Nerve Growth Factor Gene Locus Explains Elevated Renal Nerve Growth Factor mRNA in Young Spontaneously Hypertensive Rats

Fadi J. Charchar, Miroslav K. Kapuscinski, Stephen B. Harrap

Abstract—Nerve growth factor (NGF) controls the growth of sympathetic nerves and is increased in young spontaneously hypertensive rats (SHR). The NGF gene has been linked genetically with hypertension in the SHR strain and may explain high NGF mRNA levels. To test for genetic linkage between the NGF gene and its expression in vivo, we examined renal NGF mRNA levels in male SHR, control Donryu rats (DRY), and F2 rats derived from SHR and DRY at ages 2, 4, 10, and 20 weeks. Tail-cuff blood pressure was measured at 4, 10, and 20 weeks of age. NGF mRNA levels in SHR (NGF genotype: SS) were higher than those in DRY (NGF genotype: DD) at 2, 4, and 10 weeks of age (P<0.0001) but the same at 20 weeks of age. In the F2 generation, the S allele was associated with significantly (P=0.01) higher renal NGF mRNA levels at 2 weeks of age. Mean NGF mRNA levels fell (P<0.007) with age in F2 rats, and the difference between SS and DD genotype F2 rats diminished at older ages and was not significant. In F2 rats there was a positive correlation between the number of NGF S alleles inherited and tail-cuff pressure (P<0.007). Our findings indicate that the NGF locus is an important regulator of NGF mRNA levels. It is likely that mutations in or near the NGF gene explain in part high early NGF gene expression in SHR. (Hypertension. 1998;32:705-709.)

Key Words: growth substances ■ hypertension ■ rats, inbred SHR ■ gene expression ■ kidney

In both humans and the spontaneously hypertensive rat (SHR) animal model, there is convincing evidence that the sympathetic nervous system plays an important role in etiology of hypertension.1,2 During development, nerve growth factor (NGF) is required for the survival of sympathetic and some sensory neurons. We have reported previously a genetic linkage of the NGF gene of the SHR with an increment in blood pressure (BP) in segregating F2 rats derived from a cross of the SHR and the normotensive Donryu (DRY) strains.3 Studies of NGF mRNA by Northern analysis reveal higher abundance of NGF mRNA in SHR tissues than in age-matched normotensive rats.4,5 Increased NGF mRNA has been associated with increased NGF peptide levels and higher densities of sympathetic innervation6,7 and is likely to reflect enhanced NGF gene expression.

There are several possible explanations for the elevated NGF mRNA in SHR, including (1) DNA sequence differences in or near the NGF gene resulting in increased transcription or stability of mRNA, (2) genetic differences in other genes that influence NGF gene transcription, or (3) the physiological influence of increased BP on NGF gene expression in the SHR. Given the demonstrated linkage between the NGF locus and SHR hypertension,3 we studied the role of the NGF gene locus in determination of abnormal NGF mRNA levels.

The concentration of NGF mRNA in blood vessels at ages 2, 10, and 43 days8 and kidneys at ages 10 and 43 days7 was greater in SHR than in Wistar-Kyoto rats (WKY), but increased NGF gene expression seems to be developmental stage–specific. At 3 weeks of age the level of NGF in the spleen, the sciatic nerve, and the mesenteric arteries was higher in SHR than in WKY, but not at 12 weeks of age.8 Higher levels of NGF peptide levels in mesenteric arteries and aortas from young (20-day-old) SHR were not observed in adult (6-month-old) SHR.9,10 Renal sympathetic nerves in SHR are increased in density,11 and renal denervation results in a reduction in BP.12 Therefore, in the present study we examined the levels of NGF mRNA in the kidney of SHR at different ages. In addition, we performed a cross-breeding study to determine whether inheritance of the SHR NGF allele was associated with any significant effect on renal NGF mRNA levels in young animals.

Methods

Animals

Male SHR and female DRY were bred and crossed as described previously.1 Male SHR (n=5), DRY (n=5), F1 (n=4), and F2 rats were studied at the age of 2, 4, 10, and 20 weeks (F2: n=20, 23, 20, and 28, respectively). The inbred status of parental strains has been tested and reported previously.1 Systolic BP was measured twice a
week in conscious animals between 9 and 11 AM with a photoelectric
tail-cuff pulse detection system (IITC Inc, Life Science Instrument-
tion). The size of the restrainer cylinders and cuffs was matched to
the size of growing animals. Before the experimental period, the rats
were conditioned to the restraining cylinders and BP measurement.
Rats were prewarmed at 37°C for 8 to 10 minutes to facilitate tail
blood flow before BP was measured. The mean of 3 readings of
systolic tail blood pressure was used as the BP value for each rat.
Readings were not made in 2-week-old rats because their small size
precluded accurate measurements. These experiments were approved
by the University of Melbourne Animal Experimentation
Ethics Committee.

Quantification of Kidney NGF mRNA
Total RNA was isolated from 100 to 200 mg of whole kidneys
according to the acid phenol method of Chomczynski and Sacchi.13
Reverse transcription–polymerase chain reaction (RT-PCR) was
used to provide relative quantification of the amount of renal NGF
mRNA, as reported previously.14 GAPDH amplification was per-
formed to provide internal standards for relative quantification of
NGF transcripts in the isolated total RNA.

Genotyping of the NGF Locus
Genomic DNA was extracted with the use of standard methods, as
previously reported.5 Aliquots (20 μg) of genomic DNA were
digested with NsiI restriction endonuclease (New England Biolabs)
according to the manufacturer’s instructions, and 10 μg of digested
DNA was electrophoresed in 0.8% agarose gel with the use of
Tris-borate-EDTA buffer. DNA was transferred onto a charged
nylon membrane (Hybond N+, Amersham) and hybridized to mouse
NGF cDNA probe.5

Statistical Analysis
All data appearing in text, figures, and tables are reported as means
with 95% CI for the mean unless stated otherwise. When we tested
for the effects of genotype on the NGF mRNA or BP, groups were
compared using 1-way ANOVA. Differences between individual
group means were assessed with Tukey’s honestly significant dif-
fERENCE test. To assess overall relationships between NGF genotype,
BP, NGF mRNA, and age, we undertook a multiple regression
analysis in the F2 animals.

Results
Genotypes
Southern blot analysis of genomic DNA digested with NsiI
revealed single distinct bands in the parental strains. The SHR
allele (S) restriction fragment was 4.8 kb, and the DRY allele (D)
restriction fragment was 2.8 kb. A typical autoradiogram from F2
rats showed the 3 possible genotypes after NsiI digestion of
DNA and hybridization with NGF cDNA (Figure 1).

Relative Quantification of NGF mRNA
The relative NGF mRNA expression was examined in the
kidneys of SHR, DRY, F1 , and 3 possible genotypes in the F2
population at 2, 4, 10, and 20 weeks of age. Figure 2 shows
representative images of NGF and GAPDH PCR products
from kidney cDNA at 4 weeks of age in the SHR, F2, DRY,
and F1 rats. The sizes of PCR products were 440 base pairs for
NGF and 220 base pairs for the GAPDH product. Southern
blot analysis with oligonucleotide probes internal to the PCR
primers confirmed the identity of these products (data not
shown).

Renal NGF mRNA
The relative abundance of renal NGF mRNA after correction
for GAPDH at 2 weeks of age is shown in Figure 3. The SHR
showed significantly greater amounts of renal NGF mRNA compared with DRY. The mean mRNA levels in the F1 rats were intermediate and significantly different from both parental strains ($P < 0.05$). The mean NGF mRNA levels in F2 rats were similar to those in F1 animals. There was a significant difference ($P < 0.05$) in the mean NGF mRNA relative abundance between the 3 possible NGF genotypes (Figure 3). The rats homozygous for the SHR allele (SS) showed a higher level of NGF mRNA than F2 rats homozygous for the DRY allele (DD). The F2 animals heterozygous for the NGF allele showed an intermediate level of mRNA, and there was a significant ($P < 0.01$) linear association between NGF mRNA levels and the number of NGF S alleles.

At 4 weeks of age, the mean NGF mRNA abundance of the SHR parents was significantly higher than that of the DRY parents ($P < 0.0001$), with F1 values intermediate (Table 1). The relative abundance of NGF mRNA (Table 1) was also significantly higher in the 10-week-old SHR than in DRY and F1. However, at 20 weeks of age the renal NGF mRNA in SHR was less than at younger ages and was not significantly different from that in DRY (Table 1).

In F2 rats, those homozygous for the S allele had the highest levels of NGF mRNA at 4, 10, or 20 weeks of age ($P < 0.05$). Although these weekly differences were not significant alone by ANOVA, regression analysis of all F2 rats between 2 and 20 weeks of age revealed significant univariate correlations of higher NGF mRNA levels with increasing numbers of S alleles ($r = 0.23$, $P = 0.01$). In addition, there was a significant association between increasing age and a reduction in NGF mRNA ($r = -0.24$, $P = 0.01$). When all variables were entered in multiple regression analysis, both age ($P = 0.03$) and NGF genotype ($P = 0.04$) showed independent and significant associations with NGF mRNA.

**Blood Pressure**

Mean tail-cuff BP values are shown in Table 2 for ages 4, 10, and 20 weeks. At 10 and 20 weeks of age, the average BP of SHR was the highest of all groups ($P < 0.0001$) (Table 2). At 4, 10, or 20 weeks of age, F2 rats homozygous for the S allele had the highest BP values (Table 2), and although not significant by ANOVA at each week, multiple regression analysis incorporating all F2 animals aged between 4 and 20 weeks showed significant independent association between BP and the number of NGF S alleles inherited by each animal ($P = 0.007$).

**Discussion**

The present study indicates that levels of NGF mRNA are elevated from as early as 2 weeks of age in the kidneys of SHR compared with DRY. High renal NGF mRNA in SHR persists until 10 weeks of age. Between 10 and 20 weeks of age, the level of NGF in SHR falls and becomes similar to that in the DRY strain.3,4,7-10,15 However, the new finding in the present study is that this increased level of NGF mRNA is associated with the inher-

### Table 1. NGF mRNA Levels in the Kidney of SHR, DRY, and F1 Rats and the 3 Genotype Groups in F2 Rats

<table>
<thead>
<tr>
<th>Age, wk</th>
<th>DRY</th>
<th>F1</th>
<th>SHR</th>
<th>$P^*$</th>
<th>DD</th>
<th>SD</th>
<th>SS</th>
<th>$P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.37 (0.24–0.51)</td>
<td>0.55 (0.53–0.57)</td>
<td>0.81 (0.72–0.91)</td>
<td>&lt;0.0001</td>
<td>0.49 (0.23–0.76)</td>
<td>0.54 (0.32–0.75)</td>
<td>0.58 (0.35–0.81)</td>
<td>0.86</td>
</tr>
<tr>
<td>10</td>
<td>0.38 (0.31–0.47)</td>
<td>0.65 (0.63–0.67)</td>
<td>0.83 (0.66–1.01)</td>
<td>&lt;0.0001</td>
<td>0.54 (0.41–0.67)</td>
<td>0.58 (0.46–0.70)</td>
<td>0.61 (0.44–0.78)</td>
<td>0.75</td>
</tr>
<tr>
<td>20</td>
<td>0.51 (0.36–0.66)</td>
<td>0.62 (0.57–0.66)</td>
<td>0.61 (0.49–0.73)</td>
<td>0.20</td>
<td>0.52 (0.41–0.63)</td>
<td>0.53 (0.47–0.59)</td>
<td>0.58 (0.45–0.71)</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Values are mean (95% CI).

*ANOVA.
NGF in Genetic Hypertension

The elevated NGF in the SHR at young ages is important because it is a likely explanation of the sympathetic hyperinnervation implicated in this model of genetic hypertension. These early increases in NGF mRNA may augment levels of sympathetic innervation for the life of the animal. We have also recently shown that brief treatment of young SHR with an angiotensin-converting enzyme inhibitor causes a persistent reduction in both renal NGF mRNA and BP. In addition, increased NGF mRNA may contribute to the structural hypertrophy of resistance arteries that are characteristic of SHR strain.

The difference in NGF mRNA between F2 SS and F2 DD rats seemed to disappear between 2 and 4 weeks of age. We are cautious about using observations from relatively modest numbers of rats from the segregating F2 population to determine the timing of abnormal NGF gene expression. The absence of a statistically significant difference in NGF mRNA between the genotypic groups at 4 and 10 weeks of age in part reflects insufficient statistical power to detect differences that diminish as the animals age. In this respect it is relevant that when all F2 rats were combined, a significant association between NGF genotype and NGF mRNA was observed. However, it is clear that the difference in NGF mRNA between the SHR and DRY is larger and persists longer than that between F2 SS and F2 DD rats. This contrast is likely to reflect the fact that F2 rats are on average 50% SHR and 50% DRY in genetic origin. Other genes unlinked to the NGF gene locus may reduce both the magnitude and duration of NGF mRNA differences in the F2 population.

The NGF gene consists of 4 exons (IA, IB, II, IIIA, IIIB, IV) separated by introns of varying sizes, including a putative transcription unit of >45 kb. Alternative splicing gives rise to 4 types of cDNA clones with different sequences preceding the region coding for the NGF protein (exon IV). Differential expression of RNA might be explained either by specific tissue activation of a number of promoters or by the presence of regulatory sequences within a promoter recognized by tissue-specific transcriptional factors. The NGF gene transcription is thought to be mainly regulated by cis-elements in the region immediately upstream to exon IB. The cis-regulatory elements in the area preceding exon IB consist of 2 putative TATA boxes, 2 putative CAAT boxes, and a high content of G and C residues, which indicates the presence of binding sites for the regulatory protein SP1. Studies by D’Mello and Heinrich located regions in a 1-kb region close to exon IB that affect transcription of NGF in L929 cells, which include a proximal activator region containing several SP1-like elements known to increase the number of transcription-initiation complexes. They also identified in that region a transcription suppression recognition site and an AP1 binding site for the transcription factors c-fos and c-jun at the junction of exon IB and the second intron. The activation of c-fos and c-jun genes and their interaction with AP1 binding site may facilitate the expression of the NGF gene by a variety of stimuli such as angiotensin II. Therefore, the NsiI polymorphic site in SHR may be in linkage disequilibrium with a functional mutation in a region that alters tissue and developmental stage–specific gene expression.

Another mechanism by which the genotype of the NGF SHR locus may exert its influence on the NGF mRNA levels is by its effects on transcript stability. The half-life of NGF mRNA in astrocytes, Schwann cells, and fibroblasts has been shown to be 30 to 60 minutes. The half-life appears to be influenced by AU nucleotide–rich sequences including repetitive AUUUA sequences, present in the 3′-untranslated region (UTR) of the mRNA. The half-life of wild-type NGF mRNA with the 3′-UTR region averages 3 hours, whereas NGF mRNA without the AU-rich 3′-UTR region had a half-life of ∼9 hours. The rapid turnover of NGF mRNA, which acts to maintain a low level of production of the protein, may be retarded in the SHR. The finding that there is some heterogeneity of NGF transcript size raises the possibility that the difference in steady state mRNA levels may reflect differences in stability between the alternatively spliced transcripts. Sequencing of the NGF gene is required to determine the presence or absence of this region or its size in the SHR. It is also possible that the AU-rich 3′-UTR area interacts with other abnormal proteins in the SHR, which may affect the stability of NGF mRNA.

In summary, this study shows that the NGF locus implicated in BP regulation is linked to age-specific expression of

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**TABLE 2. Systolic BP in SHR, DRY, and F1 Rats and the 3 Genotype Groups in F2 Rats**

<table>
<thead>
<tr>
<th>Age, wk</th>
<th>DRY</th>
<th>F1</th>
<th>SHR</th>
<th>P*</th>
<th>DD</th>
<th>SD</th>
<th>SS</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>99</td>
<td>98</td>
<td>103</td>
<td>0.28</td>
<td>98</td>
<td>103</td>
<td>105</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>(96–103)</td>
<td>(87–109)</td>
<td>(97–110)</td>
<td>98</td>
<td>(93–102)</td>
<td>(99–108)</td>
<td>(99–111)</td>
<td>0.09</td>
</tr>
<tr>
<td>10</td>
<td>121</td>
<td>134</td>
<td>157</td>
<td>&lt;0.0001</td>
<td>134</td>
<td>137</td>
<td>145</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>(116–126)</td>
<td>(126–142)</td>
<td>(148–166)</td>
<td>(124–144)</td>
<td>(131–144)</td>
<td>(130–161)</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>127</td>
<td>157</td>
<td>201</td>
<td>&lt;0.0001</td>
<td>147</td>
<td>151</td>
<td>157</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Values are mean (95% CI), expressed in millimeters of mercury. *ANOVA.
NGF in the kidney of SHR. Comparisons of sympathetic nerve density of the cross-bred animals should provide further clues to the physiological importance of the NGF gene in SHR.

Acknowledgment
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References
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