Nerve Growth Factor Enhances Calcitonin Gene-Related Peptide Expression in the Spontaneously Hypertensive Rat

Scott C. Supowit, Huawei Zhao, Donald J. DiPette

Abstract—Calcitonin gene–related peptide (CGRP) expression is markedly reduced in dorsal root ganglia neurons in the spontaneously hypertensive rat (SHR). This decrease in such a potent vasodilator may contribute to the elevated blood pressure. Therefore, the purpose of this study was to determine whether stimulation of neuronal CGRP expression in SHR, by means of the administration of nerve growth factor, would lower the blood pressure. Nerve growth factor (10 nmol/L, per kg/d, IP) was given to 12-week SHR (n=8 to 11/group) once a day for 1, 3, and 7 days. Control SHR received vehicle only. All rats were instrumented for CGRP receptor antagonist (CGRP8–37) administration (intravenous) and mean arterial pressure recording. Both the 1- and 3-day NGF treatments lowered the mean arterial pressure to 147±5 and 147±3 mm Hg, respectively, compared with controls (166±3 mm Hg). However, by day 7, the mean arterial pressure had returned to control levels (169±5 mm Hg). CGRP8–37 administration produced a significant mean arterial pressure increase in all 3 nerve growth factor–treated groups (14±2, 10±2, and 13±2 mm Hg). CGRP mRNA levels in dorsal root ganglia were increased in the 3 neurotrophin-treated groups, whereas CGRP peptide content was higher at days 3 and 7. Therefore, nerve growth factor treatment of SHR can enhance neuronal CGRP expression. At days 1 and 3, nerve growth factor produces a depressor response that is primarily mediated by CGRP as evidenced by the pressor effect of CGRP8–37. At day 7, CGRP also plays a counterregulatory role, even though the mean arterial pressure has returned to control levels. This finding may result from a nerve growth factor–mediated upregulation of a pressor system that counteracts the hypertensive actions of CGRP. Thus, these data suggest that the decreased production of CGRP in SHR could contribute to the hypertension. (Hypertension. 2001;37[part 2]:728-732.)

Key Words: calcitonin gene–related peptide  ■ rat, spontaneously hypertensive  ■ blood pressure  ■ nerve growth factor  ■ RNA

Calcitonin gene–related peptide (CGRP) is a 37 amino acid neuropeptide that is widely distributed in the central and peripheral nervous systems in mammals.1–2 A prominent site of CGRP synthesis is the dorsal root ganglia (DRG) that contain the cell bodies of sensory nerves which terminate peripherally on blood vessels and other tissues innervated by the sensory nervous system and centrally in laminae I/II of the dorsal horn of the spinal cord. Immunoreactive CGRP (iCGRP)–containing nerve fibers are widely distributed in the cardiovascular system and in blood vessels are found at the junction of the adventitia and the media passing into the muscle layer.1,2

In vivo and in vitro studies have demonstrated that CGRP is a very potent vasodilator, approximately 100 to 1000 times more potent than other vasodilators such as adenosine, substance P, or acetylcholine.3 CGRP has been shown to dilate multiple vascular beds, with the coronary circulation being a particularly sensitive target. Systemic administration of CGRP decreases blood pressure (BP) in a dose-dependent manner in normotensive and hypertensive animals and humans.4 The primary mechanism responsible for this BP reduction is peripheral arterial dilation.

We have previously reported that in 2 rat models of acquired hypertension (deoxycorticosterone-salt, DOC-salt5,6 and subtotal nephrectomy-salt, SN-salt7), CGRP acts as a compensatory vasodilator in an attempt to counteract the BP increase. In DOC-salt rats, the mechanism that mediates this anti-hypertensive activity of CGRP seems to be through a marked increase in the neuronal (DRG) expression and presumably release of this peptide. The SN-salt model did not exhibit this increase in CGRP expression. We later demonstrated that, in this setting, the counterregulatory effects of CGRP on blood pressure were mediated by means of an enhanced sensitivity of the vasculature to the vasodilator activity of this peptide.

In contrast, we have shown that DRG CGRP mRNA and peptide production is markedly decreased in 12-week-old spontaneously hypertensive rats (SHR) compared with age-matched Wistar-Kyoto (WKY) controls.8 A significant reduction in circulating levels of CGRP have also been observed in both the SHR and the stroke-prone SHR.1,2 In addition, Kawasaki et al9 demonstrated an age-related decrease in iCGRP- and CGRP-mediated vasodilator activity in perivascular nerves associated with mesenteric vascular beds iso-
lated from SHR compared with WKY rats. This decrease in CGRP expression in the SHR is accompanied by an increase in the sensitivity of the vasculature to exogenous CGRP. Therefore, it is possible that a significant reduction in the synthesis and release of such a potent vasodilator could contribute to the elevated BP in this setting. Thus, the purpose of this study was to determine whether enhancement of neuronal CGRP production in SHR, by means of the administration of nerve growth factor (NGF) would result in a CGRP-mediated decrease in BP.

Methods

Animals and NGF Treatment
A total of 38 12-week-old male SHR (Harlan, Sprague-Dawley) were used for this study and all protocols were approved by the institutional Animal Care and Use Committee. NGF (10 nmol/L per kg/d) was administered by intraperitoneal injection once a day for 1 (n=9), 3 (n=10), and 7 (n=11) days. A separate group (n=8) of control SHR received injections of vehicle (saline) only.

CGRP Receptor Antagonist Administration and MAP Measurement
Rat α-CGRP$_{8-37}$ was synthesized by standard solid-phase T-BOC chemistry. CGRP$_{8-37}$ was dissolved in saline and has been previously shown to block the hypotensive effects of intravenously infused rat α-CGRP (Phoenix Pharmaceuticals) in normal rats. For the present studies, each rat was anesthetized with ketamine and xylazine (80 and 4 mg/kg IP, respectively). The left carotid artery was cannulated for continuous measurement of MAP using a pressure transducer coupled to a recorder (Gould Instruments). The right jugular vein was also cannulated for infusion of either vehicle (saline) or CGRP$_{8-37}$. The hemodynamic studies were performed approximately 3 hours after surgery with the rats fully awake and unrestrained.

Hybridization Probes and RNA Analysis
The α-CGRP hybridization probe was a 1.4-kb Sau 3A rat genomic restriction fragment containing CGRP exons 5 and 6. The 18S rRNA hybridization probe was a 1.15-kb BamHI-EcoRI restriction fragment of the mouse 18S rRNA gene. The DNA inserts were purified by agarose gel electrophoresis and subsequently labeled with [α-32P]dCTP using a random hexanucleotide DNA labeling kit (Amersham).

After the hemodynamic studies, the rats were deeply anesthetized by infusion of ketamine and xylazine (100 and 5 mg/kg) through the jugular vein catheter. The rats were then killed, and the cervical, thoracic, and lumbar DRG from each animal were immediately dissected and frozen in liquid nitrogen. For each animal, the DRG on one side of the spinal cord were separated from those on the other side of the cord such that half of the DGR were used for RNA analysis and the other half for CGRP peptide quantification. To determine relative CGRP mRNA levels, total cellular RNA was purified by agarose gel electrophoresis and subsequently labeled with [α-32P]dCTP using a random hexanucleotide DNA labeling kit (Amersham).

The RNA samples were subjected to electrophoresis on denaturing formaldehyde-agarose gels, and the fractionated RNAs were then transferred to nylon membranes and hybridized with the labeled CGRP probe. As a control, the CGRP probe was removed from the membranes, which were then hybridized with the 18S rRNA probe. After each hybridization reaction, the membranes were washed and placed in a cassette containing a phosphor screen. The exposed screen was put in a phosphor imager, which generates an image of each labeled RNA species and quantifies the hybridization signals.

Radioimmunoassay
To determine iCGRP content in the DGR from the experimental and control rats, a commercially available rabbit anti-rat CGRP radioimmunoassay kit (Phoenix Pharmaceuticals) was used. This antibody has 100% reactivity with rat α-CGRP and 79% reactivity with rat β-CGRP. There is no cross-reactivity with either amylin, calcitonin, somatostatin, or substance P. The assay was performed as recommended by the supplier, and the total protein content in each sample was determined by the Bradford method (Bio-Rad).

Statistical Analysis
Statistical significance was determined by the Student’s t test for unpaired data or where appropriate by ANOVA followed by the Tukey-Kramer multiple comparisons test. A value of P<0.05 was assumed to be significant. The data in the figures are expressed as the mean±SEM.

Results

Effects of NGF Treatment and CGRP$_{8-37}$ Administration on Blood Pressure
Although injection of NGF did not cause any apparent adverse affects at any of the time points studied, on the day after the first NGF injection all of the treated SHR displayed a marked increase in the intensity of the pink color of the skin that was particularly striking in the ears, which were bright red. In addition there was a very apparent deepening of the red color of the eyes. This alteration in skin and eye color was maintained through day 3. Between 3 and 7 days (the 7-day treatment group), the skin and eye color tended to return to normal, even though there was considerable variability between the individual rats.

At the end of each treatment period, each rat had arterial (for continuous MAP recording) and intravenous (for drug administration) catheters surgically implanted and were studied in a fully awake and unrestrained state. As shown in Figure 1, after a single NGF treatment (day 1, n=9), the MAP was reduced by 18 mm Hg compared with the control SHR (n=8). The MAP was still lower by 19 mm Hg on day 3.

Figure 1. MAP determinations in the NGF-treated and control SHR. Control (n=8) and NGF-treated (days 1, n=9; 3, n=10; and 7, n=11) SHR were instrumented for continuous MAP recording as described in the text. MAP determinations were made with the rats in a fully awake and unrestrained state. * P<0.05.

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however, by day 7 (n = 11), the MAP was back up to control levels (169 ± 6 mm Hg). It should be noted that the control SHR received saline injections for 1 day. In pilot studies, we observed that injection of saline for 3 and 7 days did not alter the MAP nor did it change the response to CGRP8–37 administration (unpublished observations).

To determine whether any of the MAP reduction observed on NGF treatment days 1 and 3 was due to CGRP each animal was treated acutely with bolus doses of saline (200 μL) and CGRP8–37 (800 μg/kg in 200 μL saline). Saline treatment produced a negligible increase in MAP at all the time points studied. In the control SHR, CGRP8–37 treatment resulted in a 4 ± 1 mm Hg increase in MAP that was similar to what was seen with the saline (Figure 2). In contrast, on days 1 and 3 of NGF treatment, the CGRP receptor antagonist produced a rapid (the MAP increase began approximately 15 seconds after CGRP8–37 administration) 14 ± 2 and 10 ± 2 mm Hg increase in MAP, respectively. Unexpectedly, on day 7 when the MAP had returned to control levels, antagonist treatment still caused a 13 ± 2 mm Hg increase in MAP. The pressor activity of CGRP8–37 was short-lived lasting approximately 120 seconds. The values shown in this figure represent the peak responses that lasted approximately 25 seconds. This transient effect of the antagonist has been observed by us6,7 and other investigators who have used CGRP8–37 for in vivo studies and most likely reflects the rapid proteolysis of this peptide in the circulation.1,2,14

Analysis of CGRP mRNA and Peptide Levels in the NGF Treated and Control SHR

To determine whether neuronal CGRP expression was stimulated in the NGF-treated SHR, CGRP mRNA and iCGRP levels were quantified in the DRG taken from the rats used in the blood pressure experiments described above. Figure 3 is a representative Northern blot showing both the 1.2-kb CGRP mRNA species (α- and β-CGRP) and 18S rRNA present in the DRG RNA samples. Phosphor imager analysis of the autoradiographs was then performed to quantify the hybridization signals. When the values for CGRP mRNA content were normalized to those for 18S rRNA (CGRP mRNA/18S rRNA) to control for possible differences in loading of the RNA samples between the groups, CGRP mRNA accumulation was found to be significantly increased after the 1-day (2.9 ± 0.1 arbitrary units),
Several lines of evidence suggest that NGF can stimulate CGRP expression in mature sensory neurons in vivo. Using primary cultures of adult DRG neurons, Lindsay demonstrated that NGF can directly upregulate CGRP synthesis and release. We have since confirmed and extended these results in vitro and have performed in vivo studies showing that NGF treatment of normal Sprague-Dawley rats significantly increases DRG CGRP mRNA accumulation (unpublished observations).

Administration of the NGF to SHR resulted in relatively rapid physiological changes. Although we did not measure alterations in skin blood flow in the treated and control SHR, the dramatic changes in skin and eye color that were observed are indicative of intense vasodilation in these two organ systems. This striking change in the color of the skin and eyes was also seen in the normal Sprague-Dawley rats used in preliminary NGF dose-finding experiments. We would argue that this response is caused by a NGF-mediated release of CGRP from sensory nerve terminals that are present in the skin and eyes. It is well documented that injection of exogenous CGRP into the skin, or the stimulation of endogenous CGRP release by means of subcutaneous administration of agents such as capsaicin, bradykinin, or NGF, can significantly enhance localized skin blood flows. Furthermore, we and others have demonstrated that NGF can directly enhance the synthesis and release of CGRP in primary cultures of DRG neurons, and there are several reports showing NGF-evoked release of neuropeptides from peripheral sensory nerve terminals in vivo.

NGF treatment also produced a significant decrease in MAP in the day 1 and day 3 treatment groups. The ability of the CGRP receptor antagonist to increase the MAP in the day-1 and day-3 treatment groups suggests that most of the BP decrease observed at these time points is mediated by CGRP. This pressor effect of CGRP8–37 is similar to what was observed in the DOCA-salt and SN-salt models, where CGRP is playing a compensatory vasodilator role to attenuate the BP increase. In addition, given the rapid onset of the hypertensive effect of CGRP8–37, and because this antagonist probably does not penetrate the central nervous system, it is likely that the pressor activity of CGRP8–37 results from a direct interaction of the antagonist with peripheral vascular CGRP receptors. It is also possible that the NGF-evoked decrease in BP is due to a direct vasodilator effect of this peptide on blood vessels. However, the results of CGRP receptor antagonist administration argue against this possibility, and we are not aware of any reports demonstrating that NGF can directly relax vascular smooth muscle.

The increase in DRG CGRP production on days 1 and 3 correspond well with the anti-hypertensive effects of NGF in these two groups. CGRP mRNA content is significantly elevated at these time points and iCGRP levels are increased after 3 days of NGF treatment. Unexpectedly, we did not find an increase in CGRP peptide on the day-1 time point. This finding may be explained by the fact that NGF has very potent stimulatory effects on CGRP release from peripheral sensory nerve terminals. Thus, a single injection of NGF may produce a massive release of CGRP from the sensory nerves initiating the MAP decrease and the intense vasodilation response seen in the skin and eyes. This would tend to reduce the apparent iCGRP levels in the DRG even though synthesis was increased after upregulation of CGRP mRNA content. We were unable to verify that circulating iCGRP was significantly enhanced after a single NGF injection even though CGRP mRNA accumulation was increased at this time point. However, after 3 and 7 days of NGF treatment, iCGRP levels were increased to 0.48±0.03 and 0.81±0.04 μg iCGRP/mg total protein, respectively, compared with the saline-treated SHR (0.32±0.02 μg iCGRP/mg total protein).

**Discussion**

The present study was designed to test the hypothesis that pharmacologic stimulation of neuronal (DRG) CGRP synthesis in the SHR would lower the blood pressure. This was accomplished through NGF administration. During development, NGF is essential for the survival and differentiation of sympathetic neurons and the majority of neural-crest derived sensory neurons such as those found in DRG. In mature small sensory and sympathetic neurons, NGF is required to maintain the fully differentiated phenotype. Sensory and sympathetic neurons obtain NGF from their peripheral target fields and supporting cells. NGF acts by means of a specific tyrosine kinase receptor (trkA) to control the synthesis of several neuropeptides and regulate membrane excitability. Several lines of evidence suggest that NGF can stimulate CGRP expression in mature sensory neurons in vivo. Using primary cultures of adult DRG neurons, Lindsay demonstrated that NGF can directly upregulate CGRP synthesis and release. We have since confirmed and extended these results in vitro and have performed in vivo studies showing that NGF treatment of normal Sprague-Dawley rats significantly increases DRG CGRP mRNA accumulation (unpublished observations).

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increased in these animals, because the CGRP antagonist interferes with the radioimmunoassay.

The results obtained with 7-day NGF treatment group were unexpected. That the MAP had returned to control levels even though CGRP synthesis in DRG was the highest of any of the groups studied would argue against our hypothesis. However, the magnitude of the MAP increase in response to CGRP 8–37 at this time point was not significantly different to that on days 1 and 3, suggesting that CGRP was still acting as a counterregulatory mechanism to buffer the blood pressure increase. In addition, the continued activity of the antagonist indicates that a downregulation of the CGRP receptor is not the cause of the MAP increase on day 7. One possibility is that CGRP is acting to decrease the blood pressure on days 1, 3, and 7. However, by day 7, NGF may have stimulated a pressor system to counteract the depressor effects of CGRP and bring the MAP back up to control SHR levels. Two potential candidates are the sympathetic nervous system and neuuropeptide Y, both of which are upregulated by NGF. A second possibility is that the reversal of the anti-hypertensive actions of CGRP is mediated by baroreceptors acting to return the BP to the prevailing higher pressure level. Finally, it is also possible that administration of NGF and resultant increase in neuronal CGRP production and release would acutely lower the blood pressure in any rat model of experimental hypertension. Thus, CGRP may not have a long-term impact on blood pressure homeostasis in the SHR.

Therefore, these results suggest that NGF treatment of SHR stimulates both the release and production of CGRP in DRG sensory nerves. This increase in CGRP expression, coupled with the enhanced sensitivity of the vasculature of the SHR to this peptide, produce a significant decrease in the BP after 1 and 3 days of treatment. After 7 days, the BP reduction is reversed by a yet to be determined mechanism, even though CGRP is still playing a compensatory role to buffer the increased BP, although at a higher level. Thus, these data are consistent with the argument that the decrease in neuronal CGRP that is observed in the SHR, may contribute to the elevated BP in this setting.

**Acknowledgment**

These studies were supported by National Institutes of Health grant HL-44277.
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Hypertension. 2001;37:728-732
doi: 10.1161/01.HYP.37.2.728

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

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