG Neurons

In both SHRs and WKY rats, the [Ca²⁺], increased rapidly in response to high K⁺ and fell slowly on high K⁺ removal (Figure 3A). The rate of rise (Δratio/Δtime) increased with age (Table S4), but there was no significant difference between SHRs and WKY rats in any age group. The WKY group demonstrated a significantly longer (50% and 90%) decay time in comparison with the SHR group from young and adult rats (Figure 3C). Because clear differences in both the amplitude and decay time of the [Ca²⁺], were apparent in young prehypertensive SHRs and WKY rats, further experiments to investigate the role of the ER and mitochondria focused on young rats (4 weeks old).
ER Calcium Handling in SCG Neurons

Monitoring of ER Ca^{2+} Concentration

Ca^{2+} concentration in the ER can be directly measured by monitoring the Ca^{2+} within the organelles after loading with a low-affinity Ca^{2+} indicator mag-fura-2-acetoxymethyl ester (mag-fura-2/AM; Figure S2). Baseline mag-fura-2/AM fluorescence ratios were significantly higher in sympathetic neurons of young SHRs when compared with WKY rats (Figure 4A, left group bar). This difference persisted when the suffusate had 0 calcium present (Figure 4A, right group bar). Activation of ryanodine receptors (RyRs) and depletion of Ca^{2+} from ER stores with 10 mmol/L of caffeine produced a larger drop in the mag-fura-2/AM fluorescence ratio in the SHR (−7.05±1.14% compared with the WKY rat (−2.58±0.68%; P<0.01; Figure 4B). Subsequent introduction of the cell-permeant intraluminal Ca^{2+}/heavy metal chelator, N,N,N′,N′-tetrakis(2-pyridylmethyl)-ethylendiamine (TPEN), caused a further reduction in the mag-fura-2/AM fluorescence ratio in both groups (Figure 4B). Fluorescence ratio normalized by baseline showed that the caffeine-evoked changes in ER Ca^{2+} were significantly greater in the SHRs than the WKY rats (to 0.92±0.01 versus 0.96±0.01; P<0.01; Figure 4C), whereas the TPEN response was similar (to 0.82±0.03 versus 0.85±0.03; P=0.51; Figure 4C). Releasable ER Ca^{2+} load was also estimated based on the [Ca^{2+}], responses to caffeine (10 mmol/L; 30 seconds; Figure 4D).

Ryanodine Receptor

Studies have shown that advancing age can selectively change the genetic expression and protein levels of RyR3 but not RyR2 and RyR1 isoforms in the SCG aged 6-, 12-, and 24-month rats. Therefore, we investigated whether the RyR3 receptor is altered in young SHRs. Western blots were not sensitive enough to detect RyR3 receptor protein in the SCG dissected from young rats. However, SHRs were found to have significantly more RyR3 mRNA copies than WKY rats using RT-PCR (Figure 4E).

Protein Expression of Sarco/ER Ca^{2+}-ATPase and Phospholamban

To understand the mechanism underlying the faster [Ca^{2+}], decay in the SHR, Western blot analyses were performed to assess the expression levels of sarco/ER Ca^{2+}-ATPase (SERCA) 2a and phospholamban (PLN) in SCG homogenates from young WKY rats and SHRs (Figure 5). SERCA pump is under the regulatory control of the phosphoprotein phospholamban, which inhibits the apparent affinity of SERCA for Ca^{2+} in its nonphosphorylated form. There was no difference in the protein level of SERCA2a from young SHRs and WKY rats (P=0.63; Figure 5B). However, both the total and the phosphorylated portions (PLN-Ser16) of the PLN were significantly lower in the SHR group (Figure 5C and 5D). There was no difference in the PLN-Thr17 between the 2 groups (data not shown).

ER Contribution to the Changes [Ca^{2+}], in the SHR

To evaluate the contribution of the ER to the differences in peak [Ca^{2+}], rise and recovery time, ER Ca^{2+} stores were depleted by using caffeine (10 mmol/L; 30 seconds), and Ca^{2+} reuptake from SERCA pumps was blocked with 1 μmol/L of thapsigargin. Caffeine was reintroduced at 8.5 minutes and 13.5 minutes to confirm ER Ca^{2+} depletion (Figure 6A). Under these conditions, the SHR group continued to have a significantly higher elevation of [Ca^{2+}], during high K+ depolarization when compared with the WKY group (Figure 6B). The baseline [Ca^{2+}], was increased by 0.157±0.028 μmol/L in the SHR and 0.136±0.021 μmol/L in the WKY rat after ER Ca^{2+} depletion (P=0.56, t test). Because 90% decay time for the second high K+ depolarization was not reached in some experiments, only 50% decay time was measured, and this remained significantly shorter in
Mitochondrial Calcium in SCG Neurons

Mitochondria Contribution to the \([Ca^{2+}]_i\)

The proton uncoupler carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP; 1 μmol/L) causes an immediate depolarization of the inner mitochondrial membrane, resulting in depletion of stored mitochondrial Ca\(^{2+}\) and inhibition of any further mitochondrial Ca\(^{2+}\) uptake.\(^1\) Application of FCCP produced a small transient rise in \([Ca^{2+}]_i\) (Figure 7A, center). This increase was not different between the 2 strains (SHR: 189±10 nmol/L versus WKY: 198±10 nmol/L; \(P=0.55, t\) test; \(n=17\) and 23, respectively). Subsequent exposure to high K\(^+\) in the continued presence of FCCP resulted in an increase in the amplitude of the [Ca\(^{2+}\)] transient of 23.15±1.78% in the WKY rat and 12.74±2.48% in the SHR (\(P<0.01, t\) test; Figure 7B), abolishing the difference in depolarization-induced [Ca\(^{2+}\)] increase between the 2 groups. The 50% and 90% decay times of the [Ca\(^{2+}\)] transient were significantly shortened by FCCP in both WKY and SHR neurons (\(P<0.001,\) ANOVA), and the differences in 50% and 90% decay time between SHRs and WKY rats were abolished (Figure 7C).

Measurement of the Mitochondria Membrane Potential in Sympathetic Neurons

We then measured the membrane potential of mitochondria (ΔΨ\(_{m}\)) using the fluorescent dye tetra-methylrhodamine ethyl ester (TMRE; 20 nmol/L; 5 minutes). We confirmed that TMRE uptake was indeed attributed to accumulation within the mitochondria by demonstrating that it was rapidly released on mitochondrial depolarization with FCCP (Figure 8B). TMRE fluorescence intensity was significantly higher in the WKY rat than in the SHR (Figure 8A and 8B; \(P<0.001, t\) test).

Discussion

There are 3 novel findings from this study. First, sympathetic neurons from the SHR have a significantly greater depolarization-evoked [Ca\(^{2+}\)] transient when compared with the age-matched WKY rat. This difference persisted from neonates through to young prehypertensive and fully develop-

![Figure 4](http://hyper.ahajournals.org/)

**Figure 4.** Enhanced endoplasmic reticulum (ER) calcium load in superior cervical ganglion (SCG) neurons in the young prehypertensive spontaneously hypertensive rat (SHR). A, Direct measurement of resting ER Ca\(^{2+}\) concentration using mag-fura-2-acetoxymethyl ester (mag-fura-2/AM). B, ER Ca\(^{2+}\) depleted with caffeine followed by \(N_2N_2N_2-N_4\)-tetraakis(2-pyridylmethyl)-ethylenediamine (TPEN). C, Normalization of ratio by baseline. D, Caffeine evoked Ca\(^{2+}\) release from the ER (measured using fura-2/AM) was significantly higher in SHRs either in normal or 0 calcium solution. The inset is an example of the fluorescence ratio in the SCG neurons in response to 10 nmol/L of caffeine for 30 seconds. E, The SCG from the SHR has significantly more copies of ryanodine receptor (RyR3) mRNA after 46 cycles of semi-quantitative RT-PCR when compared with the Wistar-Kyoto (WKY) rat (n=8 SCGs per group). *P<0.05, **P<0.01, t test, SHR compared with WKY rat.

![Figure 5](http://hyper.ahajournals.org/)

**Figure 5.** A, Representative immunoblots using antibodies specific for sarco/ER Ca\(^{2+}\)-ATPase (SERCA) 2a, total phospholamban (PLN), Ser\(^{16}\)-phosphorylated PLN, and GAPDH (as a loading control), using superior cervical ganglion (SCG) homogenates from young spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) rats. Equal amounts of protein were loaded in each line. B through D, Bar graphs showing protein level of SERCA2a, total PLN, and Ser\(^{16}\)-phosphorylated PLN contents from the young SHRs and WKY rats (n=10 SCGs per group; *P<0.05, t test).
opened adult hypertensive animals. Furthermore, the rate of decay of $[\text{Ca}^{2+}]_i$ was faster in young and adult SHRs. Second, increased ER $\text{Ca}^{2+}$ content, upregulated SERCA (because of reduced PLN inhibition), and RyR activity were seen in the young SHR. Third, mitochondrial membrane potential was reduced in the young SHR sympathetic neurons, and this may contribute to the differences in depolarization-evoked $[\text{Ca}^{2+}]_i$, transients and the rate of decay between the 2 groups. When all of the results are taken together, they suggest that alteration of $[\text{Ca}^{2+}]_i$, in sympathetic neurons from the SCG (and, by extrapolation, stellate ganglion cells), precedes the actual onset of hypertension and thereby maybe responsible for the enhanced sympathetic responsiveness seen at this developmental stage.

**Intracellular Free Calcium Transients in the SHR**

Calcium is an important ubiquitous secondary signaling messenger that is involved in both short-term and long-term regulation of cell function, metabolism and growth. In neurons, an increase in $[\text{Ca}^{2+}]_i$ forms the pivotal link between the action potential and neurotransmitter release. It is well established that hypertension is strongly associated with noradrenergic hyperactivity, with increased central sympathetic output, and with elevated plasma epinephrine and NE levels. This dysregulation occurs at several levels of the cardiovascular neural axis, where peripheral sympathetic hyperresponsiveness in the adult hypertensive rat results in enhanced evoked cardiac NE release and also increased $\beta$-adrenergic responses in myocytes. Abnormal calcium handling properties have been reported in SHR vascular smooth muscle cells and endothelial cells, and it is conceivable that this also occurs in sympathetic neurons, thus providing a possible molecular link to enhanced noradrenergic neurotransmission.

The resting $[\text{Ca}^{2+}]_i$ level in the SCG neurons from the neonates through to fully developed hypertensive animals was higher than that observed in the age-matched WKY rat. Similarly, the $[\text{Ca}^{2+}]_i$ handling expressed as a percentage change from baseline was also greater in the young SHRs. However, this was not evident in the neonatal or adult cells from the SHRs and may be related to poor statistical power. Nevertheless, when taken together with the absolute $[\text{Ca}^{2+}]_i$, responses and the area under the curve measurement for $[\text{Ca}^{2+}]_i$ (Figure S1), the data are consistent with the hypothesis that prehypertensive SHRs have abnormal $[\text{Ca}^{2+}]_i$ handling properties compared with age-matched WKY animals. The overall magnitude of the evoked $[\text{Ca}^{2+}]_i$ transient increased with age in both groups, and the increase in the SHR group was larger, thus maintaining a difference between the 2 groups. This supports the idea that abnormal calcium regula-

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**Figure 6.** A. Typical experimental protocol presenting fluorescence ratio of fura-2-acetoxymethyl ester (fura-2/AM) to assess the contribution of the endoplasmic reticulum (ER) to the increase in intracellular free calcium concentration ($[\text{Ca}^{2+}]_i$) on high KCl exposure in the young Wistar-Kyoto (WKY) rats. B. Statistical data showing the peak evoked $[\text{Ca}^{2+}]_i$, transient both before (S1) and after (S2) ER $\text{Ca}^{2+}$ store depletion between young spontaneously hypertensive rats (SHRs) and WKY rats. C. The difference between SHRs and WKY rats with regard to 50% decay time before (S1) and after (S2) ER $\text{Ca}^{2+}$ store depletion. Caff indicates caffeine; Thap, thapsigargin. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

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**Figure 7.** A. Typical experimental protocol showing fura-2-acetoxymethyl ester (fura-2/AM) fluorescence ratio to assess the contribution of mitochondria to intracellular free calcium concentration ($[\text{Ca}^{2+}]_i$) in superior cervical ganglion (SCG) neurons from young spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) rats. B. Statistical data showing the peak evoked $[\text{Ca}^{2+}]_i$, both before (S1) and after (S2) mitochondrial $\text{Ca}^{2+}$ store depletion with FCCP between young SHRs and WKY rats. C. Time taken for the $[\text{Ca}^{2+}]_i$ from peak to 50% and 10% of the increase (triangles, 50% decay time; circles, 90% decay time) after high K+ challenge. *$P<0.05$, **$P<0.01$, ***$P<0.001$. 

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and heart failure.\textsuperscript{29} In the present study, before neurons were re-exposed to high KCl, ER Ca\textsuperscript{2+} stores were depleted by caffeine, and Ca\textsuperscript{2+} reuptake from SERCA pumps were prevented by thapsigargin. Under these conditions, the increased [Ca\textsuperscript{2+}], should represent a non-ER contribution. We found that the SHR group continued to have a higher increase in depolarization-evoked [Ca\textsuperscript{2+}], even after ER Ca\textsuperscript{2+} depletion. This result suggests that the non-ER sources may underlie the significantly higher depolarization-evoked [Ca\textsuperscript{2+}] transients seen in the SHR.

The larger ER Ca\textsuperscript{2+} store in the SHR was confirmed by directly measuring ER-free Ca\textsuperscript{2+} concentration using mag-fura-2/AM, followed by ER Ca\textsuperscript{2+} release/depletion with caffeine and the ER-Ca\textsuperscript{2+} chelator, TPEN. This finding itself does not necessarily indicate a larger ER contribution toward depolarization-evoked [Ca\textsuperscript{2+}], in the SHR, because the net ER contribution depends on the relative release and reuptake of Ca\textsuperscript{2+} by the ER.\textsuperscript{30,31} It does, however, imply a larger ER Ca\textsuperscript{2+} store that is potentially available for release on appropriate stimulation.

The rate at which SERCA moves Ca\textsuperscript{2+} across the ER membrane can be reduced by PLN, whereas phosphorylation of the PLN relieves its inhibition.\textsuperscript{32,33} Our results showed that the difference in total PLN was much larger than the difference in PLN-Ser\textsuperscript{16}, suggesting that there was less nonphosphorylated PLN in the SHR than in the WKY rat and, therefore, less PLN-dependent inhibition of SERCA activity in the prehypertensive SHR. This is in keeping with the functional data showing shorter 50% and 90% decay times in [Ca\textsuperscript{2+}].

RyRs are Ca\textsuperscript{2+} permeable channels that open in response to increase in [Ca\textsuperscript{2+}].\textsuperscript{34} We introduced the RyR activator caffeine to deplete Ca\textsuperscript{2+} from ER stores. Prehypertensive SHRs had a significantly higher caffeine-induced [Ca\textsuperscript{2+}] amplitude when compared with the age-matched WKY rat, indicating that RyRs in sympathetic neurons were upregulated in the young SHR. This was confirmed by more copies of RyR3 mRNA in the SHR. These data are consistent with observations by others in cardiac myocytes.\textsuperscript{35} Taken together, these findings suggest that sympathetic neurons from prehypertensive SHRs have more active/dynamic ER Ca\textsuperscript{2+} handling machinery.

**Mitochondrial Calcium Signaling in the SHR**

Mitochondria take up Ca\textsuperscript{2+} primarily through the mitochondrial calcium uniporter,\textsuperscript{36} which is modulated by both [Ca\textsuperscript{2+}] and the mitochondrial membrane potential (\(\Delta \Psi _{m}\)).\textsuperscript{37} The uniporter transports Ca\textsuperscript{2+} down the electrochemical gradient, and this gradient is maintained across the mitochondrial inner membrane without direct coupling to ATP hydrolysis or transport of other ions. In this study we found that TMRE uptake was reduced in the SHR neurons, which suggests that the membrane potential of mitochondria (\(\Delta \Psi _{m}\)) was more depolarized in the SHR. Depolarization of \(\Delta \Psi _{m}\) could lead to a reduced Ca\textsuperscript{2+} uptake by the mitochondria in the SHR.

A significant association of hypertension with mitochondrial uncoupling proteins has been reported both in experimental\textsuperscript{38} and human hypertensive states.\textsuperscript{39} Moreover, in experimental hypertension, a decreased activity of complex
IV has been observed in the hypertrophied myocardium from the SHRs.40 However, we could not find any differences in protein levels of mitochondrial uncoupling protein 2, citrate synthase (used as a quantitative enzyme marker for the presence of intact mitochondria), and complexes I to V in SCG homogenates from young SHRs and WKY rats (Figure S3). This indicates that mitochondrial number and the electron transport chain were not changed in young SHRs. The differences in depolarization-evoked \( [Ca^{2+}]_i \) transient and the 50% and 90% decay times between SHRs and WKY rats wereabolished by application of the proton uncoupler FCCP. These results suggest that mitochondria play a major role in the depolarization-evoked \( [Ca^{2+}]_i \) difference observed between the SHR and the WKY groups. Although we have no direct proof to show whether the ER and mitochondria contributed to the differences with age in \( [Ca^{2+}]_i \) transients in the SHR, our results are supportive of this idea when taken together with others41,42 that showed an age-related decline in SERCA function with a subsequent increased reliance on mitochondria to control high K\(^{+}\)-evoked \( [Ca^{2+}]_i \) transients.

**Perspectives**

Results from this study suggest that the difference in \( Ca^{2+} \) homeostasis between sympathetic neurons of SHRs and WKY rats occurs early in the development and before the actual onset of hypertension itself. Impairment of \( [Ca^{2+}]_i \) handling was observed at 2 neural sites in the sympathetic nervous system, suggesting that this impairment may be widespread. A close link between faulty mitochondria \( Ca^{2+} \) release and reuptake appears to be central to the enhanced \( [Ca^{2+}]_i \) transients observed in prehypertensive SHRs. The precise molecular pathway underpinning this is not firmly established but warrants further investigation. Moreover, it would be desirable to see whether the changes that we report here are also seen in other animal models of hypertension. When all of the observations are viewed together with the current data, there is compelling evidence to suggest that alterations of \( Ca^{2+} \) homeostasis are central to sympathetic hyperactivity in the SHR, resulting in enhanced sympathetic neurotransmission at the end organ. The resultant chironotropic and inotropic actions of NE will contribute to raising cardiac output and arterial blood pressure. These pathways may be important targets to prevent sympathetic dysregulation that occur before the onset of hypertension.

**Acknowledgments**

We thank Prof Sergey Kasparov and Dr Haibo Xu for providing neonatal SHRs and Dr Lisa Heather for her kind donation of primary antibody. We also thank Dr Mary McMenamin, Julia Shanks, Chieh-ju Lu, and Kate Wannop for technical assistance.

**Sources of Funding**

This work was supported by a project grant from the British Heart Foundation (PG/08/0061) and the Wellcome TrustOXION initiative, N.H. and D.J.P. acknowledge additional support from the British Heart Foundation Centre of Research Excellence, Oxford.

**Disclosures**

None.

**References**


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Hypertension. published online January 17, 2012;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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Abnormal Intracellular Calcium Homeostasis in Sympathetic Neurons from Young Prehypertensive Rats

Short title: Sympathetic neural calcium regulation in the SHR

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Supplemental Materials and Methods

Heart rate and arterial blood pressure measurement

The heart rate and arterial blood pressure (ABP) were measured invasively as a terminal procedure on some rats to confirm their ABP given the genetic background. Briefly the rat was ventilated under anaesthesia (1-3% isoflurane and 100% oxygen) and its left carotid artery was cannulated with a 3F portex cannula. The cannula was then connected to a pressure transducer and data were acquired using a Biopac M150 system connected to a Macbook Pro computer running AcqKnowledge 3.9.2 software. The heart rate and blood pressure readings were taken as an average over 30 seconds once blood pressure had stabilized over a 5 minute period. 200 data points per second were acquired during the recording period.

Sympathetic neuron isolation and tissue culture

Rats were humanely killed by an approved Home Office schedule 1 method: overdose of pentobarbital (>200mg/kg) followed by exsanguination. Each experiment used 2-4 rats. Isolation and culture of the superior cervical ganglia (SCG) or cardiac stellate ganglia were carried out using modifications of the methods as previously described 1, 2. Briefly, SCG or cardiac stellate ganglia were dissected and placed in cold L-15 medium and desheathed carefully under a dissection microscope to remove all surrounding connective tissue. Ganglia were cut into a number of pieces and digested with collagenase and trypsin. The ganglia were then rinsed twice in L-15 blocking medium (96.8% L-15 medium supplemented with 0.6% D-(-)-Glucose solution, 2 mmol/L L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10% Fetal bovine serum), and rinsed two more times in N2 medium (49% DMEM, 49% F-12 nutrient mixture (HAM), 0.5 mg/ml Bovine serum albumin, 2 mmol/L L-glutamine, 1% N2 supplement and 100 ng/ml Nerve growth factor) to remove any residual fetal bovine serum. The ganglia were dissociated by sequential mechanical trituration using a fire-polished glass pipettes. Dissociated neurons were purified by seeding on a collagen coated dish for 1.5 h to minimize the number of fibroblasts and Schwann cells in the culture. The supernatant containing mostly neurons were plated onto poly-D-lysine/ laminin coated 6 mm cover slips and cultured in N2 medium, then kept at 37°C in 5% CO2. Media were changed every day and experiments were performed 2-3 days after plating.

Measurement of free intracellular calcium and ER calcium concentration

\[ [\text{Ca}^{2+}]_i \] was determined in single SCG neurons using Fura-2/AM fluorescence ratio imaging. Subconfluent neurons were loaded with 2.5 µmol/L Fura-2/AM at 37 °C for 30 min. For analysis of calcium in ER compartments, SCG neurons were incubated with 5 µmol/L mag-fura-2/AM for 120 min at 37 °C as previously described 3. Loaded neurons were visualized using a Nikon Diaphot microscope with a 40 × oil immersion lens, equipped with a Cairn Optoscan monochromator and photomultiplier tube. Image was captured with a deep cooled QImaging Retiga-SRV CCD camera connected to a Cairn Optosplit II.

For measuring \([\text{Ca}^{2+}]_i\), from cardiac sympathetic neurons, Fura-4F/AM fluorescence ratio imaging was used. Neurons were incubated with 2.5 µmol/L Fura-4F/AM at 37 °C for 30 min, and captured with a CoolSnap digital CCD camera connected to a PTI easy ratio pro
fluorescence imaging system housed on an inverted Nikon microscope equipped with a 60x, oil-immersion objective.

The cover slip containing the neurons was placed into a temperature-controlled (37 °C), gravity fed chamber (volume: 100 µl), superfused with Tyrode solution gassed with 5% CO2/Balance air at a flow rate of 3 ml/min. The evoked [Ca²⁺]i transient was evaluated by 30 s exposure to 100 mM KCl (with equimolar reduction in NaCl) in the Tyrode solution. Neurons were excited alternately at 340 nm and 380 nm at interval of 3500 ms and the emitted fluorescence measured at 510 nm. Fluorescence excitation ratios were transformed into [Ca²⁺]i concentrations using the equation derived by Grynkiewicz et al ( [Ca²⁺]i=Kd×(Sf²/Sb²)×(R−Rmin)/(Rmax−R) ).

Measurement of mitochondrial membrane potential

The mitochondrial membrane potential (Δ⁰Vm) was monitored using the fluorescent indicator tetra-methylrhodamine ethyl ester (TMRE). TMRE is a lipophilic, cationic fluorescent dye that accumulates within mitochondria according to their Δ⁰Vm in a Nernstian fashion. SCG Neurons were incubated with 20 nmol/L TMRE for 5 minutes at room temperature, and excited at 530 nm at interval of 2500 ms and the emitted light at wavelengths longer than 560nm was measured.

Immunohistochemistry

Cultured primary SCG neurons were fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton X100 and 1% BSA. Cells were then processed for immuno-reactivity with sympathetic neuron specific marker, mouse anti tyrosine hydroxylase (TH), 1:200 (Sigma). Fixed cells were incubated sequentially with 10% normal horse serum, primary antibody and biotinylated secondary antibody. Finally, the immunofluorescent signals were detected by Texas Red Streptavidin (SA-5006, Vector Labs) for TH and VECTASHIELD DAPI (H-1200, Vector Labs) for nucleus.

Western blot analysis

SCG homogenates were analyzed by Western blotting to compare SERCA2, PLN, phosphor-Ser16-PLN and phosphor-Thr17-PLN (Badrilla). Briefly, liquid nitrogen-frozen SCG isolated from 4-7 weeks SHR and WKY were ground to a fine powder under liquid nitrogen, homogenized in 100 ul CelLytic buffer (containing 2.5 µl protease inhibitor cocktail and 1 µl phosphatase inhibitor cocktail, Sigma). 30 µg of protein from each sample was loaded into polyacrylamide gel. The proteins were separated by electrophoresis and transferred to a polyvinylidene fluoride membrane. The non-specific binding sites were blocked with 5% non-fat skim milk in phosphate buffered saline containing 0.05% Tween-20. The membrane was then probed with specific antibodies overnight at 4 °C. Binding of the primary antibody was detected with the use of peroxidase-conjugated secondary antibodies for 1 h at room temperature. The relevant protein signal was amplified with luminol based chemiluminescence (Western Lightning Plus, Perkin Elmer Life Science) and detected using light-sensitive film (CL-XPosure film, Pierce). The film was digitized and the relative densities were determined with a computer software (UN-SCAN-IT, gel 6.1). The results were normalized to GAPDH (Sigma) that served as loading control.

RT-PCR quantification of RyR3 mRNA levels
Total RNA was isolated from the dissected SCG using the RNaseasy Mini extraction kit (Qiagen) according to the manufacturer’s instruction. The integrity of the RNA was determined by assessing the 260/280 ratio. RNA samples with ratios of 1.8-2.0 indicate high purity of RNA, and samples yielding these values were used for all RT-PCR reactions. cDNA is synthesized from total RNA by Stratagene AccuScrip High-Fidelity Reverse Transcriptase (RT) in a reaction primed with oligo (dT). A portion of the cDNA synthesis reaction was then transferred to a new tube and amplified by PCR using PfuUltra II fusion HS DNA Polymerase (Stratagene). The primers used for RT-PCR analysis were specific for the RyR3 and GAPDH, as determined by the NCBI blast database for *Rattus norvegicus*. The sequence of each primer were as follows: RyR3 FWD 5’ GAACCGAGA TGTTGCTGTG 3’. RyR3 REV 5’ GTCTTGGCCAGCAAAAATG 3’. GAPDH FWD 5’ ACCACAGTCCATGCCATC 3’. GAPDH REV 5’ AGTTGGGATAGGGCCTCTT 3’. Primers were synthesized commercially by Sigma-Aldrich Company Ltd, UK. To test for DNA contamination we performed negative controls, which consisted of the same RT-PCR reaction mix contained in the biological samples minus reverse transcriptase. After running the PCR reaction in a DNA Engine thermal cycler with heated lid (MJ Research), each reaction was separated on 1.5% agarose gel containing 0.3 µg/ml ethidium bromide in 0.5% TBE buffer and DNA visualised under UV illumination (Syngene).

**Solutions and materials**

Unless otherwise stated, all chemicals were purchased from Sigma (St. Louis, MO) and Invitrogen (Eugene, OR). Experiments were performed with a normal Tyrode solution containing (in mmol/L) 117 NaCl, 4.5 KCl, 1 MgCl₂, 23 NaHCO₃, 2.5 CaCl₂ and 11 glucose, gassed with 5%CO₂/Balance air. For Ca²⁺-free solutions (0 Ca²⁺), the CaCl₂ was omitted and EGTA (0.5 mmol/L) added to buffer contaminating any Ca²⁺. All fluorescent dyes were obtained from Molecular Probes (Eugene, OR).

**Statistical Analysis**

Data were expressed as means ± s.e.m. and n indicates the number of SCG neurons used. All statistical calculations were performed using the SigmaPlot 11 software package (Systat Software Inc.). For comparison of two groups, an unpaired t-test was performed, or a Mann-Whitney Rank Sum Test if the data were not normally distributed. To compare more than two groups, One-Way analysis of variance (ANOVA) was performed and the Holm-Sidak method was used as post-hoc test. For all experiments, statistical significance was accepted at *P*<0.05.

**Supplemental References**


**Supplemental Results**

**Supplement Table S1. Cardiac autonomic phenotype of SHR and WKY rats**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Young WKY</th>
<th>SHR</th>
<th>Adult WKY</th>
<th>SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventricular weight /body weight (×10⁻³)</td>
<td>4.36±0.17 (n=9)</td>
<td>4.23±0.12 (n=9)</td>
<td>2.94±0.03 (n=12)</td>
<td>3.23±0.03* (n=15)</td>
</tr>
<tr>
<td>Mean ABP (mmHg)</td>
<td>75±5 (n=8)</td>
<td>71±4 (n=7)</td>
<td>86±3 (n=8)</td>
<td>173±4* (n=11)</td>
</tr>
<tr>
<td>Heart rate <em>in-vivo</em> (bpm)</td>
<td>277±12 (n=8)</td>
<td>298±7 (n=7)</td>
<td>287±5 (n=8)</td>
<td>353±11* (n=11)</td>
</tr>
</tbody>
</table>

*\(p<0.05\), *-test, for comparison between age matched WKY and SHR.*
## Supplement Table S2. Baseline free $[\text{Ca}^{2+}]_i$ in SCG neurons

<table>
<thead>
<tr>
<th>Age</th>
<th>WKY</th>
<th>SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neonatal</td>
<td>$72.07 \pm 3.77 \ (n=9)$</td>
<td>$120.35 \pm 5.21 \ (n=14)$†</td>
</tr>
<tr>
<td>Young</td>
<td>$98.70 \pm 3.26 \ (n=50)$‡</td>
<td>$110.39 \pm 4.25 \ (n=48)$*</td>
</tr>
<tr>
<td>Adult</td>
<td>$72.06 \pm 5.42 \ (n=11)$</td>
<td>$89.54 \pm 4.50 \ (n=12)$* §</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. *p<0.05, †p<0.001 for comparison between age matched WKY and SHR, t-test. ‡ Young WKY vs. Neonatal (p<0.01) or Adult (p<0.001) WKY; § Adult SHR vs. Neonatal (p<0.01) or Young (p<0.01) SHR, one way ANOVA.

## Supplement Table S3. Percentage change of $[\text{Ca}^{2+}]_i$ from the baseline.

<table>
<thead>
<tr>
<th>Age</th>
<th>WKY</th>
<th>SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neonatal</td>
<td>$2354 \pm 136% \ (n=9)$</td>
<td>$2055 \pm 137% \ (n=14)$</td>
</tr>
<tr>
<td>Young</td>
<td>$1724 \pm 174% \ (n=23)$</td>
<td>$2392 \pm 189% \ (n=22)$*</td>
</tr>
<tr>
<td>Adult</td>
<td>$3153 \pm 309% \ (n=11)$</td>
<td>$3631 \pm 221% \ (n=12)$</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. *p<0.05, for comparison between age matched WKY and SHR, t-test.

## Supplement Table S4: Rate of $[\text{Ca}^{2+}]_i$ rise in response to high $K^+$ $[\Delta\text{ratio}/\Delta\text{time}(\text{sec})]$

<table>
<thead>
<tr>
<th>Age</th>
<th>WKY</th>
<th>SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neonates</td>
<td>$20.27 \pm 2.69 \ (n=9)$</td>
<td>$19.14 \pm 2.56 \ (n=14)$</td>
</tr>
<tr>
<td>Young</td>
<td>$33.36 \pm 2.70 \ (n=23)$*</td>
<td>$37.29 \pm 2.55 \ (n=22)$‡</td>
</tr>
<tr>
<td>Adult</td>
<td>$35.93 \pm 2.64 \ (n=19)$†</td>
<td>$36.78 \pm 5.28 \ (n=13)$†</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. *p<0.05, †p<0.01, ‡p<0.001. Compared with neonates within the same group, one way ANOVA.
Figure S1: Calculated the area under the curve of the Ca\textsuperscript{2+} response to KCl (during 30 seconds, A), and the period from stimulation with KCl to recovery to the base line (150 seconds, B, this covered the whole curve). The values of the areas under the curve were all significantly higher in the SHR from neonatal to the fully developed hypertensive rat when compared with the age matched WKY (except Fig. B, young rats \(p=0.075\), \(*p<0.05\), \(**p<0.01\), \(t\)-test. \(\dagger p<0.05\), \(\ddagger p<0.01\), one way ANOVA).
Figure S2: SCG neurons loaded with a fluorescent indicator mag-fura-2/AM, which was used to measure the free calcium concentration changes in the internal stores. Loaded with 2.5 µmol/L at 37 ºC for 30 min, fluorescence was diffusely distributed through the cytoplasm (A). In contrast, when neurons were loaded with 5 µmol/L at 37° C for 120min, discrete fluorescence labeling identified the putative ER calcium stores (B). Excitation at 340 nm.
**Figure S3, A&B:** Immunoblots using specific antibodies for UCP2 and Citrate synthase using SCG homogenates from young SHRs and WKY rats. Equal amounts of protein were loaded in each line (n=10 SCGs per group). The expression of UCP2 or Citrate synthase was not significantly different. **C-G:** Immunoblots using specific antibody for complexes I-V (CI-CV). Protein levels of complexes I-V in SCG from young SHRs and WKY rats were not significantly different between two strains. n=5 SCGs per group.