Chemoreceptor Hypersensitivity, Sympathetic Excitation, and Overexpression of ASIC and TASK Channels Before the Onset of Hypertension in SHR

Zhi-Yong Tan,* Yongjun Lu,* Carol A. Whiteis, Annabel E. Simms, Julian F.R. Paton, Mark W. Chapleau, François M. Abboud

Rationale: Increased sympathetic nerve activity has been linked to the pathogenesis of hypertension in humans and animal models. Enhanced peripheral chemoreceptor sensitivity which increases sympathetic nerve activity has been observed in established hypertension but has not been identified as a possible mechanism for initiating an increase in sympathetic nerve activity before the onset of hypertension.

Objective: We tested this hypothesis by measuring the pH sensitivity of isolated carotid body glomus cells from young spontaneously hypertensive rats (SHR) before the onset of hypertension and their control normotensive Wistar–Kyoto (WKY) rats.

Methods and Results: We found a significant increase in the depolarizing effect of low pH in SHR versus WKY glomus cells which was caused by overexpression of 2 acid-sensing non–voltage-gated channels. One is the amiloride-sensitive acid-sensing sodium channel (ASIC3), which is activated by low pH and the other is the 2-pore domain acid-sensing K⁺ channel (TASK1), which is inhibited by low pH and blocked by quinidine. Moreover, we found that the increase in sympathetic nerve activity in response to stimulation of chemoreceptors with sodium cyanide was markedly enhanced in the still normotensive young SHR compared to control WKY rats.

Conclusions: Our results establish a novel molecular basis for increased chemotransduction that contributes to excessive sympathetic activity before the onset of hypertension. (Circ Res. 2010;106:536-545.)

Key Words: carotid body glomus cells ▪ pH sensitivity ▪ ion channels ▪ sympathetic nerve activity ▪ prehypertensive SHR

Increased sympathetic nerve activity (SNA) has been linked to the pathogenesis of hypertension in humans with essential hypertension,¹-⁴ with borderline hypertension⁵ and with obstructive sleep apnea.⁶ Similarly, exaggerated sympathoadrenal drive is a major determinant of the elevated arterial pressure in the spontaneously hypertensive rat (SHR) model of genetic hypertension.⁷,⁸ There is also evidence that in young SHR there are regional increases in sympathetic activity⁹ and changes in norepinephrine levels¹⁰ that occur at 4 to 5 weeks of age before the onset of hypertension. In young SHR sympathectomy and prazosin abrogate the subsequent development of hypertension in adult rats.¹⁰ Recent studies by Simms et al¹¹ also support the view that increased SNA is already present in neonatal SHR before the onset of hypertension as evident in the significant enhancement of respiratory-coupled bursts of sympathetic activity compared to Wistar–Kyoto (WKY) rats. Our goal was to identify mechanisms that could result in the initiation of an early increase in SNA in the SHR before hypertension.

We considered the possibility that enhanced chemoreceptor activity may be such a mechanism for 2 reasons. First, the chemoreceptor reflex is enhanced in established hypertension¹²-¹⁵ and, it increases SNA and blood pressure when activated by hypoxemia, hypercapnia, and acidosis,¹²,¹³,¹⁶-¹⁹ Second, the increases in SNA reported in the young SHR⁸,¹⁰ have been associated with disturbances in acid-base balance before the onset of hypertension.²⁰,²¹ Such changes in acid-base balance may activate peripheral chemoreceptors and exaggerate sympathetic drive.

Therefore, in this study, we tested the hypothesis that carotid body chemoreceptors are hypersensitive in SHR before the onset of hypertension. Cellular and molecular studies on isolated carotid body glomus cells allowed us to define an enhanced responsiveness of specific acid-sensing ion channels and their overexpression in SHR. Additionally, functional studies revealed augmented SNA in response to...
chemoreceptor stimulation with intraarterial sodium cyanide (NaCN) in prehypertensive SHR compared to WKY rats. The results establish a novel molecular basis for increased chemotransduction that contributes to the initiation of excessive SNA.

Methods

The work was performed on SHR and WKY rats, 4 to 6 weeks of age. All animal handling and experimental protocols were approved by the animal care and use committee of the University of Iowa. Published protocols for isolation of glomus cells from rat carotid bodies were followed.22,23 Conventional whole-cell perforated patch clamp recordings from glomus cells provided data on membrane currents and voltage potentials during fast exchanges of various pH solutions extracellularly, whereas intracellular pH was buffered at pH 7.2. Carotid body mRNA and proteins were measured at 1 month of age using real-time RT-PCR. Western blots, and immunohistochemistry to determine the expression of acid-sensing sodium channels (ASICs) and 2-pore domain acid-sensitive K⁺ (TASK) channels as reported.24 Using the working heart–brainstem preparation as previously described,11 responses of thoracic sympathetic and phrenic nerve activities to intra-aortic NaCN were measured.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org. This section includes Online Figure I showing age-dependent increases in blood pressure in SHR and WKY; Online Table I showing the PCR primer sequences; and Online Table II showing the antibodies used in the study.

The Online Data Supplement also describes results that include Online Figure II showing a blockade of pH-induced rapid inward currents with amiloride; Online Figure III showing voltage-dependent outward currents blocked by low pH and quinidine; Online Table III showing selective effects of amiloride and quinidine on low pH-induced depolarizations; and Online Table IV showing hemodynamic, autonomic, and respiratory responses to chemoreceptor stimulation with NaCN.

Results

There was no significant difference in the size (cell diameter), membrane capacitance, membrane resistance, and resting membrane potential between SHR and WKY glomus cells. Values were 9.1±0.2 μm, 4.8±0.3 pF, 3.0±0.3 GΩ and −60±1mV (n=28) in SHR, and 9.1±0.1 μm, 4.7±0.3 pF, 2.6±0.3 GΩ and −57±1mV (n=36) in WKY.

Acid-Evoked Rapid Inward ASIC-Like Currents

Extracellular solutions at pH 7.0, 6.5, 6.0 and 5.5 triggered pH-dependent initial rapid inward currents that were markedly enhanced in SHR compared with WKY glomus cells (Figure 1A). Both the amplitude and density of these currents were significantly higher in SHR compared with WKY glomus cells (Figure 1B). The threshold for activation occurred at about pH 7 or higher, and the currents were half-activated at pH 6.5 (Figure 1A). Right, Currents were normalized to the peak response induced by pH 6.0; curves are best fit of Hill equation, I=I_0 /[1+(1+([H⁺]^n/[H⁺]^θ)])], half-activation values were pH 6.5 for both groups; Hill coefficients were 2.6 and 2.4 for SHR and WKY cells, respectively. Values are means±SE.

Acid-Inhibited TASK-Like Outward Background Potassium Currents

A slow ramp depolarization from −100 to −40 mV over a period of 2 seconds was used to record the background outward current (Figure 2). Outward currents recorded at pH 7.4 in glomus cells from a SHR and a WKY rat were increased by an alkaline pH of 8.0, and were progressively inhibited by acidic pHs of 7.0, 6.5, and 6.0 (Figure 2A and 2B).
At pH 8.0, the currents were significantly higher in SHR cells compared to WKY, and the decrease in current as pH was lowered from 8.0 to 6.5 was significantly greater in SHR versus WKY cells (ANOVA) (Figure 2B). The TASK channel blocker quinidine (1 mmol/L) reversibly inhibited the outward currents during ramp depolarizations as did low pH (Figure 3A and 3C). A residual outward current after quinidine was completely inhibited by quinidine plus low pH (Figure 3A). Figure 3B shows the pH-inhibited and quinidine-inhibited currents during the ramp depolarizations. The calculated reversal potentials of the pH-inhibited currents in both SHR and WKY coincide with the known reversal potential for K⁺ channels. Limited contributions by voltage-gated currents to pH responses are discussed and presented in the Online Data Supplement (Online Figure III).

**Acid-Induced Depolarizations**

The activation of inward ASIC currents and inhibition of outward TASK currents caused early rapid and subsequent sustained depolarizations, respectively. Under current-clamp conditions, graded pH levels of 7.0, 6.5, and 6.0 were applied. The rapid “initial depolarization,” which peaked within 5 seconds, was followed by a more “sustained depolarization” that reached a plateau over 15 to 20 seconds (Figure 4A). Both the initial and the sustained depolarizations were pH-dependent and were significantly enhanced in glomus cells from SHR versus WKY rats (ANOVA, Figure 4A and 4B).

The ASIC blocker amiloride (200 μmol/L) did not alter resting membrane potential of glomus cells of SHR (−1.5±0.3 mV, n=6) and WKY (−0.8±0.6 mV, n=6) rats, but it specifically and reversibly inhibited (P<0.01) the initial rapid depolarization in response to low pH, leaving the slower

---

**Figure 2.** Effect of pH on TASK-like outward background leak current seen with slow ramp depolarization from −100 to −40 mV. A, Representative recordings show pH-dependent inhibition of the currents from a WKY and a SHR glomus cell with progressive reductions in pH. The pH-sensitive currents were calculated by subtracting values obtained at pH 6.0 from those at pH 8.0. The reversal potentials of these pH-inhibited currents averaged −80.3±4.2 mV (n=10) and −87.2±4.5 mV (n=11) for WKY and SHR, respectively, and were not statistically different. These values coincide with the reversal potential of the outward leak K⁺ currents that have a theoretical reversal potential of −85.7 mV. B, Means±SE of outward background currents recorded at −40 mV during exposure to progressively lower levels of pH ranging from pH 8.0 to pH 6.0. The acid-inhibited outward current was significantly enhanced in SHR cells compared to WKY (ANOVA) and the current at pH 8.0 was significantly larger in SHR than in WKY (Bonferroni). Values are means±SE. *P<0.05 for post hoc comparison (Bonferroni) between SHR and WKY cells.

**Figure 3.** Effect of quinidine on the pH-sensitive outward background leak current (TASK). A, Representative recordings from an SHR glomus cell show that both pH 6.0 (left tracing) and 1 mmol/L quinidine (middle tracing) reversibly inhibited the outward background currents during ramp depolarization from −100 to −40 mV. The small residual outward current seen after quinidine was completely inhibited by quinidine plus low pH (right tracing). The rightward shift of the curve with pH 6.0 compared to control is not seen with quinidine, suggesting the presence of a pH-insensitive, but quinidine-sensitive, inward current that is blocked by quinidine. B, The tracings represent a pH-sensitive current calculated by subtracting values obtained during the ramp depolarizations at pH 6.0 from those obtained at pH 8.0; the quinidine-sensitive current was calculated by subtracting the values during ramp depolarizations at pH 7.4 after quinidine from those before quinidine. The reversal potential of the pH-sensitive current was −82 mV, which coincides with that of K⁺ current. The quinidine-sensitive net current had a reversal potential of −64 mV, reflecting blockade of both the outward K⁺ current and an inward current which is not sensitive to pH. The mean group value for the quinidine-sensitive current reversal potential was 63.4±2.4 mV (n=5). C, The bar graphs show that the outward currents measured at −40 mV (means±SE) are completely inhibited by pH 6.0 followed by recovery (left) and markedly reduced by quinidine followed by recovery (right). **P<0.01 for differences from control and from recovery.
sustained depolarization intact (Figure 5 and Online Table III). In contrast to amiloride, the TASK blocker quinidine (1 mmol/L) caused depolarizations of the resting membrane potential that were greater in SHR glomus cells ($-20.6 \pm 1.7$ mV, $n=5$) compared to WKY ($-16.0 \pm 0.8$ mV, $n=5$) ($P<0.05$) (Figure 5). Moreover, quinidine reversibly inhibited ($P<0.05$) the sustained depolarization in response to low pH, while the initial depolarization with low pH remained intact (Figure 5 and Online Table III). The inhibitory effects of both blockers were similar in SHR and WKY.

**Expression of ASIC and TASK Channels in Carotid Bodies**

Carotid bodies were obtained from 1 month old rats. Figure 6A shows the bands of real time PCR products of carotid bodies of WKY rats. Only one band in each lane was observed verifying the specificity of the PCR primers for each gene expressed. mRNA expression of TASK1, TASK3, ASIC1b and ASIC3 were significantly higher in SHR than in WKY carotid bodies (Figure 6B).

Protein expressions were detected by Western blotting. Carotid bodies from rats were pooled and homogenized as one sample for extracting membrane proteins. Three such samples were analyzed from SHR and four from WKY rats. TASK1 and ASIC3 protein expression were each increased significantly in SHR over WKY carotid bodies by 100±48% and 36±0.4% respectively (Figure 6C and 6D), but the brain values of both channels were not different between SHR and WKY rats. Despite increased mRNA levels of ASIC1b in SHR versus WKY, the protein expression was not significantly increased (data not shown).

Clusters of glomus cells from SHR are shown in Figure 7. The expression of tyrosine hydroxylase (TH) identified type I glomus cells. TH, TASK1 and ASIC3 were detected from the immunofluorescence signals. TASK1 and ASIC3 were each expressed in type I glomus cells, and each colocalized with TH (Figure 7A and 7B). The majority of cells expressing TASK1 and ASIC3 were also TH positive and ASIC3 and TASK1 were colocalized in several cells (Figure 7C).

**Increased SNA in Response to NaCN in SHR Versus WKY Rats**

Using the working heart–brainstem preparation, the changes in thoracic SNA, heart rate, perfusion pressure and phrenic nerve activity were measured in response to stimulation of carotid chemoreceptors with intraarterial injections of NaCN. NaCN increased SNA in both SHR and WKY rats (Figure 8A). The increase was significantly enhanced in the expiration phase of respiration in SHR ($P<0.001$) (Figure 8B and Online Table IV). In SHR, the bradycardia was less pronounced than in WKY rats ($P<0.05$), whereas the increased frequency of phrenic nerve activity bursts and perfu-
sion pressure were not significantly different in the 2 groups (Figure 8A and Online Table IV).

Discussion

It is increasingly evident that exaggerated SNA is an essential component of the pathogenesis of hypertension in humans and in animal models.\(^1\)\(^\text{-}^{10}\) Two important issues have been unclear. One is the mechanism that leads to excessive SNA and the second is whether the increase in SNA occurs before the onset of hypertension.

We addressed these 2 issues in a genetic model of neurogenic hypertension, the SHR, because it exhibits early signs of increased SNA before the onset of hypertension at 4 to 6 weeks of age\(^9\)\(^\text{-}^{11}\) and there is evidence that chemical-sympathectomy plus prazosin abrogate the development of hypertension in the adult rats.\(^10\)

Our results show that in young SHR compared to WKY: (1) carotid body glomus cells are hyperresponsive to low pH; (2) activation of amiloride-sensitive ASIC-like inward sodium currents and inhibition of quinidine-sensitive TASK-like outward potassium currents by low pH are enhanced, resulting in greater rapid and sustained depolarizations respectively; (3) carotid body mRNA and protein expression of ASIC3 and TASK1 are significantly increased at 1 month of age; and (4) activation of peripheral chemoreceptors with intraarterial NaCN causes significantly greater increases in thoracic SNA.

These results demonstrate exaggerated SNA responses to peripheral chemoreceptor activation before the development of hypertension and provide a molecular basis for enhanced chemoreceptor activity. We speculate that the genetic overexpression of ASIC and TASK channels in carotid bodies results in increases in SNA that may initiate increases in vascular resistance and contribute to structural changes that are seen in chronic hypertension.

In this discussion, we address: (1) the relative roles of ASIC and TASK channels versus other ion channels and molecular modulators of chemoreceptors; (2) the mechanisms that may be involved in overexpression of acid-sensing ion channels versus other genetic factors that may contribute to the initiation of hypertension in young prehypertensive SHR; and (3) the functional implications of enhanced chemoreceptor activity and consequent increases in SNA.

Figure 6. A and B, mRNA expression of ASIC and TASK channels in carotid bodies from 1 month old WKY and SHR. A, mRNA expression of TASK1, TASK3, ASIC1b, and ASIC3 in carotid bodies from WKY rats. GAPDH was used as control. B, Expression levels of ASIC and TASK mRNAs were higher in SHR carotid bodies compared to WKY. The results from SHR carotid bodies (n=5) are expressed relative to those from WKY carotid bodies (n=5) for corresponding channels (dashed line). \(**P<0.01\) and \(*P<0.05\), SHR vs WKY. The relative mRNA expression of ASIC3 was confirmed when \(\beta\)-actin and 18S were used as housekeeping genes in addition to GAPDH. Comparison of ASIC3 expression obtained with GAPDH vs \(\beta\)-actin, GAPDH vs 18S, and \(\beta\)-actin vs 18S gave probability values of 0.81, 0.89, and 0.79, respectively. C and D, Comparison of protein expression of ASIC and TASK channels in carotid bodies (CB) and brain from WKY and SHR at 1 month of age. Carotid bodies of WKY were used as reference. Protein expressions were detected by Western blotting from carotid bodies and brain of WKY and SHR. There was a selective increase in expression of both TASK1 and ASIC3 in carotid bodies of SHR vs WKY rats (\(P<0.01\) and \(P<0.05\), respectively) but not of ASIC1. Expression did not differ in brain between SHR and WKY rats.

Figure 7. Immunocytochemical localization of TH, TASK1, and ASIC3 proteins in clusters of glomus cells from 2 SHRs. A and B show localization of TASK1 and ASIC3, respectively, in TH-containing cells. C shows colocalization of ASIC3 and TASK1. Only a qualitative assessment of the distribution of fluorescence could be obtained, which showed occasional TASK1 or ASIC3 fluorescence in the absence of TH. Scale bar=10 \(\mu\)m.
Ionic Mechanisms of Chemoreceptor Activation

The depolarization of glomus type I cells in carotid bodies is recognized as the primary event in the chemotransduction of hypoxemia, hypercapnia, and acidosis. The resulting increase in intracellular Ca\(^{2+}\) causes the release of neurotransmitters (ATP and acetylcholine) that activate the adjacent carotid nerve endings triggering both hyperventilation and sympathetic activation. Hypoxemia has been reported to induce closure of K\(^{+}\) channels including the large-conductance Ca\(^{2+}\)-activated potassium (BK) channels. The transduction of acidosis includes a drop in intracellular pH which may inhibit voltage-gated K\(^{+}\) channels but may also be mediated by extracellular pH-sensitive ion conductances such as Cl currents and the non–voltage-gated TASK-like currents. We have reported that extracellular acidosis activates the non–voltage-gated acid-sensing sodium channels (ASICs) causing a rapid early depolarization of glomus cells followed by inhibition of the TASK channels that results in a sustained depolarization. Acid-evoked inward current and depolarizations is in contrast with the effect of hypoxia which may induce depolarization by inhibition of BK, as well as inhibition of voltage-gated Kv1.2, and of TASK-like currents.

A clear definition of ion channels involved in the transduction of hypoxia, hypercapnia and acidosis remains a challenge because of the complexity of channel interactions. It appears, however, that extracellular acidosis involves predominately non–voltage-gated ASIC3 and TASK-like currents.

Assessment of Contributions of the ASIC and TASK Currents to the Depolarization of Glomus Cells

Role of ASICs

The rapid kinetics of activation and inactivation of ASIC may limit its functional contribution to acid chemosensing. However, the complementary contribution of TASK channels and the coexpression of both ASIC and TASK in glomus cells allow the rapid early depolarization by ASICs to be followed by the slower more sustained depolarization by TASKs in response to low pH. The ASIC desensitization which is much slower in the glomus cells than reported in eg, dorsal root ganglion neurons may also be modulated under certain conditions to sustain a more persistent response to prolonged stimuli as was reported in myocardial ischemia. Although there is a significant variability in the ASIC response to low pH, the

![Figure 8. A, Responses to peripheral chemoreceptor stimulation with cyanide (NaCN) in young prehypertensive SHR and normotensive WKY rats recorded in situ. The arrows indicate the time of intraarterial injection of NaCN. The vertical shaded bars coincide to phrenic nerve activity during inspiration before and immediately after NaCN. The subsequent expirations are bracketed within the dashed rectangles. The response of thoracic SNA (tSNA) was analyzed separately for inspiratory and expiratory phases. Note the greater expiratory-related increases in tSNA in the SHR compared to the WKY rat. The bradycardia in beats per minute (heart rate [HR]) was less pronounced in SHR than in WKY. PNA indicates phrenic nerve activity; PP, perfusion pressure. B, Mean grouped data showing the enhanced thoracic sympathetic nerve activity (tSNA) response evoked after peripheral chemoreceptor stimulation during the expiratory versus inspiratory respiratory phase in the SHR compared to the WKY rat as recorded in situ. #P<0.001, *P<0.05.](http://circres.ahajournals.org/fig8)
percentage of responsive glomus cells is enhanced significantly in the presence of low Ca^{2+} and high lactate, and in glomus cells from SHR. Moreover, the current density is high especially in SHR glomus cells as is the ASIC message and protein expression with immunofluorescence of a majority of cells.

Role of TASK
Buckler and colleagues were the first to point to the role of TASK-like non–voltage gated K^+ channels in the pH response of neonatal rat glomus cells. Biophysical and metabolic regulation of this potassium channel in glomus cells indicates that it provides a background leak outward current to maintain a more negative resting membrane potential. Reduction of this background current by low oxygen or low pH depolarizes and activates the glomus cell. Also, the quinidine-induced depolarization of glomus cells may be explained in part by inhibition of the TASK leak current. In contrast, ASICs are not active at resting membrane potentials and their inhibition by amiloride does not alter the resting membrane potential.

In this study, we were able to quantify the magnitude of the outward leak background current with a slow ramp depolarization from −100 to −40 mV. There was a significant pH-dependent regulation of the TASK outward current with enhancement at pH 8.0 and progressive inhibition as pH was lowered to 6.0. The calculation of pH-inhibited outward current during the ramp depolarization revealed a reversal potential of −82.0 mV (Figure 3B) that coincides with that of a selective K^+ channel (ie, −85.7 mV). That reversal potential was similar in SHR (−87.2±4.5 mV) and WKY (−80.3±4.2 mV), suggesting the engagement of comparable pH-sensitive outward currents in both SHR and WKY. Although the magnitude of the inhibited current was greater in SHR. In contrast, the calculated quinidine-sensitive net current during ramp depolarization had a reversal potential of −64.0 mV, which is significantly less negative than that of K^+ indicating a contribution of an inward current that is pH-insensitive, but quinidine-sensitive.

The concentration of quinidine used in the present study (1 mmol/L) was insufficient to block totally the sustained depolarization. A higher concentration of 2 to 5 mmol/L of quinidine was also tested. The higher doses of quinidine caused additional inhibition of the depolarization but often disturbed the stability of membrane potential and its recovery (data not shown). Thus, quinidine effects on the sustained depolarization may not be specific solely to its inhibition of TASK.

Role of Voltage-Gated Currents
Using various blockers of voltage-gated currents in our previous report, we concluded that the primary contributions to pH-induced depolarizations in glomus cells was determined by the activity of the 2 non–voltage-gated ion channels ASICs and TASKs. In this study, we pursued further the possible role of voltage-gated channels in the pH-sensitivity of SHR and WKY glomus cells. The results are presented and discussed in the Online Data Supplement (Online Figure III).

Briefly, we used patch-clamp measurements of outward currents during the slow ramp depolarizations of glomus cells from −100 mV to 0 mV as well as during rapid step (50ms) depolarizing pulses from a holding potential of −60 mV to −40 mV and beyond up to +20mV. We did detect large outward currents at very low negative potentials as well as at positive potentials. These were equally inhibited by low pH in SHR and WKY. We question their functional significance because glomus cells rarely develop action potentials and the outward currents were most pronounced during marked slow ramp depolarizations or step depolarizations to positive membrane potentials (refer to Online Figure III).

Mechanisms Involved in Overexpression of Acid-Sensing Ion Channels
Several mechanisms have been identified as potential regulators of expression of ASICs. Inflammation has been shown to induce large increases in ASIC expression in sensory neurons through proinflammatory mediators nerve growth factor, serotonin, interleukin-1, and bradykinin. An analysis of the promoter region of the ASIC3 encoding gene reveals that gene transcription is controlled by nerve growth factor and serotonin. It is interesting that the tissue levels of both nerve growth factor and bradykinins are increased in young SHR. Moreover, the disturbance of acid-base balance in young SHR might affect the expression of ASICs because chronic metabolic acidosis has been found to upregulate expression of ENaCs, another member of the DEG/ENaC superfamily, as well as other genes involved in acid-base, sodium, and water transport and in cell proliferation.

More than 25 transcripts encoding proteins involved in urine acidification were coregulated during acidosis. We speculate that the adjustment to metabolic acidosis in the young SHR may not only include the kidney, but the cardiovascular system through upregulation of acid-sensing ion channels in the glomus cells of the peripheral chemoreceptors, which are the critical sites for reflex adjustments to pH changes.

Functional Significance and Mechanisms of Enhanced Chemoreceptor Activity
The peripheral chemoreceptor reflex contributes to exercise hyperpnea in humans and provides a respiratory compensation for the metabolic acidosis of exercise. Enhanced peripheral chemoreceptor sensitivity and sympathetic activity are often coupled with a reduction in baroreceptor activity and are found in patients with borderline hypertension. In animal models of hypertension such as SHR and chronic intermittent hypoxia, and in heart failure. In congestive heart failure, the enhanced SNA is thought to be a consequence of the heart failure but it also contributes greatly to the morbidity and mortality. The reported mechanisms involved in increased carotid body nerve activity in heart failure include overexpression of AT1 receptors, enhanced NADPH oxidase-derived superoxide anion production, inhibition of voltage-gated potassium channels (K_v(O)) and the downregulation of carbon monoxide and nitric oxide in carotid body. The blunted outward K_v(O) current in CB glomus cells and the chemoreceptor sensitivity to hypoxia have been normalized by gene transfer of CuZn superoxide dismutase to carotid body.

In hypertension, enhanced SNA is recognized as an important component of the disease that sustains hypertension and, an exaggerated chemoreceptor activity contributes to the process. In contrast to heart failure, the molecular mechanisms involved in the exaggerated chemoreceptor activity in
hypothesis that activated chemoreceptors may promote increased SNA and elevated arterial pressure in normal rats.68,69 We have herein provided support for that scenario. The significant increase in SNA in the young prehypertensive SHR following intraarterial injection of NaCN supports the conclusion that the chemoreceptor reflex is enhanced. Cyanide is a potent stimulus of chemoreceptors which simulates a hypoxic response by inhibition of mitochondrial oxidative respiration.73 Its inhibitory effect on TASK-like currents in glomus cells causing their depolarization74 is among the mechanisms by which it stimulates chemoreceptors possibly through a drop in intracellular pH. Our in vivo findings of chemoreceptor hyperactivity in young prehypertensive SHR reflected in increased SNA and others have reported that the increased SNA to the expiration phase of respiration may also contribute to the enhanced reflex. The coupling of the enhanced SNA to the expiration phase of respiration (Figure 8) demonstrates such an interaction.

Other Genetic Factors That May Contribute to the Development of Hypertension in SHR

Several other abnormalities in gene expression have been described in prehypertensive SHR and may be linked to the development of hypertension. Young SHR demonstrate an increase in sodium retention. Enhanced expressions of AT1R mRNA75 and Na+/K+ ATPase76 were found in proximal renal tubule of 4-week-old SHR compared to WKY. Vascular smooth muscle cells derived from aortas of 4-week-old SHR also show exaggerated growth, increased production of Ang II, and increased expression of several growth factors, adhesion molecules and cytokines.77 Phospholipase C isozymes and phospholipase activity are also upregulated in systemic and renal vasculatures in 6-week-old SHR.78

Functional adhesion molecule-1 is differentially expressed in multiple regions of the brain and in all peripheral vascular beds. More specifically, its expression in the nucleus tractus solitarius was significantly higher in SHR both at 3 weeks of age (prehypertensive), as well as in the adults (15 weeks old), suggesting a prohypertensive role for this protein in the brain stem.79

Limitation of Interpretations

Our results provide the first demonstration of chemoreceptor hyperactivity in young prehypertensive SHR reflected in increased SNA in response to intraarterial NaCN. The overexpression of acid-sensing channels in glomus cells is likely an important factor in the initiation of this carotid body hyperactivity. Because we showed that this hyperactivity leads to increased SNA and others have reported that the elimination of SNA at this stage10 prevents the development of hypertension, future studies may consider if targeted inhibition of expression of ASIC and TASK in the carotid body will suppress the development of hypertension in SHR.

Of particular interest has been the recognition of the wide variability in responses between glomus cells, which we report in Figure 1. Early reports on ultrastructure of type 1 glomus cells80–82 reveal that some had very large dense microvesicles, whereas others had less dense or clear vesicles, and some were heavily innervated and others were not and may explain the wide variability in responses to low pH. These differences led some investigators to classify type 1 glomus cells into 2 or more subtypes. The differences in structure and innervation may denote different transmitters and the expression of different ion channels and certainly suggest selective sensitivities to different chemical stimuli such as pH, hypoxia or hypercapnia.

A quantitative correlation between the varying electrophysiological responses of individual glomus cells and the magnitude of gene or protein expressions in the whole carotid bodies with its diverse composition of glomus cells of different types, other cells, vascular tissue, axons or neurons would be very unlikely. Moreover, the ASIC channel is probably a heterotrimer and although ASIC3 is the predominately acid-sensitive subunit, the association with other subunits, rather than the amounts of ASIC3 protein, may determine the current magnitude.

Although the glomus cell is the site of initial chemotransduction and the enhanced chemoreceptor reflex may certainly reflect the carotid body hyperactivity, neural interactions at other sites involving afferent, central, and efferent neurons may also contribute to the enhanced reflex. The coupling of the enhanced SNA to the expiration phase of respiration (Figure 8) demonstrates such an interaction.

Acknowledgments

The studies on the effects of intravenous NaCN on SNA in young normotensive SHR and WKY rats were carried out by coauthor A.E.S. in the laboratory of Dr Andrew Allen (Department of Physiology, University of Melbourne, Australia). We thank Sarah Porter and Courtney Cable (Department of Internal Medicine, University of Iowa) for assistance preparing the manuscript. We thank Shawn Roach (Department of Internal Medicine, University of Iowa) for assistance in creating and formatting the figures for this article and the Central Microscopy Core Facility of the Carver College of Medicine for providing the confocal microscope used in these studies.

Sources of Funding

The work at the University of Iowa was supported by NIH grant HL14388. The work at the University of Melbourne was supported by the Australian National Health and Medical Research Council (project grant 454432).

Disclosures

None.

References


25. Immke DC, McLeskey EW. Protons open acid-sensing ion channels by catalyzing relief of Ca(2+) blockade. *Neuroscience.* 2003;37:75–84.


Increased sympathetic nerve activity has been linked to the pathogenesis of hypertension; however, 2 important issues have been unclear: first, what the cause of increased sympathetic nerve activity (SNA) is; and second, whether the increase in SNA contributes to hypertension. We report for the first time that in genetically hypertensive rats (SHR) hypersensitivity of chemoreceptors causes a greater increase in SNA at a very young age before the onset of hypertension. We have found that the isolated glomus type 1 cells of the carotid body, where chemotransduction occurs, have enhanced depolarization in response to low pH in the prehypertensive SHR compared to normotensive WKY because of increased expression of 2 ion channels. One, which conducts an inward sodium current, is a strain-specific sodium channel that closes at low pH. Thus, early overexpression of these channels in normotensive WKY contributes to hypertension. Future basic and translational studies need to consider if targeted inhibition of expression of ASIC and TASK in the carotid bodies by genetic or pharmacological means could suppress the development of hypertension.
Chemoreceptor Hypersensitivity, Sympathetic Excitation, and Overexpression of ASIC and TASK Channels Before the Onset of Hypertension in SHR
Zhi-Yong Tan, Yongjun Lu, Carol A. Whiteis, Annabel E. Simms, Julian F.R. Paton, Mark W. Chapleau and François M. Abboud

Circ Res. 2010;106:536-545; originally published online December 17, 2009;
doi: 10.1161/CIRCRESAHA.109.206946
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/106/3/536

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2009/12/17/CIRCRESAHA.109.206946.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
SUPPLEMENT MATERIAL

Chemoreceptor hypersensitivity, sympathetic excitation, and overexpression of ASIC and TASK channels prior to hypertension in SHR

This online supplement includes:

1) Detailed Materials and Methods
   • Online Figure I—Age dependent increases in blood pressure in SHR and WKY
   • Online Table I—PCR primer sequences
   • Online Table II—Antibodies used in the study

2) Results
   • Online Figure II—Effect of amiloride on rapid inward current
   • Online Table III—Selective effects of amiloride and quinidine on initial and sustained depolarizations in response to low pH
   • Online Figure III—Voltage-dependent outward currents blocked by low pH and quinidine
   • Online Table IV—Responses to chemoreceptor stimulation with NaCN in SHR vs. WKY

3) References

Materials and Methods

Male SHR and WKY rats (ages 4-6 weeks) were used in these experiments. At that age, the SHR are prehypertensive (1-4); their blood pressure begins to increase progressively after 6-8 weeks and exceeds values in WKY rats reaching a maximal sustained level after approximately 15 weeks (Online Figure I).

Isolation of carotid bodies

Rats were deeply anesthetized by isoflurane inhalation and decapitated. The carotid bifurcation was exposed and both carotid bodies removed. Carotid bodies (CB) were placed in ice cold PBS until preparation for PCR and in ice cold Hibernate A (Braintree) prior to glomus cell isolation for electrophysiologic studies. The project had been approved by the University of Iowa Animal Care and Use Committee.

Culture of carotid body glomus cells

Established protocols describing the isolation of glomus cells from rat carotid bodies were followed (S5, 6). Two carotid bodies were excised from each of 54 SHR and 71 WKY rats, 4-6 weeks of age. The isolated carotid bodies were incubated in 1 cc of nominally Ca²⁺- and Mg²⁺-free Tyrode solution containing collagenase (2 mg ml⁻¹; Type IV; Worthington), trypsin (0.4 mg ml⁻¹; Worthington) and deoxyribonuclease (DNase II, 0.5 mg ml⁻¹; Worthington) at pH 7.4 for 31 minutes at 37°C. At 25 and 31 minutes the carotid bodies were mechanically disrupted for 1 minute by repeated aspiration through a fire polished Pasteur pipette treated with Aquasil Siliconizing Fluid (Pierce). Digestion was terminated at 31 minutes by dilution of the enzymes with 3 ml of a DMEM/0.1% BSA solution and cells were pelleted (2000 RPM for 8 minutes). The supernatant was discarded, the cells resuspended in 2 ml of the same solution and centrifuged for 5 minutes again. After discarding the supernatant the pellet was finally resuspended in 200 μl (400 μl for 2 rats) of culture medium (F-12/DMEM (1:1) plus, 5% fetal calf serum supplemented with 10,000 U/ml Pen V/Streptomycin and 10mM HEPES. 100 μl aliquots were plated onto
small poly-D-lysine-coated coverslips placed in 35 mm Petri dishes and incubated at 37°C with an atmosphere of 5% CO2 in air. Two hours later, when the cells are attached, 2 ml of culture medium was added to the Petri Dish. Spherical glomus cells ranging in size from 7 to 12 μm in diameter and without processes were chosen for recording 2-8 hours after plating.

**Perforated patch clamp recording**

Coverslips were transferred into a 1.0 ml recording chamber on the stage of an inverted microscope (Nikon). Whole-cell patch clamping was performed as described previously (S5) except that the perforated whole-cell mode was used in this study to maintain the integrity of the intracellular milieu. Membrane current or membrane potential were recorded with an Axopatch 200A amplifier (Axon Instruments, CA) interfaced with a personal computer. Cells with a resting membrane potential positive to -40 mV were discarded. All the cells accepted in this study had significant voltage-gated outward currents. Voltage was held at -60 mV to record ASIC currents. The patch pipettes (4 - 7 MΩ) were pulled from borosilicate glass (BF100-50-10, Sutter instrument, CA). The pipette solution consisted of (mmol/L): 35 KCl, 57.5 K2SO4, 2 MgCl2, 1 Na2ATP, 10 EGTA, 20 HEPES with pH adjusted to 7.2 by KOH. Amphotericin B at a final concentration of 240 μg/mL was used for membrane perforation. Recordings were filtered at 5 kHz and digitized at 10-50 kHz using a Digidata 1200 interface (Axon Instruments, CA), and pCLAMP9.0 (Axon Instruments, CA) was used for data acquisition and data analysis. Low pH – induced inward currents and membrane depolarizations were filtered at 1 kHz using Clampfit. The ramp currents were filtered at 50 Hz. To test the effects of extracellular acidosis on glomus cells, we used brief applications of acidic solutions while maintaining a high intracellular buffering capacity using HEPES to prevent intracellular pH changes (5). Solutions of varying pH were delivered topically to patch-clamped cells through a pipette (500 μm in diameter) using a valve controller (VC-6, Warner Instrument Corp). Fast exchange of the varying pH solutions was achieved within 20 ms and tested by measuring a change in the liquid-junction potential between the recording pipette and bath solutions. The standard bath solution contained (mmol/L): 140 NaCl, 5 KCl, 2 CaCl2, 2 MgCl2, 20 HEPES, 5 Dextrose, pH was adjusted to 7.4 by NaOH. Acidic solutions from pH 5.5 to pH 7.0 were buffered using a combination of 10 mmol/L HEPES and 10 mmol/L MES. For TASK current recordings, the bath solution contained (mmol/L): 140 NaCl, 5 KCl, 2 MgCl2, 20 HEPES, 5 Dextrose, 0.5 EGTA, pH was adjusted to 7.4 by NaOH. Acidic solutions from pH 8.0 to pH 6.0 were buffered using a combination of 10 mmol/L HEPES and 10 mmol/L 2-(N-morpholino) ethanesulfonic acid.

**Data analysis**

Data are expressed as means ± SEM. pH dose–response curves were fit to sigmoidal dose–response functions using the following Hill equation:  Y = Y_0 + A / (1 + ([H^+]_{50}/X)^b), where X is the concentration of proton and Y is the response. The H+ concentration causing half maximal activation is presented as [H^+]_{50}. pH_{50} was then calculated from [H^+]_{50}. Statistical differences were analyzed by Student’s t test, Chi Square or ANOVA. Significance was set at p<0.05.

**Real-time RT PCR.**

Measurements were made on tissue from 1 month old rats. Carotid bodies were removed as described above. Total RNA was extracted from the two carotid bodies of each of five SHR and each of five WKY rats by using Trizol reagent (Invitrogen), and reversely transcribed into cDNA by using AffinityScriptTM QPCR cDNA Synthesis Kit (Stratagene) according to the manufacturer. The quantitative PCR was carried out by using Model 7000 real-time PCR system (ABI), Brilliant SYBR Green QPCR Master Mix (Stratagene) and gene specific primers.
according to the protocol provided by the manufacturer. The primers were designed in the lab, and their sequences are given in Online Table I. The oligomers were purchased from Integrated DNA Technologies (IDT, IA). The expression of GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was used to normalize the measurements.

Online Table I. PCR primer sequences

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Genbank Accession</th>
<th>Primer Sequences</th>
</tr>
</thead>
</table>
| TASK1 62 bp | AF031384          | Forward: 5’-CTT CGC CGG TCT CTA CTA CT-3’  
Reverse: 5’-CCG CAT GAC CAT AGC CGA TT-3’ |
| TASK3 112 bp| AF192366          | Forward: 5’-AGC TGG TAA TCC TGC AGT CT-3’  
Reverse: 5’-GCA GCA TGT CCA TAT CCG ATAG-3’ |
| ASIC1b 80-bp| AJ006519          | Forward: 5’-GCT GGA GGA CAT GCT GCT CTA T-3’  
Reverse: 5’-ACC GAG TGA AGA CCA CTG AGA A-3’ |
| ASIC3 163-bp| NM_173135         | Forward: 5’-CTG CAC CAC CAA CTG CTT AG-3’  
Reverse: 5’-GGC CAT CCA CAG TCT TCT GA-3’ |

Western blotting.

Eight carotid bodies were pooled from four WKY rats and eight were pooled from four SHR at 1 month of age. They were washed twice with PBS, and homogenized in lysis buffer: 50 mmol/L Tris-HCl, 0.1% sodium dodecyl sulphate (SDS), 100 μg/mL phenyl methyl sulphonial fluoride, 150 mmol/L NaCl, pH 8.0) plus 1% Triton X-100, 3X Halt Protease Inhibitor Cocktail and 1X EDTA (Pierce). The lysates were then vortexed, and sonicated on ice with three bursts of 20 s ultrasonic irradiation (Microron Ultrasonic Cell Disruptor). A membrane protein extraction kit (Pierce) was used to extract the cellular membrane proteins. The total cellular protein concentration was determined by using a Bio-Rad Protein Assay kit (Bio-Rad). Protein (60 μg) was denatured with an equal volume of 2X gel SDS loading buffer at 95°C for 5 min, and run on a SDS-7.5% Tris-HCl polyacrylamide gel for 1 hr at 170 volts in Biorad Cell with DS-Tris-glycine running buffer. The proteins in gel were equilibrated in 20% methanol transfer buffer and then transferred electrophoretically to a nitrocellulose membrane at 100 volts for 75 min. Membrane was blocked with 5% (wt/vol) nonfat milk in Tween-Tris-buffered saline (TTBS: 0.02 M Tris and 0.15 M NaCl buffer, containing 0.1% Tween 20, pH 7.45) for one hour at room temperature. The antibodies used are listed in Online Table II. The membrane was then incubated in 1:200 antibody TTBS buffer of the anti-TASK1, ASIC1 and ASIC3 antibodies overnight, and then for one hour at room temperature with horseradish peroxidase- conjugated secondary IgG antibodies matched to the hosts, primary antibodies. For example, for the primary antibody goat anti-ASIC1, we used the secondary antibody donkey anti-goat IgG HRP; and for rabbit anti-ASIC3, used mouse anti-rabbit IgG HRP. The antibody activities were detected with an enhanced chemiluminescence detection system (Pierce Chemical) and exposure to X-ray film. After this step, the membrane was stripped and reprobed with anti-β-actin antibody for the purpose of normalizing protein loading according to the density of the β-actin band. The protein standard marker (BenchMark Pre-Stained Protein Ladder, Invitrogen) was used to evaluate the transfer efficiency and to locate the ranges of protein molecular weights after electrophoresis. The bands
taken for analysis were ASIC1 (~ 60kDa), ASIC3 (~ 60 kDa), TASK1 (50 ~ 65 kDa) and β-actin (~ 42 kDa). The densities of the bands were analyzed by using software ImageJ 1.40g. Similar Western blot analyses were carried out on brain tissue from WKY and SHR for contrasting brain to carotid body expression.

Online Table II. Antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Maker &amp; Cat #</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary ab</td>
<td></td>
<td>WB</td>
</tr>
<tr>
<td>Goat anti-ASIC1</td>
<td>Santa Cruz, sc-16009</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-TH</td>
<td>Santa Cruz, sc-25269</td>
<td>-</td>
</tr>
<tr>
<td>Goat anti-TASK1</td>
<td>Santa Cruz, sc-11309</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-ASIC3</td>
<td>Abcam, ab49333</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-β-actin</td>
<td>Sigma, A5441</td>
<td>1:2000</td>
</tr>
<tr>
<td>Secondary ab</td>
<td></td>
<td>IF</td>
</tr>
<tr>
<td>Donkey anti-goat IgG HRP</td>
<td>Santa Cruz, sc-2020</td>
<td>1:10000</td>
</tr>
<tr>
<td>Mouse anti-rabbit IgG HRP</td>
<td>Santa Cruz, sc-2357</td>
<td>1:10000</td>
</tr>
<tr>
<td>Goat anti-mouse IgG HRP</td>
<td>Santa Cruz, sc-2005</td>
<td>1:10000</td>
</tr>
<tr>
<td>Donkey anti-mouse IgG TEAS RED</td>
<td>Santa Cruz, sc-2098</td>
<td>-</td>
</tr>
<tr>
<td>Donkey anti-goat IgG TEXAS RED</td>
<td>Santa Cruz, sc-2783</td>
<td>-</td>
</tr>
<tr>
<td>Donkey anti-goat IgG FITC</td>
<td>Santa Cruz, sc-2024</td>
<td>-</td>
</tr>
<tr>
<td>Mouse anti-rabbit IgG FITC</td>
<td>Santa Cruz, sc-53806</td>
<td>-</td>
</tr>
</tbody>
</table>

**Immunohistochemistry of glomus cells in clusters**

The methods for isolation and culture of glomus cells in clusters were as described above except for a shorter period of trituration to reduce the dispersion of individual cells.

Clusters of glomus cells in culture for 20 hours were fixed with ice-cold fixative, 4% paraformaldehyde in phosphate buffered saline (PBS, GIBCO). Slides were subjected to a staining procedure of double-antibodies labeling for immunochemical analysis using a confocal microscope (Bio-Rad 1024).

After washing with PBS plus 0.1% Tween 20, the slides were blocked in solution containing 3% BSA (Bovine Serum Albumin) plus 0.5% Triton X-100 in PBS for 1 hour, and then incubated for 16-18 hours at 4°C with primary antibodies in block solution (1:10 or 1:50). After rinsing three times for 5 minutes in PBS, slides were incubated for 1 hour with secondary antibodies diluted 1:200 in blocking solution.

Primary antibodies used were anti-enzyme tyrosine hydroxylase (anti-TH), anti-TASK1 purchased from Santa Cruz, and anti-ASIC3 from Abcam as listed in Online Table II. Fluorescent stain was obtained by using fluorescein (green) or Texas Red (red) conjugated secondary antibodies matched to the hosts’ primary antibodies (for example, we used the secondary antibody mouse anti-rabbit IgG FITC for the primary antibody rabbit anti-ASIC3; and donkey anti-mouse IgG TEAS RED for mouse anti-TH). Preparations without the primary antibody were used for controls. Sample slides were washed 3 times using PBS (5 min each) and a drop of Vectashield Mounting Medium (Vector Laboratories, Burlington, Ontario, Canada) was added before applying the coverslip. The samples were viewed using the Bio-Rad 1024 Laser Scanning
Confocal Microscope, equipped with the lasers of Argon (488 and 514 nm) and helium-neon (543 nm).

Hemodynamic, autonomic and ventilatory responses to peripheral chemoreceptors stimulation in prehypertensive SHR and normotensive WKY.

The working heart-brainstem preparation was used as described previously (4, 7). Rats (SHR and WKY, 4-5 weeks old) were anesthetized with isoflurane and decerebrated. They were bisected sub-diaphragmatically and exsanguinated and anesthesia withdrawn. The head and thorax was placed in ice-chilled Ringer’s (composition in mM: 125 NaCl, 24 NaHCO₃, 5 KCl, 2.5 CaCl₂, 1.25 MgSO₄ and 1.25 KH₂PO₄, 10 dextrose, pH 7.4 after carbogenation (5% CO₂, 95% O₂)). The descending aorta was isolated and retrograde perfusion of the thorax and head was achieved via a double-lumen catheter (1.25mm, DLR-4, Braintree Scientific, MA, USA) inserted into the descending aorta. The perfusate was the Ringer’s solution (see above) containing Ficoll (1.25%) warmed to 31°C and gassed with carbogen. The second lumen of the cannula was connected to a transducer to monitor perfusion pressure in the aorta. Flow was adjusted until an augmenting (i.e. eupneic) pattern of phrenic nerve activity (PNA) was achieved (15-19 mL.min⁻¹ gave 45-70 mmHg). Neuromuscular blockade was established using vecuronium bromide added to the perfusate (4 µg.ml⁻¹, Organon Teknica, Cambridge, UK). Simultaneous recordings of PNA and thoracic sympathetic nerve activity (tSNA, T8-T10) were obtained using glass suction electrodes, amplified (20kHz, Neurolog), filtered (50-1500kHz, Neurolog), digitized (CED, Cambridge, UK) and recorded to hard disk using Spike 2 (CED). Heart rate was derived from the R-wave of the electrocardiogram (ECG) recorded simultaneously with the phrenic nerve activity. The peripheral chemoreceptors were stimulated using sodium cyanide (NaCN; 0.05% solution; 100 µl bolus) injected into the aorta or carotid arteries. The increase in central respiratory rate, maximum bradycardia and the percentage increase in tSNA were all analyzed. tSNA was rectified and integrated with a time constant of 100ms and the noise level subtracted as assessed after applying xylocaine (0.5%) to the sympathetic chain at the end of each experiment. Reflexly evoked sympathetic nerve discharge was averaged separately during the phrenic burst and the inter-phrenic interval (3 bursts/intervals in control and during the response) as previously described (4, 7).

Data analysis.

Data are expressed as means ± SEM. Statistical differences were analyzed by unpaired Student’s t test, Chi Square or ANOVA. Posthoc test with a Bonferroni correction was further used. Significance was set at P<0.05.

Results

Effect of amiloride on the rapid inward current (Online Figure II).

Representative tracings and group data shown in Online Figure II indicate a greater rapid inward current in SHR than in WKY at pH 6.0 and its blockade with essentially a complete recovery after amiloride.

Effects of amiloride and quinidine on the acid-induced depolarizations (Online Table III).

Figure 5 in the main text shows individual tracings of the initial rapid and sustained depolarizations in 4 cells: 2 from WKY and 2 from SHR. They portray the selective inhibition of the initial depolarizations by amiloride and the selective inhibition of the sustained
depolarizations by quinidine. The Online Table III shows the mean ± SE of the group data which confirm the effectiveness and selectivity of the blockers and indicate that full recovery of the responses was achieved.

**Online Table III: Selective effects of Amiloride and Quinidine on initial and sustained depolarizations in response to low pH**

<table>
<thead>
<tr>
<th></th>
<th>Initial depolarization (ΔmV)</th>
<th>Sustained depolarization (ΔmV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number Control</td>
<td>Amiloride</td>
</tr>
<tr>
<td>WKY</td>
<td>6</td>
<td>15.7±2.9</td>
</tr>
<tr>
<td>SHR</td>
<td>6</td>
<td>24.2±4.7</td>
</tr>
<tr>
<td></td>
<td>Control Quinidine</td>
<td>Recovery</td>
</tr>
<tr>
<td>WKY</td>
<td>5</td>
<td>13.8±4.6</td>
</tr>
<tr>
<td>SHR</td>
<td>5</td>
<td>20.4±6.7</td>
</tr>
</tbody>
</table>

Responses (ΔmV) to low pH 6.0 in WKY vs. SHR glomus cells. Values are means±SEM. Amiloride selectively and significantly inhibited the initial depolarization (†P<0.01) in both WKY and SHR glomus cells. Conversely, quinidine selectively and significantly inhibited the sustained depolarizations (*P<0.05).

**Effect of low pH on voltage-gated channels in glomus cells (Online Fig. III).**

We have shown (Circ. Res. 2007) that low pH depolarizes glomus cells predominantly by altering conductances of two non-voltage-gated channels. ASIC channels are activated while TASK channels are inhibited resulting in rapid early depolarization followed by sustained depolarization respectively. In this study we report that these responses are enhanced in prehypertensive SHR. Following pharmacologic blockade of these two channels with amiloride and quinidine the majority of the pH-induced depolarization is abrogated.

To further test the effect of pH on voltage gated currents in glomus cells we used patch clamp recordings of currents induced by a slow ramp depolarization from -100 mV to 0 mV over a period of two seconds. An exponential increase in outward current was noted when the cells were almost fully depolarized between -30 and 0 mV. This large current was reversibly inhibited by both low pH and quinidine (Online Fig. IIIA).

Moreover, when we applied rapid incremental step depolarizations of 50 msec. duration from a holding potential of -60 to -40 mV and then progressively up to +20 mV large outer currents were induced. These currents were increased by raising pH from 7.4 to 8.0 and then reduced by stepwise lowering of pH from 8.0 to 6.0. The partial blockade of these currents was reversed when pH was restored from 6.0 to 7.4 (Online Fig. IIIB). The magnitude of inhibition of these currents by lowering pH from 8.0 to 6.0 was comparable in SHR and WKY glomus cells (Online Fig. IIIC). We question the functional relevance of these currents since glomus cells rarely develop action potentials and these outward currents are largest when the cells are markedly depolarized or at a positive membrane potentials.
Effects of intraarterial NaCN in SHR and WKY Rats (Online Table IV).

Stimulation of peripheral chemoreceptors with intraarterial cyanide caused increases in tSNA, in perfusion pressure, and in central respiratory drive (frequency of phrenic nerve activity bursts) and decreases in heart rate. These are portrayed in Figure 8A in the main text, which contrasts responses in an SHR and a WKY rat. The online Table IV shows the mean ±SE of the group data. Ventilatory, autonomic, and hemodynamic responses to NaCN were not statistically different in SHR vs. WKY rats with the exception of the increase in tSNA, which was significantly greater in SHR during expiration and the bradycardia, which was less pronounced in SHR.

Online Table IV. Responses to chemoreceptor stimulation with NaCN in SHR vs. WKY

<table>
<thead>
<tr>
<th></th>
<th>HR (Δbeats/min.)</th>
<th>PP (ΔmmHg)</th>
<th>tSNA (Insp.) (%Δ)</th>
<th>tSNA (Expir.) (%Δ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>-286 ± 21.9</td>
<td>9.0 ± 0.89</td>
<td>109 ± 29.1</td>
<td>78.5 ± 16.8</td>
</tr>
<tr>
<td>SHR</td>
<td>-197* ± 23.2</td>
<td>7.3 ± 1.3</td>
<td>102 ± 25.9</td>
<td>237*# ± 37.5</td>
</tr>
</tbody>
</table>

Phrenic Nerve Activity

<table>
<thead>
<tr>
<th></th>
<th>Frequency (bursts/min)</th>
<th>Inspr. Length (s)</th>
<th>Expir. Length (s)</th>
<th>% Δ freq. (Δ interburst interval)</th>
<th>% Δ in amplitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>19.9 ± 1.9</td>
<td>0.69 ± 0.06</td>
<td>2.7 ± 0.34</td>
<td>111 ± 28.7</td>
<td>9.7 ± 2.7</td>
</tr>
<tr>
<td>SHR</td>
<td>17.5 ± 0.97</td>
<td>0.79 ± 0.06</td>
<td>2.8 ± 0.25</td>
<td>96.9 ± 20.8</td>
<td>11.7 ± 2.2</td>
</tr>
</tbody>
</table>

Δ indicates the response to NaCN; HR = heart rate; PP = perfusion pressure; tSNA = thoracic sympathetic nerve activity; Inspr. = inspiration; Expir. = expiration; s = seconds; freq. = frequency. * = P<0.05 for WKY vs. SHR; # = P<0.01 for expiration vs. inspiration in SHR.
References


Online Figure I: Age-dependent increases in blood pressure in SHR and WKY. (Adapted from ref. #1 Andresen et. al, Circ. Res. 1980;47:821-828.)

Values are means of systolic pressure from 10–30 rats each and the SE were smaller than the symbols. At 6 weeks SHR had a pressure that was still equivalent to that of the normotensive adult WKY. Before 6 weeks of age blood pressure in SHR was not significantly different than that in WKY and considered to be in the prehypertensive phase.
Online Figure II: Amiloride blocks rapid inward current evoked by extracellular acidosis in glomus cells from WKY rats and SHRs.

A) Representative currents from a WKY and a SHR glomus cell show inhibition of inward current by pH 6.0 and recovery. A residual small sustained inward current was noted at low pH after amiloride;

B) Mean maximum current ± SE from WKY and SHR glomus cells show inhibition and recovery of the rapid inward current

(* P<0.05 for amiloride vs. control and recovery).
Online Figure III: A) Effect of low pH (6.0) and quinidine on the outward current induced by a slow ramp depolarization over a 2 second period from -100 mV to 0 mV. An exponential increase in pH and quinidine-sensitive currents occurs with pronounced depolarization; B) Effect of pH on voltage-gated outward currents evoked by incremental depolarizing pulses of 50ms duration from a holding potential of -60 to -40 mV and then progressively up to +20 mV in 5 mV increments. The currents increased with pH 8.0, then declined with low pH, and recovered at pH 7.4; C) pH 6.0-induced reduction of voltage-gated outward currents seen during step depolarizing pulses from -60 to -40 mV and then progressively to +20 mV. Values are mean ±SE of the magnitude of decline in outward currents from levels obtained at pH 8.0 to levels at pH 6.0 (ΔpA). The values increased progressively with greater depolarizations reaching a plateau at about +10 mV. There were no significant differences between SHR and WKY.