Relation of Blood Pressure Quantitative Trait Locus on Rat Chromosome 1 to Hyperactivity of Rostralventrolateral Medulla

Kamon Iigaya, Hiroo Kumagai, Toru Nabika, Yuji Harada, Hiroshi Onimaru, Naoki Oshima, Chie Takimoto, Tadashi Kamayachi, Takao Saruta, Hiroshi Itoh

Abstract—Genetic factors that induce essential hypertension have been examined using genome-wide linkage analyses. A quantitative trait locus (QTL) region that is closely linked to hypertension has been found on chromosome 1 in stroke-prone spontaneously hypertensive rats (SHRSPs). We used 2 congenic rats in which the blood pressure QTL on rat chromosome 1 was introgressed from SHRSP/Izm to Wistar-Kyoto (WKY)/Izm (WKYpch1.0) and from WKY/Izm to SHRSP/Izm (SHRSPwch1.0) rats by repeated backcrossing. Previous studies reported that the intermediate phenotype of this QTL for hypertension is characterized by the hyperactivity of the sympathetic nervous system in response to physiological and psychological stress. We performed intracellular patch-clamp recordings of rostral ventrolateral medulla (RVLM) neurons from WKY, WKYpch1.0, SHRSPwch1.0, and SHRSPs and compared the basal electrophysiological activities of RVLM neurons and the responses of these neurons to angiotensin II. The basal membrane potential of RVLM neurons from WKYpch1.0 was significantly “shallower” than that of the neurons from WKY. The depolarization of RVLM neurons from WKYpch1.0 in response to angiotensin II was significantly larger than that in neurons from WKY rats, whereas the depolarization of RVLM neurons from SHRSPwch1.0 was significantly smaller than that in neurons from SHRSPs. The response to angiotensin II of RVLM neurons from WKYpch1.0 and SHRSPs was sustained even after the blockade of all of the synaptic transmissions using tetrodotoxin. The QTL on rat chromosome 1 was primarily related to the postsynaptic response of RVLM bulbospinal neurons to brain angiotensin II, whereas both the QTL and other genomic regions influenced the basal activity of RVLM neurons. (Hypertension. 2009;53:00-00.)

Key Words: sympathetic nervous system | congenic rat | angiotensin II | stress | RVLM neurons

The stroke-prone spontaneously hypertensive rat (SHRSP) is a useful model for the study of human essential hypertension.1 Previous genome-wide analyses identified a potent quantitative trait locus (QTL) on rat chromosome 1 (Chr-1) that is responsible for hypertension in SHRSPs; this trait was confirmed in congenic strains for the QTLs.2–5 Further analyses of the congenic strains suggested that this QTL harbored a gene (or genes) that regulated sympathetic responses to various stresses, such as restraint, cold, and air-jet stress.3–7 Because the stressors used were either physical or emotional in nature, we hypothesized that a common pathway regulating sympathetic responses to stress might be responsible for this phenomenon. In this regard, the genetic effects of the Chr-1 QTL on the neuronal activity of the rostral ventrolateral medulla (RVLM), which is thought to determine the basal sympathetic nervous tone in response to various inputs from higher brain centers,3,9 were explored in this study. In addition, among various modulators of RVLM activity, we particularly focused on the role of angiotensin II (Ang II) based on the following observations: physiological studies on rabbits showed that cardiovascular responses to air-jet stress were attenuated by the infusion of Ang II receptor blockers into the RVLM, implying a pivotal role of angiotensinergic neurons in the RVLM on the responsiveness to emotional stress.10 Thus, we studied the stimulatory effect of Ang II on RVLM neurons in a previous study using brain stem-spinal cord preparations from neonatal rats.11

In the present study, we used reciprocal congenic strains constructed to evaluate the effect of the blood pressure QTL on Chr-1 on the electrophysiological activities of RVLM neurons in the absence of any influence from blood pressure and the higher brain center in the hypothalamus. The basal activity and response to Ang II superfusion were recorded in a single neuron using the whole-cell patch-clamp technique.
Materials and Methods

Animals

Two congenic rat strains, WKY.SHRSP-(D1Wox29-D1Arb21)/Izm (abbreviated as WKYpch1.0) and SHRSP.WKY-(D1Wox29-D1Arb21)/Izm (abbreviated as SHRSPrwch1.0) were provided by the Disease Model Cooperative Research Association (Kyoto, Japan). The genomic constructs of the 4 strains used in this study are shown in References 4 and 5. The congenic strains were maintained in the Keio University School of Medicine Animal Laboratory Center. All of the rats were fed a standard laboratory chow and tap water ad libitum and kept in a room maintained at a constant temperature of 25°C. The experimental protocols were approved by the Keio University School of Medicine Animal Research Committee, in compliance with Japanese Law (No. 105).

Recording of Electrophysiological Activities

Experiments were performed on brain stem-spinal cord preparations obtained from 0- to 4-day-old rats. Under deep ether anesthesia, the brain stem and spinal cord were isolated and sectioned at the second thoracic nerve root (Th2) level, as described previously.15 The preparation was continuously superfused at 2 to 3 mL/min with a standard solution consisting of (in mmol/L) 124.0 NaCl, 5.0 KCl, 2.4 CaCl2, 1.3 MgCl2, 26.0 NaHCO3, 1.2 KH2PO4, and 30.0 g glucose and equilibrated with 95% O2 and 5% CO2 (pH7.4), at 26°C to 27°C. Intracellular recordings using the whole-cell patch-clamp technique were performed as follows: a patch electrode was filled with the following pipette solution (mmol/L): 130 K-gluconate, 10 EGTA, 10 HEPES, 2 Na2-ATP, 1 CaCl2, 1 MgCl2, and 0.5% lucifer-yellow (Aldrich Chemical [pH 7.2 to 7.3], adjusted with KOH). A patch-clamp amplifier (Axopatch 1D, Axon Instruments) was used to record the membrane potential. Before starting the intracellular recordings, the firing patterns were checked using extracellular recordings. RVLM neurons exhibiting discharges that were synchronized with the simultaneously recorded phrenic nerve activity were assumed to be respiratory neurons and were excluded from the study. The membrane potential was recorded using the current-clamp technique (20-pA increments from −100 to 20 pA, 500-ms duration) and was corrected for the junctional potential at the tip of the pipettes (−11 mV). The basal membrane potential and the firing rate were recorded over 10 minutes. The input resistance was calculated from the current-voltage curve. The membrane potential was shifted to −50 mV because of the negative current. One RVLM neuron per preparation was used in the experiment.

To evaluate the responses to Ang II, we superfused the brain stem-spinal cord preparation for 20 minutes with Ang II (6 μmol/L, Sigma-Aldrich) dissolved in a standard solution and recorded the changes in the membrane potential and in the input resistance of the RVLM neurons. Li and Guyenet15 used a bath application of 0.3 to 1.0 μmol/L of Ang II in their slice preparations. In an earlier study from our laboratory, we used 1, 3, and 12 μmol/L of Ang II to examine the dose responsiveness of RVLM neurons in a brain stem-spinal cord preparation, in which the target neurons were located 100 μm from the surface of the preparation.14 Based on these results, we selected the dosage of 6 μmol/L of Ang II, because the distance from the surface of the preparation to the RVLM neurons makes it difficult for Ang II to reach the target neurons. We then performed superfusion with a mixture of Ang II (6 μmol/L) and tetrodotoxin (50 μmol/L; Wako Pure Chemical Industries). Tetrodotoxin was used to block every synaptic input to the RVLM neurons. The intermediolateral cell column neurons were stimulated with a stainless-steel electrode (5 to 15 V, 100 ms, single pulse) to identify the RVLM bulbospinal neurons. RVLM bulbospinal neurons showing antidromic action potentials after intermediolateral cell column stimulation were used in the experiments.15 Lucifer yellow was allowed to diffuse spontaneously or by iontophoresis into the neurons during the intracellular recordings to verify the location of the neurons examined.16 All of the data were recorded and analyzed using PowerLab (AD Instruments). After the addition of tetrodotoxin, we first confirmed the disappearance of phrenic nerve activity (∼15 to 20 minutes after the addition of tetrodotoxin) and then added Ang II to the superfusate. Superfusion with Ang II induced depolarization in the bulbospinal neurons of the RVLM after a 1- or 2-minute latency period.

Statistics

All of the data were represented as the means±SDs. Differences between the WKY and WKYpch1.0 rats or between the SHRSPrwch1.0 and SHRSPr rats were examined using independent t tests. A value of P<0.05 was considered statistically significant.

Results

Basal Electrophysiological Activities of the RVLM Neurons

The RVLM neurons are classified into 3 types: regularly firing neurons, irregularly firing neurons, and silent-type neurons; however, the physiological roles of these neurons have not yet been fully elucidated.14 The irregularly firing neurons exhibited many excitatory postsynaptic potentials, whereas the regularly firing neurons rarely showed such behavior (Figures 1 and 2).

In the regularly firing neurons, the basal membrane potential was significantly less negative in the WKYpch1.0 rats (−46.4±2.3 mV) than in the WKY rats (−55.4±5.6 mV). The basal membrane potential of the SHRSPrwch1.0 rats (−47.8±2.6 mV) was similar to that of the SHRSP rats (−45.4±3.6 mV). The firing rate did not differ between the WKYpch1.0 and WKY rats or between the SHRSPrwch1.0 and SHRSPr rats.

As shown in Figure 2, the same trend was observed in the irregularly firing neurons of the RVLM; the basal membrane potential was significantly less negative in the WKYpch1.0 rats (−49.3±3.0 mV) than in the WKY rats (−57.0±1.0 mV; P<0.01). The basal membrane potential of the SHRSPrwch1.0 rats (−51.3±3.2 mV) was similar to that of the SHRSP rats (−50.0±0.0 mV). The firing rate did not differ between the WKYpch1.0 and WKY rats or between the SHRSPrwch1.0 and SHRSPr rats.

The input resistance of the regularly and irregularly firing neurons was also not significantly different among the 4 strains (data not shown).

Response to Ang II

Superfusion with Ang II (6 μmol/L) induced depolarization in the bulbospinal neurons of the RVLM after a 1- or 2-minute latency period. Repolarization toward the basal level was then observed over the next 2 or 3 minutes; this repolarization was considered to represent the desensitization of the neurons. We, thus, quantified the magnitude of the maximal depolarization.
The magnitude of the depolarization of the RVLM neurons from WKYpch1.0 rats during Ang II superfusion was 4.1 ± 2.3 mV, which was significantly larger than that of the neurons from WKY rats (Figure 3). The depolarization of the RVLM neurons from SHRSPs was significantly larger than that of the neurons from SHRSPwch1.0 rats. Of note, the depolarization was statistically significant in the 2 strains (WKYpch1.0 and SHRSP) that possess the SHRSP-derived fragment of the Chr-1 QTL.

In the next experiment, which used a potent inhibitor of synaptic transmission, tetrodotoxin, we examined whether the Ang II effect was mediated through presynaptic or postsynaptic pathways. After the administration of tetrodotoxin, we first confirmed the disappearance of phrenic nerve activity and then added Ang II to the superfusate. The average magnitude of the depolarization of the neurons from WKYpch1.0 rats during superfusion with tetrodotoxin and Ang II was significantly larger than that of the neurons from WKY rats (Figure 4). Meanwhile, the average magnitude of the depolarization of the neurons from SHRSPs was also significantly larger than that of the neurons from SHRSPwch1.0 rats. These differences in depolarization were basically the same as those obtained in the experiments without tetrodotoxin, and the neurons from SHRSPs and WKYpch1.0 rats showed a significantly greater depolarization than those from the respective counterpart rats.

**Discussion**

The major finding of the present study was that the QTL on rat Chr-1 affected the electrophysiological activity of RVLM neurons, particularly their responsiveness to Ang II. Because isolated brain stem-spinal cord preparations from neonatal rats were used in this study, we would like to emphasize that the results were not caused by the secondary effects of hypertension or by the influence of the higher brain centers, such as the hypothalamus. Therefore, this observation strongly suggests that a gene (or genes) responsible for the difference in the activity of the RVLM neurons is (are) located in this genomic region.

In regularly firing neurons, the basal membrane potential differed significantly between neurons from WKY and WKYpch1.0 rats (Figure 1). Considering the genomic composition of the congenic strain (WKYpch1.0), this observation suggests that genes responsible for this interstrain difference are located both inside and outside of the QTL. Furthermore, the lack of a difference between neurons from SHRSPs and SHRSPwch1.0 rats implies that the effects of these genes are not additive, suggesting that they may be
involved in the same biological process, just as in the case of
the blood pressure QTLs in Dahl salt-sensitive rats.\textsuperscript{19}

In the irregularly firing neurons, we obtained a similar
result regarding the difference in the basal membrane poten-
tial and no differences in the firing rate (Figure 2). These
results can probably be explained by the fact that a larger
number of synaptic inputs modulates the firing rate in
irregularly firing neurons.\textsuperscript{14} Nevertheless, a similar interstrain
difference in the membrane potential was observed in neu-
rons with different properties, suggesting that this genetic
effect is ubiquitous in RVLM neurons.

The Ang II–induced depolarization and increase in the
firing rate are shown in Figure 3. Of particular importance,
these responses depended largely on the genotype of the
congenic fragment, irrespective of the background genome;
the 2 strains with the congeneric fragment originating from
SHRSPs (WKYpch1.0 and SHRSP) showed a significantly
greater depolarization than the rats containing the congeneric
fragment originating from WKY rats (WKY and SHRSPwch1.0). This observation suggests that the responsiveness of
the RVLM neurons to Ang II was largely determined by the
gene(s) located in the QTL.

WKYpch1.0 rats have been shown repeatedly to have
exaggerated sympathetic responses to different types of
stress.\textsuperscript{5–7} Considering a recent observation that the Ang II
type 1 receptor in the RVLM mediates activating the sympa-
thetic nervous system by emotional stress, resulting in an
increase in blood pressure in conscious rabbits,\textsuperscript{10} it is quite
attractive to hypothesize that a gene (or genes) in the Chr-1
QTL affects the responsiveness of the sympathetic nervous
system to stress through an Ang-II–mediated system
in the RVLM. Supporting this hypothesis, Yamazato et al\textsuperscript{7}
reported that the hyperresponsiveness of blood pressure and
renal sympathetic nerve activity to air-jet stress in
WKYpch1.0 rats was inhibited by the intracerebroventricular
injection of an Ang II receptor blocker.

Based on the results of the present study, we suspect that
the basal membrane potential in RVLM neurons is affected
primarily by age and background genome, whereas the
response to Ang II in RVLM neurons depends on the blood
pressure QTL of Chr-1. The depolarization of the RVLM
neurons by Ang II superfusion was statistically significant in
the neonatal WKYpch1.0 and SHRSPs but not in the neonatal
WKY and SHRSPwch1.0 rats. Our data are supported by the
results reported by Yamazato et al,\textsuperscript{7} who showed that in-
creases in renal sympathetic nerve activity in response to
air-jet stress were significantly larger in 4-week–old
WKYpch1.0 and SHRSPs than in 4-week–old WKY rats and
that the sympathoexcitation of WKYpch1.0 rats was similar
to that of SHRSPs. The depolarization of RVLM neurons by
Ang II shown in our study may account for the increases in
sympathetic nerve activity and blood pressure in response to
air-jet stress, because the intracerebroventricular injection of
Ang II receptor blocker candesartan reduced the sympatho-

\begin{figure}
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\caption{Basal membrane potential and firing rate of irregularly firing neurons in the RVLM. A, Representative traces from the 4 strains.
B, Top and bottom panels show the basal membrane potential and the firing rate (mean ± SD of 8 neurons from different rats).}
\end{figure}
excitatory and pressor responses.\textsuperscript{7} Also, a report from Cui et al.\textsuperscript{5} demonstrated that the increase in systolic blood pressure in response to restraint stress in 16- to 20-week-old WKYpch1.0 rats was larger than that of WKY rats. These compatible data for different rat ages and from 3 different laboratories strongly suggest that the genes in the Chr-1 QTL of SHRSPs are responsible for the depolarization response of RVLM neurons to Ang II in neonatal WKYpch1.0 and SHRSPs, the increase in peripheral sympathetic activity in response to air-jet stress in 4-week-old WKYpch1.0 and SHRSPs, and the pressor response to restraint stress in 16- to 20-week-old WKYpch1.0 rats. However, these data do not suggest that age is responsible for the stress responses. In contrast, the basal blood pressures of WKY and WKYpch1.0 rats seem to be determined largely by age and also by genes both inside and outside the QTL.

In some cases, the effects of the QTL region on blood pressure were asymmetrical in reciprocal congenic strains for hypertensive QTLs. As Rapp\textsuperscript{20} pointed out previously, strains with congenic fragments from the Dahl salt-sensitive rat on a background of normotensive strains did not show a significant increase in blood pressure, whereas congenic rats with the reversed genotype showed an obvious reduction. Consistent with this finding, WKYpch1.0 rats showed little, if any, increase in blood pressure, whereas SHRSPwch1.0 rats showed a large decrease.\textsuperscript{3,13} This asymmetrical nature of the effects of QTLs on blood pressure was probably caused by gene-gene interactions, which convoluted the analyses. In
contrast, reciprocal genetic effects like the one observed in the present study suggest a mendelian control of phenotypes: the genotype (or the haplotype) of a single gene (or a cluster of genes) had a major effect on the phenotype, independent of the genetic background.\textsuperscript{21,22} Such a phenotype may be regulated by the gene(s) in a more direct manner and may be useful for speculating the functions of the gene(s).

Because tetrodotoxin blocks all synaptic transmissions, the observation that the addition of tetrodotoxin did not change the responsiveness to Ang II further implied that the interstrain difference in the responsiveness was intrinsic (or postsynaptic) in the RVLM neurons. A previous study of Summers et al\textsuperscript{23} reported that the exaggerated reactivity of RVLM neurons to Ang II in spontaneously hypertensive rats was because of an increase in the number of Ang II receptors on the RVLM neurons, which then induced oxidative stress and activated signal transduction via G proteins. It would be interesting to investigate the underlying intracellular mechanisms of the Ang II–dependent activation of RVLM neurons using congenic strains.

The blood pressure QTL on rat Chr-1 is quite large and contains hundreds of genes and expressed sequence tags.\textsuperscript{5,13} Among them, however, several interesting candidates were found when the putative roles of the genes in the sympathetic nervous system were considered. These genes include Arix, a transcription factor regulating the development of the sympathetic nervous system, as well as the expression of dopamine β-hydroxylase\textsuperscript{24–26}; Ntrk3, a receptor for neurotrophin 3\textsuperscript{27}; Arrb1, a cofactor regulating the internalization of the β-adrenergic receptors and angiotensin receptors\textsuperscript{28}; Nox4, a subunit of NADPH oxidase\textsuperscript{29}; and Homer 2, a regulator of the metabolic glutamate receptors.\textsuperscript{30,31} In future studies, it will be necessary to reduce the number of candidate genes using both their positional and functional information to identify the responsible gene(s) and to confirm the roles of increased RVLM activity in the pathogenesis of hypertension.

The main limitation of this study is the lack of data regarding how RVLM neuron activity may (or may not) differ between strains in older animals and how this possible difference may be correlated with blood pressure. We hope to measure the RVLM neuron activity of both young and old rats in the future. To demonstrate that these early differences in RVLM neuron activity are causally related to stress-related differences in blood pressure, we should have compared the RVLM activity of older congenic and parental rats. Ideally, the electrophysiological properties of RVLM and the blood pressure, heart rate, and renal sympathetic nerve activity should be simultaneously examined in neonatal and older rats in vivo, and the changes in these parameters in response to various stressors should be determined. However, because the contact between the targeted neurons and the patch pipette is impaired by the increase in glial cells and astrocytes that occurs in rats older than 2 weeks of age, intracellular patch-clamp recordings of RVLM neurons are technically impossible to perform in older rats.

**Perspectives**

Several lines of evidence have indicated that sympathetic nerve activity differs significantly between spontaneously hypertensive rats/SHRSPs and WKY rats, and this difference is one of the putative causes of hypertension in spontaneously hypertensive rats/SHRSPs. Because membrane potential of RVLM neurons determines the sympathetic tone, the present observation suggests that a gene (or genes) in the Chr-1 QTL is (are) responsible for the greater sympathetic tone observed in SHRSPs. Identification of the responsible gene(s) will improve our understanding of the role of the sympathetic nervous system in the pathogenesis of hypertension and promote the development of new therapeutic and preventive strategies for essential hypertension.

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**Disclosures**

None.

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