Nicotinic receptor gene cluster on rat chromosome 8 in nociceptive and blood pressure hyperresponsiveness

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Received 27 June 2002; accepted in final form 21 August 2002

Khan, Imran M., Erin Singletary, Adamu Alemayehu, Shanaka Stanislaus, Morton P. Printz, Tony L. Yaksh, and Palmer Taylor. Nicotinic receptor gene cluster on rat chromosome 8 in nociceptive and blood pressure hyperresponsiveness. Physiol Genomics 11: 65–72, 2002. First published September 3, 2002; 10.1152/physiolgenomics.00079.2002.—Spontaneously hypertensive rats (SHR) exhibit enhanced pressor, heart rate, and nociceptive responses to spinal nicotinic agonists. This accompanies a paradoxical decrease in spinal nicotinic receptor number in SHR compared with normotensive rats. The congenic strain, SHR-Lx, with an introgressed chromosome 8 segment from the normotensive Brown-Norway-Lx strain (BN-Lx) exhibits reduced blood pressure. This segment contains a gene cluster for three nicotinic receptor subunits expressed in the nervous system. We examined the implication of this gene cluster in the enhanced responsiveness of the SHR. Pressor and nociceptive responses to spinal cytisine, a nicotinic agonist, were diminshed in SHR-Lx. Moreover, with repeated administration, these responses desensitized faster in SHR-Lx and progenitor BN-Lx than in progenitor SHR/Ola. This implicates the gene cluster in both cardiovascular and nociceptive responses to spinal nicotinic agonists. Since diminished responsiveness to agonist stimulation is greater than the basal blood pressure differences between the strains and the introgressed rat chromosome maps to a quantitative trait locus in human hypertension, polymorphisms in the three nicotinic receptor genes become candidates for altered central control of blood pressure.

spinal nicotinic receptor; intrathecal cytisine; spontaneously hypertensive rats; sensory responses; genetics of hypertension

THE CHOLINERGIC NERVOUS SYSTEM is intimately involved in control of arterial pressure and may influence the hypertensive state at several locations. Peripheral cholinergic control is intrinsic to the cardiac response associated with baroreflexes, and from animal models, cholinergic pathways in the spinal cord and higher brain centers are known to modulate cardiovascular responses that influence basal blood pressure and pressor responses induced by stress and pharmacological agents (4). The Okamoto spontaneously hypertensive rat (SHR) has been documented to have altered central cholinergic activity compared with normotensive Wistar-Kyoto (WKY) rats (4, 24, 25). Early studies pointed to a role for muscarinic acetylcholine receptors in the pathogenesis of hypertension. However, more recently, the nicotinic acetylcholine receptor (nAChR) system in the central nervous system (CNS) has been also shown to exhibit altered activity in the SHR model of hypertension (8, 14).

Our continuing objective has been to determine whether neurotransmission involving the spinal nicotinic receptor system has a role in the development and/or maintenance of high blood pressure in the SHR genetic hypertensive model. Expression of nAChR subtypes in the spinal cord parallels that of higher centers (14, 17, 18). In the rat brain, nAChR receptors can be identified at presynaptic as well as postsynaptic sites. In the spinal cord, the capacity of nicotinic agonists to release other transmitters, as detected by microdialysis, suggests that the receptors may regulate transmitter release from presynaptic sites (14), as appears to be the case elsewhere in the CNS (19, 28, 31, 32).

The spinal cord has well-defined tracts of cardiovascular control leading from peripheral afferent and higher brain centers and extending to efferent outputs that emanate to the periphery and back to higher centers. Moreover, pharmacological agents can be administered to the conscious animal at discrete segmental levels of the spinal cord and act at localized levels of the cord. Hence, the spinal cord offers a well-defined CNS system for examining gene expression and responsiveness of gene products that potentially control cardiovascular function.

Nicotinic agonists, when administered in small doses intrathecally, elicit dose-dependent increases in blood pressure, heart rate, and nociceptive responses upon binding to specific nicotinic receptors in the spinal cord (14, 16, 17). We previously demonstrated that intrathecal nicotinic agonists elicited greater pressor and nociceptive responses in 12- to 14-wk-old SHR compared with age-matched WKY or Sprague-Dawley (SD) rats (12). Paradoxically, we observed a decrease in spinal nAChR number in adult SHR compared with age-matched WKY, with a lower density of nAChRs being particularly evident in the superficial layer of the dor-
The hyperresponsiveness to spinal nicotinic agonists in the SHR was also observed when prehypertensive 6-wk-old SHR and WKY rats were compared (13). In addition, chronic lowering of blood pressure in SHR by hydralazine (per os from 6 to 12 wk of age) or captopril (from in utero to 12 wk of age) did not alter the exaggerated responses to spinal nicotinic agonist in SHR rats. Similarly, chronic elevation of systolic blood pressure in WKY rats by desoxycorticosterone acetate (DOCA) salt treatment (from 7 to 12 wk of age) yielded a hypertensive state without augmented responses to spinal cytisine (13). In brief, these observations indicate that the pathway mediating responsiveness to the nicotinic receptors in the dorsal lumbar spinal cord is altered in the SHR, and the altered activity of these receptors is not causally related to the hypertensive state of the animal. Rather, these observations are consistent with the cardiovascular and behavioral responses being inherited traits.

The SHR strain was derived by selective inbreeding of Wistar rats (33). Recent linkage studies in recombinant inbred rat strains and F2 populations derived from SHR and normotensive strains suggest that blood pressure quantitative trait loci (QTL) may exist on several chromosomes (35). More recently, Kren et al. (22), using a congenic strain, SHR-Lx, in which a segment of chromosome 8 from the normotensive Brown-Norway-Lx (BN-Lx) strain was transferred onto the genetic background of the SHR/Ola, confirmed the presence of a blood pressure QTL on chromosome 8. This congenic strain was of special interest to us since the replaced chromosomal segment contains a cluster of neuronal nicotinic receptor subunit genes, encoding the α3-, α5-, and β4-subunits (2, 30) that are expressed in moderate to high abundance in brain and spinal cord (6, 20, 38, 39). Accordingly, we sought to determine whether this specific region on chromosome 8 encodes gene products linked with the hypersensitivity to spinal nicotinic agonists in SHR. To this end, we examined the responses to spinal cytisine in the progenitor SHR/Ola, BN-Lx normotensive rats, and the congenic strain SHR-Lx.

**RESULTS**

Basal cardiovascular parameters in SHR/Ola and SHR-Lx rat strains. Arterial systolic (SBP), diastolic (DBP), and mean (MAP) arterial pressures recorded the normotensive BN-Lx strain was introgressed onto the genetic background of the progenitor SHR/Ola. The BN-Lx donor strain was originally derived by introgressing the mutant Lx gene of the polydactylyous (PD/Cub) rat onto the BN background (22). The mutant Lx gene gives rise to the polydactyly luxate syndrome (PLS) in which extra digits develop on the hind feet and sometimes the front feet along with variable luxation of the hind limbs. Because the PLS maps to rat chromosome 8, the Lx mutation was used as a morphogenetic marker for the inheritance of this region of chromosome 8 during the introgression process.

Surgical procedures, arterial blood pressure, and heart rate recording. Catheters were chronically implanted into the thecal space for spinal drug delivery under halothane anesthesia as described previously (14). Four to five days following catheter implantation, the tail artery was catheterized for measurement of blood pressure and heart rate as previously described, and the rat was placed in an open wire restrainer cage where it could maintain a typical resting position but could not turn around (14). Drugs were administered intrathecally to awake restrained animals according to a previously reported cumulative dosing procedure (14). In a typical experimental paradigm, an individual rat received three sequential doses of cytisine followed by two consecutive highest dose (5 μg) of cytisine. To allow responses to return to baseline values between dosings, 25–30 min elapsed between administrations. Only rats naïve to administered cholinergic agonists were employed in the study, and rats were euthanized following the conclusion of the experimental protocol.

Cardiovascular parameters were recorded with a Gould polygraph, and the data were analyzed with the Ponemah Physiology Platform-3 (Gould Instruments Systems, Valley View, OH).

Nociceptive or irritation responses to intrathecal nicotinic agonists were quantified by a visual scoring index as previously described (14). In brief, a score of 1 was given for each of the following behavioral responses: 1) movement of the head and the front limbs; 2) movement of the hind limbs; 3) whole body movement, twisting and turning movement; and 4) tail erection and high-pitched squeaking. The responses occurred in the sequence described, and the maximum assignable score was 4. In the present study, we incorporated an irritation intensity parameter to provide further quantitation of the irritation response following nicotinic agonist administration. The irritation intensity was determined as the sum of the irritation scores of each 1-min time interval over 10-min period following nicotinic agonist administration, i.e., irritation intensity index = sum of (specific irritation score × frequency of the particular score within a specific time period).

Chemicals. Cytisine was obtained from Sigma (St. Louis, MO).

Statistics. All values presented are means ± standard error of mean (SE). Student’s t-test for unpaired data was used to determine differences between two treatment groups. Differences between multiple groups were compared using ANOVA. Irritation intensity scores between treatment groups for a particular dose or a time were compared by a nonparametric test (Mann-Whitney U). All statistical tests were performed with the aid of Prism software (GraphPad, San Diego, CA).

**DISCUSSION**

The SHR strain was derived by selective breeding in which a segment of chromosome 8 from the normotensive BN-Lx strain was introgressed onto the genetic background of the progenitor SHR/Ola. The BN-Lx donor strain was originally derived by introgressing the mutant Lx gene of the polydactylyous (PD/Cub) rat onto the BN background (22). The mutant Lx gene gives rise to the polydactyly luxate syndrome (PLS) in which extra digits develop on the hind feet and sometimes the front feet along with variable luxation of the hind limbs. Because the PLS maps to rat chromosome 8, the Lx mutation was used as a morphogenetic marker for the inheritance of this region of chromosome 8 during the introgression process.

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**RESULTS**

Basal cardiovascular parameters in SHR/Ola and SHR-Lx rat strains. Arterial systolic (SBP), diastolic (DBP), and mean (MAP) arterial pressures recorded...
from the tail artery in resting congenic SHR-Lx and normotensive BN-Lx were significantly lower compared with corresponding pressures in SHR/Ola rats (Fig. 1). However, there was no difference in basal heart rate among the three strains of rats. This finding of a 20- to 30-mmHg reduction in MAP is consistent with the study of Kren et al. (22); however, they did not report the basal heart rate of SHR-Lx and SHR/Ola rats. It should be noted that substitution of the BN-Lx chromosomal segment onto the SHR/Ola background resulted in a phenotype that is still hypertensive relative to the BN-Lx rat strain.

**Responses to intrathecal cytisine.** Spinal administration of cytisine resulted in dose-dependent increases in SBP, heart rate, and nociceptive responses in all three strains of rats with the maximal response attained presented in Fig. 2. In this sequential dose-response curve protocol, the lower dose of cytisine did not identify significant strain-dependent differences in the change in SBP; however, at the highest dose of cytisine the pressor response was significantly greater in SHR/Ola compared with BN-Lx strains. Although the absolute change in SBP following 5 µg cytisine was higher in SHR/Ola than SHR-Lx, the difference did not reach statistical significance. No significant differences in heart rate response to any of the doses of cytisine were observed between SHR/Ola and SHR-Lx rats; however, the tachycardia resulting from 0.5 and 5 µg of cytisine was significantly higher in BN-Lx than in SHR-Lx rats. The irritation intensity index to cytisine was significantly higher in both SHR/Ola and BN-Lx rats compared with the congenic rats for the 5-µg dose of cytisine only.

**Temporal analysis of spinal cytisine-elicited responses in the SHR/Ola and SHR-Lx strains.** Since measurements of maximal increases in response may weight spiking activity over short intervals, we also performed temporal analyses of the cardiovascular and nociceptive responses following cytisine administration. The responses record the maximum change in systolic BP (SBP) and heart rate, and area under the curve (AUC) for maximum irritation intensity over a specific period following agonist administration (4 min for the lowest dose and 10 min for higher two doses of cytisine). Sequentially increasing doses of cytisine were administered with a 25- to 30-min interval between administrations. *P < 0.05 SHR/Ola vs. BN-Lx; †P < 0.01 SHR-Lx vs. SHR/Ola or BN-Lx; and ‡P < 0.05 SHR-Lx vs. BN-Lx (two-way ANOVA with Bonferroni posttests). Each value represents the mean ± SE.
As shown (Fig. 3), sustained differences in pressor or irritation responses were not observed among the three groups of rats following 0.05 or 0.5 μg cytisine administration. In contrast, the pressor and irritation responses to 5 μg cytisine were significantly higher in the SHR/Ola than in SHR-Lx during the first 2 min. Interestingly, the temporal response profiles indicate that, in contrast to what is observed in SHR/Ola, the pressor response to 5 μg cytisine in the SHR-Lx or BN-Lx was not increased over the 0.5-μg dose. Similar observations hold for tachycardia and the irritation responses. The data indicate that responses to spinal cytisine may desensitize at different rates for the SHR/Ola compared with SHR-Lx or BN-Lx rats.

Desensitization of cytisine-elicited responses in SHR/Ola and SHR-Lx rats. Repeated administrations of 5 μg cytisine at 25- to 30-min time intervals resulted in dampened cardiovascular and nociceptive responses in all three strains of rats. Figure 4 shows the cumulative responses for the 10-min period following each dose of 5 μg cytisine shown as areas under the curve for the response over a specified interval. The profiles illustrate that the pressor response to cytisine desensitizes in SHR-Lx at a slower rate than that for BN-Lx. However, the residual pressor response in SHR-Lx following second and third dose of cytisine appeared to be intermediate between those for SHR/Ola and BN-Lx. Similar to the pressor response, the increase in heart rate also desensitized in the three strains of rats. The most dramatic decrement occurred in BN-Lx rats. According to the temporal profile of the heart rate response, tachycardia following the second dose of cytisine was substantially more elevated in SHR/Ola than in both SHR-Lx and BN-Lx rats, with both of the latter strains being nearly equivalent in response.

Similar to the cardiovascular responses, the nociceptive responses to successive doses of cytisine also desensitized in these three strains of rats; yet, irritation intensity remained significantly higher in SHR/Ola than in both SHR-Lx and BN-Lx for the second and third consecutive doses of cytisine. As with the change in heart rate, the response of the SHR-Lx patterned
organ system level as well as alterations in neurotransmitter systems (5, 9). In this regard, abnormalities in the cholinergic activity have been implicated in the development of hypertension in this genetic rat model of hypertension (4). The primary focus of this laboratory has been toward evaluating the potential contributions of CNS nicotinic receptors to genetic hypertension.

**Comparative cardiovascular responses.** Our previous studies established that spinal administration of various nicotinic agonists resulted in exaggerated pressor, tachycardia, and nociceptive responses in SHR compared with normotensive rats (12, 13, 18). Studies under nongenetic hypertensive conditions or employing systemic drug treatments over time, to approach a normotensive state, provided evidence that hypersensitivity to nicotinic agonists in SHR is independent of increased blood pressure and the conditions associated with the general hypertensive state (13, 18). Moreover, our data imply that the hyperresponsiveness of SHR to spinal nicotinic agonists may have a genetic origin in this rat strain (13).

**Neuronal nicotinic receptor composition and function.** Neuronal nicotinic receptors are pentameric ion channels with the heteromeric forms usually consisting of two α-subunits and three β-subunits expressed in discrete combinations with α3 and α4 being the predominant α-subunits (27). An exception to heteromeric assemblies appears to be the α7- and α9-subunits, which may predominate as homomeric pentamers (27). Several studies show that the mRNA encoding α4 and β2 are diffusely distributed in the CNS (3, 6, 20, 26, 38, 39); however, α3-, α5-, and β4-subunits are expressed in more localized regions of the nervous system (40).

Although several studies have identified nicotinic receptor subunit transcripts in spinal cord tissues or sensory ganglia (3, 11, 20, 26, 38, 39), data that examine and define expression of the individual receptor subunits in spinal neurons and terminals are far more limited. Flores et al. (6) demonstrated that nicotinic receptors composed of α4β2-, α3β2-, and α3β4-subunits are present in the trigeminal ganglia of rats. Our recent studies demonstrate that in the rat spinal cord α4-, α5-, and β2-subunits are expressed primarily in postsynaptic neurons, whereas α3 and β4 are found on mainly presynaptic sites including the primary afferent terminals (Khan IM, unpublished observations). However, some α3-subunit protein expression is also found in the postsynaptic neurons in dorsal lumbar spinal cord. In addition, α5- and β2-subunit proteins are also expressed on the primary afferent terminals, whereas the α4-subunit is sparsely localized on primary afferent terminals. Our studies also indicate that nicotinic receptors on the primary afferent terminals play a role in mediating the nociceptive response to spinal nicotinic agonists. This is supported by studies where ablation of the primary afferent terminals significantly modifies the pain response to spinal nicotinic agonists as well as reduces nicotinic agonist binding sites in the superficial dorsal lumbar spinal cord (Khan IM, unpublished observations).
The SHR-Lx rat strain. The development of hypertension in the SHR is thought to be related to a phenotype arising from multiple gene products. Several blood pressure QTL have been identified in the rat genome and can be compared with syntenic regions in man (35, 37). The SHR-Lx congenic strain has a genetic background identical to the SHR/Ola except for an ~30-cM homozygous region of chromosome 8 that has been replaced with the homologous region from normotensive BN-Lx rats, whereas another 40 cM may be heterozygous with respect to the transferred region (Fig. 5). The region of rat chromosome 8 under study shows an area of synteny with human chromosome 15q, a region reported to be homologous region from normotensive BN-Lx rats, whereas another 40 cM may be heterozygous with re- spect to the transferred region (Fig. 5). The region of rat chromosome 8 under study shows an area of synteny with human chromosome 15q, a region reported to be linked to a systolic blood pressure in genomic scans of humans (23). Quite fortuitously a unique cluster of nico- tinic receptor subunit genes exists in the ~30-cM region. The α3, α5, and β4 genes lie in tandem (2, 7, 29, 30), and a common transcriptional element may exert a global control on gene expression (Fig. 5). This congenic strain has a resting blood pressure, which is ~20–30 mmHg lower than the SHR/Ola strains, when measured by te- lemometry (22). We obtained similar differences when the blood pressure was measured via the tail artery.

Strain sensitivity to nicotine and cytisine. Spinal nicotinic agonists elicit a pressor response primarily via direct stimulation of preganglionic sympathetic neurons in the intermediolateral cell column (IML) region of thoracic spinal cord (15). Our previous studies demonstrated that the pressor response to intrathecal cytisine, in contrast to nicotine, might reflect, in part, a reflex action to its nociceptive response (15). Moreover, spinal nicotinic agonists appear to be more potent in eliciting nociceptive than cardiovascular responses in rats. Furthermore, nicotinic receptor binding sites on primary afferent terminals appear to mediate a significant portion of the nociceptive response elicited by spinal cytisine (36; and Khan IM, unpublished obser- vations). Thus the above findings are consistent with the mode of action of intrathecal cytisine, which has been reported to be relatively selective for the αβ4 nAChR subtype (1, 34).

In the present study, alike to our previous observa- tions, we observe that pressor and nociceptive re- sponses to spinal cytisine have similar dose-response profiles. We also document that SHR/Ola rats exhibit augmented cardiovascular and nociceptive responses compared with normotensive BN-Lx rats. Thus the hypertensive SHR strain not only shows augmented sensitivity to nicotinic agonists compared with WKY rats (12), but also to another inbred normotensive strain, the BN-Lx. In addition, both the pattern of the maximal responses (in a 10-min period following dos- ing) and the temporal responses suggest that the chro- mosome 8 nicotinic receptor gene cluster is involved in mediating cardiovascular and nociceptive responses to cytisine. This is implied by the fact that the SHR-Lx strain, with its introgressed segment from the BN-Lx rat, exhibits significantly diminished pressor and nociceptive responses within the first 2 min, at the highest doses of cytisine, compared with progenitor SHR/Ola strain (Fig. 3). The maximal responses to 0.5 μg of cytisine in both SHR-Lx and BN-Lx appear maximal with no further increase seen at 5.0 μg. By contrast, 5.0 μg cytisine elicits greater cardiovascular and irritation responses in SHR/Ola strain (Fig. 3). This may be, in part, due to desensitization of the spinal nicotinic receptors by the lower doses of cytisine, which appears to have a slower onset in the SHR than in normotensive rats (12). Hence, differences in pressor or nociceptive response between SHR/Ola and SHR-Lx only become evident with the administration of the highest dose of cytisine (5 μg). Thus our data suggest that similar to WKY and BN-Lx normotensive rats, SHR-Lx exhibits more rapid and greater desensitization of pressor and nociceptive responses to cytisine than the SHR/Ola, with the more rapid desensitization occurring with nociception.

With repeated high dosing, the heart rate and nociceptive responses of SHR-Lx and BN-Lx pattern each other more closely than with SHR/Ola, whereas the pressor response for SHR-Lx is intermediate between the two progenitors. Repeated administration of a 5-μg
dose of cytisine did not result in a significant difference in the pressor response between SHR/Ola and SHR-Lx rats. In contrast, although the nociceptive response to cytisine was desensitized in both strains, the response still remained significantly greater in SHR/Ola compared with SHR-Lx. This disparity in the magnitude of desensitization of the pressor and nociceptive responses between SHR/Ola and SHR-Lx indicates that these two responses are not fully linked. However, an alternative explanation may lie in the masking of a reflex pressor response from nociceptive stimulation by the stimulation of preganglionic sympathetic neuron through direct activation of the nicotinic receptors in thoracic IML region (15).

Our results are consistent with our original hypothesis that the observed end-organ effects on the cardiovascular system from intrathecal administration of nicotinic agonists arise from two parallel effects: 1) a direct stimulation of preganglionic sympathetic neurons through activation of nicotinic receptors in thoracic IML region (15), and 2) an indirect stimulation of preganglionic sympathetic neurons through an ascending nociceptive pathway leading to a bulbospinal reflex sympathetic activation. Thus interpretation of effects of intrathecal nicotinic agonists on arterial pressure responses is complicated by these two temporally dissociable contributions to sympathetic activation, which influence arterial pressure through common spinal preganglionic sympathetic neurons. The similar pattern of heart rate and irritation response to the second and third doses of cytisine between SHR-Lx and BN-Lx, and their contrast with that for SHR/Ola, also argue that the chromosome 8 gene cluster encoded genes controlling these responses.

Overall the data presented in this study indicate that hypersensitivity of SHR/Ola to spinal nicotinic agonists is linked to a specific gene segment of chromosome 8 containing a blood pressure QTL. The three genes in the cluster encoding the nicotinic receptor subunits become obvious candidate genes, and a difference in desensitization characteristics conferred by any of the three subunits in SHR/Ola may be a mechanism for differences in sensitivity to spinal nicotinic agonists.

Although the enhanced response to direct nicotinic administration and a cluster of three nicotinic receptor genes expressed in the spinal cord is an attractive coincidence, we have yet to show that these specific genes in the introgressed region are responsible for the enhanced responsiveness. Other gene products encoded in this region and perhaps involved in the signaling pathway also remain as candidates. Also, it is not yet established that the diminished basal blood pressure in the SHR-Lx arises from altered functions of the nicotinic receptor. Although both the basal blood pressure reduction and reduced responsiveness localized with the introgressed 30-cM region, the actual response genes could localize in different regions of this segment. A study utilizing congenic substrains harboring smaller segments of the original introgressed segment of chromosome 8 (21) offers the prospect of further delimiting the responsive introgressed region of the chromosome.

In conclusion, our results point to a cluster of genes on chromosome 8 that are important both for basal blood pressure and for cardiovascular and behavioral responses to nociceptive stimuli. The probable consequence of these observations on the cluster of nicotinic receptor genes in this location of rat chromosome 8 raises the possibility that spinal nicotinic cholinergic mechanisms are aberrant in genetic hypertension.

This work was supported by National Heart, Lung, and Blood Institute Grant HL-35018 and the California Tobacco Related Disease Research Program (to P. Taylor).

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