Calcitonin Gene–Related Peptide Gene Expression in the Spontaneously Hypertensive Rat

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Calcitonin gene–related peptide, a product of the calcitonin gene, is a potent vasodilator neuropeptide. We have demonstrated that dietary calcium deficiency decreased the neuronal (laminae I/II of the dorsal horn of the spinal cord) content of immunoreactive calcitonin gene–related peptide in the normal rat. Neuronal calcitonin gene–related peptide levels are also reduced in the spontaneously hypertensive rat, a model characterized by calcium deficiency. However, the mechanism of this reduction in neuronal calcitonin gene–related peptide could be due to decreased synthesis or increased release. To determine if neuronal calcitonin gene–related peptide messenger RNA (mRNA) levels are also decreased in the spontaneously hypertensive rat, we measured relative calcitonin gene–related peptide mRNA levels (using a genomic hybridization probe specific for α- and β-calcitonin gene–related peptide mRNA) in dorsal root ganglia from spontaneously hypertensive and Wistar-Kyoto control rats. Dorsal root ganglia neuronal cell bodies are a prominent site of calcitonin gene–related peptide synthesis and send axons to peripheral blood vessels and central spinal cord sites (laminae I/II). After normalization of calcitonin gene–related peptide mRNA levels to 18S RNA, the calcitonin gene–related peptide mRNA/18S RNA ratio was significantly decreased approximately threefold in the spontaneously hypertensive rats compared with controls. This alteration in calcitonin gene–related peptide mRNA levels is specific for dorsal root ganglia, because no strain differences in calcitonin gene–related peptide mRNA content were detected in heart or brain. In conclusion, the neuronal levels of calcitonin gene–related peptide mRNA and immunoreactive calcitonin gene–related peptide are decreased in the spontaneously hypertensive rat. Therefore, decreased neuronal synthesis and release of this potent vasodilator may be associated with hypertension. (Hypertension 1993;21:1010–1014)

KEY WORDS • calcitonin gene–related peptide • gene expression • ganglia, spinal • rats, inbred SHR • hypertension, genetic

Calcitonin gene–related peptide (CGRP), a product of the calcitonin/CGRP gene, is a potent vasodilator neuropeptide.1,2 Altered calcium homeostasis has been reported in both human essential hypertension and experimental animal models of hypertension, such as the spontaneously hypertensive rat (SHR) and mineralocorticoid-salt–induced hypertension.3–5 Such alterations include decreased serum ionized calcium and calcitonin levels and increased serum parathyroid hormone and 1,25-dihydroxyvitamin D₃ levels.3 Because CGRP is a product of the calcitonin gene and these changes in calcium metabolism are observed, it is logical to speculate that alterations in CGRP may also be present in hypertension.

Systemic administration of CGRP decreases blood pressure in a dose-dependent manner.6,7 The mechanism of blood pressure reduction is by peripheral arterial dilation, which reduces total peripheral resistance.7 In addition to its vasodilator properties, CGRP has marked positive chronotropic and inotropic effects.8 Immunochemical techniques have identified CGRP-containing nerve fibers throughout the cardiovascular system, particularly in association with blood vessels; the coronary circulation appears to be especially sensitive to the vasodilator effects of CGRP.10

We have previously demonstrated that altered dietary calcium intake changes the neuronal content of immunoreactive CGRP in laminae I/II of the dorsal horn of the spinal cord: low dietary calcium, which decreases serum ionized calcium, decreases CGRP; high dietary calcium, which increases serum ionized calcium, increases CGRP content.11 Laminae I/II of the dorsal horn of the spinal cord are the termination sites for incoming primary afferents rich in CGRP content. These afferents synapse with the intermediolateral cell column of the spinal cord containing the sympathetic preganglionic neurons. In addition, evidence is accumulating suggesting that efferent release of peptides from primary afferents occurs.12 Therefore, changes in the neuronal content of CGRP may play a cardiovascular...
role by both efferent and afferent mechanisms. We have also recently reported that the SHR, which has decreased serum ionized calcium levels, has decreased neuronal levels of CGRP in laminae I/II. Thus, lower neuronal CGRP content may play a role in the blood pressure elevation in this experimental model of hypertension. In light of these results, the present study was undertaken to determine if the levels of CGRP messenger RNA (mRNA) were also decreased in the dorsal root ganglia (DRG) of the SHR. The DRG was chosen for study because it contains the cell bodies of CGRP-producing neurons, which send axons to both peripheral tissues (i.e., blood vessels) and centrally to the dorsal horn of the spinal cord (laminae I/II).

Methods

Animals

All studies were approved by the institutional Animal Care and Use Committee. A total of 15 male rats (12 to 14 weeks old) were available for study for the SHR group and 13 for the Wistar-Kyoto (WKY) control strain (Harlan, Houston, Tex.). Four different rats from each group were weighed and had their blood pressures recorded by indirect tail cuff in the nonheated state using a photoelectric sensor (IITC Inc., Woodland Hills, Calif.), and four different rats from each group were used to determine serum ionized calcium levels using an ion-selecting electrode (Radiometer America, Inc., Westlake, Ohio). Blood was obtained with the orbital puncture technique. Seven SHR and five WKY rats were killed, and the tissues were immediately dissected. Thoracic and lumbar DRG from each animal were quickly frozen on a dry-ice plate and transferred to liquid nitrogen for later tissue analysis. The kidneys, heart, and brain from each animal were also immediately dissected and placed directly in liquid nitrogen. Different groups of rats were used for each determination to avoid any possible influence of the blood sampling and blood pressure procedures on CGRP synthesis.

Plasmids and RNA Analysis

The $\alpha$-CGRP hybridization probe was a 1.4-kb Sau3A rat genomic restriction fragment containing CGRP exons 5 and 6, cloned into the SP64 plasmid vector.1 The 18S ribosomal RNA hybridization probe was a 1.15-kb BamHI–EcoRI restriction fragment of the mouse 18S ribosomal DNA, cloned into pBR322.14 The DNA inserts were excised from the plasmid vectors with the appropriate restriction endonucleases and purified by agarose gel electrophoresis. The hybridization probes were subsequently labeled with [$\alpha$-32P]deoxyctydine triphosphate using a random hexanucleotide DNA labeling kit (Amersham, Arlington Heights, Ill.). For the ribonuclease protection assay, a CGRP-specific 180-bp Sau3A–Rsa I genomic fragment (3,842 to 4,022) was subcloned into the SP6–T7 plasmid vector (BRL). The plasmid was linearized with EcoRI in the polylinker region, and the anti-sense RNA was synthesized with T7 RNA polymerase as described elsewhere.15

Total cellular RNA was isolated from the DRG, heart, brain, and kidney tissues by the guanidinium–cesium chloride method, and RNA samples were subjected to electrophoresis on denaturing formaldehyde–agarose gels.16 The fractionated RNAs were transferred to Nytran nylon membranes and hybridized with the 32P-labeled CGRP DNA probe, which hybridizes to both the $\alpha$- and $\beta$-CGRP mRNA species.17 As a control, the CGRP probe was removed from the membrane, which was then rehybridized with the 18S ribosomal DNA probe. After hybridization, the membranes were washed and exposed to X-OMAT X-ray film (Eastman Kodak Co., Rochester, N.Y.) at −70°C with an intensifying screen. After autoradiography, the relative levels of CGRP mRNA and 18S RNA were quantitated by computerized scanning laser densitometry. Where indicated, the ribonuclease protection assay was performed as described elsewhere.15,16

All data are expressed as mean±SEM. Student's $t$ test for unpaired data was used to determine statistical significance where appropriate. A value of $p<0.05$ was considered statistically significant.

Results

There were no differences in body weight between the SHR and WKY rats (SHR, 307±9 g; WKY, 311±15 g), and as anticipated, the SHR had a significantly higher tail-cuff indirect systolic blood pressure than the WKY rats (214±5 versus 147±4 mm Hg, $p<0.001$). Serum ionized calcium levels obtained from the SHR and WKY rats were significantly lower in SHR versus WKY rats (1.31±0.01 versus 1.36±0.01 mmol/L, $p<0.01$).

Before quantifying the relative CGRP mRNA levels in DRG from the SHR and WKY rats, we developed an assay for CGRP mRNA by Northern hybridization analysis. Furthermore, we established that our assay was specific for CGRP mRNA and that the intensity of the hybridization signal was proportional to the amount of RNA in each sample. As shown in Figure 1, decreasing amounts of total cellular DRG RNA obtained from a normotensive male adult Sprague-Dawley rat were fractionated on a denaturing formaldehyde–agarose gel and analyzed for CGRP mRNA content. All of the lanes with DRG RNA displayed a 1.2-kb band that corresponds to CGRP mRNA, whereas no hybridization was detected in the control kidney RNA or transfer RNA samples. We have previously used the ribonuclease protection assay to control for the presence of CGRP mRNA in DRG, brain, and heart.18 Denisometric analysis demonstrated that the intensity of the CGRP mRNA hybridization signal was proportional to the quantity of RNA loaded onto the gel. Because we routinely normalize the levels of CGRP mRNA in each sample to 18S RNA, a similar analysis was performed using the 18S ribosomal DNA as the hybridization probe. The intensity of the 18S RNA bands was linear with respect to the amount of RNA present in each sample (data not shown).

After developing and standardizing the Northern blot hybridization assay, we examined DRG CGRP mRNA and 18S RNA levels in DRG tissue from three of the seven SHR and three of the five WKY normotensive rats (Figure 2). Laser densitometric analysis revealed that the SHR had significantly lower levels of CGRP mRNA than did the WKY control rats (8.5±3 versus 24.8±4 arbitrary units, respectively, $p<0.05$). As an internal control for possible differences in RNA loading or blotting artifacts between the groups, 18S RNA levels were similarly determined. There were no significant differences in 18S RNA levels between these SHR and...
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FIGURE 1. Panel A: Standardization of Northern blot hybridization assay of calcitonin gene–related peptide (CGRP) messenger RNA (mRNA). Total cellular RNA samples isolated from either dorsal root ganglia (DRG) or kidney from a single control animal were fractionated on a denaturing formaldehyde–agarose gel and transferred to a Nytran nylon membrane. The membrane was hybridized with the 32P-labeled CGRP genomic DNA insert (10^6 cpm/mL) and subjected to autoradiography. Lane 1 was loaded with control kidney (KID) RNA (15 μg), lane 2 with yeast transfer RNA (tRNA) (5 μg), and lanes 4 to 8 with decreasing amounts of DRG RNA (2.0 to 0.125 μg). Lane 3 is blank. Panel B: Plot shows autoradiogram as analyzed by computer-assisted laser densitometry.

WKY rats (21.7±2.0 versus 14.9±3.7 arbitrary units, respectively). The ratio of CGRP mRNA to 18S RNA, shown in Figure 3, was significantly lower in the SHR compared with the WKY rats (4.0±0.8 versus 16.9±1.0 arbitrary units, respectively, p<0.02). These values represent approximately a fourfold decrease in DRG CGRP mRNA content in the SHR.

To confirm these results, we performed a second experiment using DRG RNA samples from an additional four SHR and two WKY rats. Similar to the first experiment, the SHR exhibited a threefold decrease in the CGRP mRNA/18S RNA ratio compared with the WKY rats. To combine the data from the two experiments, we normalized each CGRP mRNA/18S RNA ratio to the highest value in each group and expressed it as a percent of that value. After this normalization, the CGRP mRNA ratio was significantly decreased in the SHR compared with the WKY rats (32.6±18% versus 94.0±7%, p<0.001).

FIGURE 2. Northern blot analysis of dorsal root ganglia calcitonin gene–related peptide (CGRP) messenger RNA from spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Total cellular dorsal root ganglia RNA samples isolated from three SHR and three WKY rats were fractionated on denaturing formaldehyde–agarose gels and transferred to a Nytran nylon membrane. The membrane was hybridized with the 32P-labeled CGRP genomic DNA insert (top, 10^6 cpm/mL) and subjected to autoradiography. The membrane was subsequently hybridized with the 32P-labeled 18S ribosomal DNA probe (bottom, 5×10^5 cpm/mL). Lanes 1 to 3 were loaded with RNA samples (2 μg) from the WKY rats; lanes 4 to 6 had RNA samples (2 μg) from SHR.

To determine if the observed decrease in neuronal CGRP mRNA in the SHR was tissue specific, we also examined CGRP mRNA content in brain and heart. It is well documented that specific regions of the brain synthesize CGRP mRNA, and we have recently reported that rat heart contains low but detectable levels of CGRP mRNA.2,18 Ribonuclease protection analysis of RNA samples isolated from these tissues from three SHR and

FIGURE 3. Bar graph shows densitometric analysis of Northern blot hybridization assay. The calcitonin gene–related peptide (CGRP) messenger RNA (mRNA)/18S RNA ratios were determined by computer-assisted laser densitometry from Wistar-Kyoto (WKY) control rats (n=3) and spontaneously hypertensive rats (SHR) (n=3). **p<0.01 SHR vs. WKY rats.
three WKY rats indicated that there were no significant differences in either brain or heart CGRP mRNA content between the two groups (data not shown).

**Discussion**

Our results demonstrate that CGRP mRNA levels are decreased approximately threefold in DRG from SHR when compared with WKY controls. Because the CGRP hybridization probe used in these studies hybridizes to both α- and β-CGRP mRNA, both of which are synthesized in the DRG, we do not yet know if the reduction in CGRP mRNA content results from the decrease of one or both CGRP mRNA species. Although differential expression of the α and β genes has been reported in neuronal tissues, its significance is not clear, because in the rat and in humans the α and β protein sequences differ by one and three amino acids, respectively, and there are no significant differences in the biological activities of the two peptides. This decrease in CGRP mRNA content appears to be specific for the DRG, because there were no significant differences in CGRP mRNA levels in either heart or brain between the SHR and WKY rats. However, whole hearts and brains were used for this analysis, so we cannot rule out the possibility that there are subtle differences in specific regions of these tissues between SHR and WKY rats that cannot be detected by Northern blot analysis of RNA isolated from the whole organ.

Abnormalities of calcium homeostasis have been shown to be present in both human essential hypertension and animal models of hypertension. Decreased serum ionized calcium and calcitonin levels accompanied by increased serum parathyroid hormone and 1,25-dihydroxyvitamin D₃ levels have been described in human low renin essential hypertension. The SHR and doxycorticosterone-salt hypertensive animals also exhibit decreased serum ionized calcium levels and increased serum parathyroid hormone levels. Because abnormalities of calcium homeostasis are present in certain forms of hypertension, it is possible that alterations in CGRP, a product of the calcitonin/CGRP gene, also may occur in hypertension. If CGRP gene expression is regulated by calcium, either directly or indirectly, decreased ionized calcium levels could, in turn, decrease CGRP gene expression and the neuronal content of CGRP. Likewise, increased ionized calcium levels could increase CGRP gene expression and the neuronal content of CGRP. In support of this hypothesis, we have previously shown that dietary calcium intake directly changes the neuronal content of CGRP in the normal rat. Thus, low dietary calcium intake, which significantly decreased serum ionized calcium levels, significantly decreased the neuronal content of CGRP and vice versa.

The results presented herein also support our previous findings that the SHR has significantly decreased neuronal content of CGRP compared with WKY rats. In the SHR and in the dietary calcium studies, laminae I/II of the dorsal horn of the spinal cord were examined for immunoreactive CGRP content, because the central processes of afferent CGRP neurons innervating the heart and blood vessels terminate in laminae I/II. To evaluate CGRP mRNA levels, it was necessary to study DRG, because they are the site of the cell bodies to the afferent axons that terminate peripherally on blood vessels and centrally in laminae I/II of the dorsal horn of the spinal cord. These afferents make connections with the intermediolateral cell column of the spinal cord, which contains the sympathetic preganglionic neurons. This connection could influence the activity of the sympathetic nervous system and thus vascular tone. Evidence is also accumulating to suggest the efferent release of peptides from primary afferents. Local factors such as vascular wall tension, bradykinin, and other mediators of inflammation as well as interactions with the sympathetic nervous system may promote CGRP release. If basal neuronal CGRP content is increased or decreased, then these local factors would be expected to release more or less CGRP, respectively, resulting in a greater or lesser degree of vasodilatation. Therefore, CGRP could modulate vascular tone via both efferent and afferent neuronal activity. Thus, the reduction in the neuronal content of CGRP in the SHR could contribute to the development or maintenance of the elevation of blood pressure in this model of experimental hypertension through the reduction of such a potent vasodilator. Alternatively, we cannot rule out the possibility that the changes in neuronal CGRP mRNA levels are secondary to the increase in blood pressure.

In summary, we have demonstrated that DRG CGRP mRNA levels are significantly reduced in the SHR compared with WKY control rats and that this alteration appears to be tissue specific. These data complement our earlier studies which showed that immunoreactive CGRP content in laminae I/II of the dorsal horn of the spinal cord is decreased in the SHR. The reduced CGRP mRNA levels coupled with the reduced immunoreactive peptide levels suggest decreased synthesis and release of CGRP and not increased release. Future studies are required to identify and characterize the factors and mechanisms responsible for this decrease in neuronal CGRP gene expression in the SHR and to elucidate the roles that CGRP may play in the pathology of blood pressure elevation and regional blood flows in this setting.

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**References**

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