Persistent Reduction in Renal Nerve Growth Factor mRNA After Perindopril Treatment of Young Spontaneously Hypertensive Rats

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

Abstract—Nerve growth factor (NGF) determines sympathetic innervation of target tissues, and NGF levels are increased in young spontaneously hypertensive rats (SHR). Angiotensin can affect NGF levels, and the persistent reduction in blood pressure after brief angiotensin-converting enzyme inhibition in young SHR may involve long-term changes in NGF and sympathetic innervation. We measured the relative abundance of renal NGF mRNA by reverse transcription–polymerase chain reaction in SHR during and after treatment from 6 to 10 weeks of age with vehicle, perindopril (3 mg/kg per day), the bradykinin B₂ antagonist Hoe 140 (0.5 mg/kg per day), both perindopril and Hoe 140, or angiotensin II (Ang II; 200 ng/kg per minute). Glomerular filtration rates were estimated at 10 and 20 weeks of age. At 10 weeks of age, Ang II caused a significant (P<.01) increase and perindopril caused a significant (P<.01) decrease in renal NGF mRNA levels. Blockade of the bradykinin B₂ receptor during perindopril treatment attenuated (P<.05) the reduction in NGF mRNA levels. Renal NGF mRNA (P=.05) and blood pressure (P<.001) remained significantly lower than control 10 weeks after perindopril treatment was stopped. The partial reduction in blood pressure at 20 weeks of age in rats that had received perindopril and Hoe 140 was not associated with any difference in renal NGF mRNA. Perindopril-induced long-term reduction in renal NGF mRNA levels may decrease sympathetic innervation and thereby contribute to the long-term posttreatment blood pressure reduction. (Hypertension. 1998;31:678-683.)

Key Words: nerve growth factor ■ angiotensin-converting enzyme ■ sympathetic nervous system ■ bradykinin ■ genetics

The renin-angiotensin¹ and sympathetic nervous²,³ systems contribute to the development of hypertension in the SHR. These two control systems also show significant interaction⁴ that may be relevant, particularly in the kidneys of young SHR, in which increased renin gene expression⁵ and heightened renal sympathetic nerve activity have been demonstrated.⁶,⁷

Treatment with the ACE inhibitor perindopril is followed by a long-term reduction in BP.¹,⁸ Surgical ablation of the renal sympathetic nerves has also been shown to prevent the development of hypertension in young SHR.⁹ ACE inhibition may have both functional and structural effects on the sympathetic nerves. For example, angiotensin can enhance sympathetic nerve transmission and ACE inhibitors have sympatholytic effects.¹⁰ In addition, angiotensin may modulate sympathetic innervation by effects on NGF in key tissues. Angiotensin increases the secretion of NGF from vascular smooth muscle cells in vitro,¹¹,¹² and Ang II receptor blockade reduces tissue levels of NGF.¹³

In most tissues, including the kidney, the density of sympathetic innervation correlates closely with tissue levels of NGF peptide¹⁴ and mRNA,¹⁵ both of which are intercorrelated in target tissues.¹⁶ It seems likely that the increased renal sympathetic innervation in SHR¹⁷ is related to the increased levels of NGF peptide and NGF mRNA that have been described in young SHR.¹⁸-²¹

Our hypothesis was that ACE inhibition, by reducing NGF in key tissues (ie, the kidneys) at a critical time (ie, in youth), would reduce renal sympathetic innervation in a long-term manner. In effect, early ACE inhibition would produce a partial pharmacological sympathectomy. To test this hypothesis, we measured renal NGF mRNA levels during and after treatment of young SHR with the ACE inhibitor perindopril. Renal levels of NGF mRNA have been shown to correlate with altered sympathetic innervation in SHR,²² and perindopril treatment causes a significant reduction in renal angiotensin peptide levels.²³ Because the persistent reduction in BP after ACE inhibition may also be explained in part by the accumulation of bradykinin during treatment,²⁴ the effect of bradykinin antagonism on renal NGF mRNA was also studied.

Methods

Animals

Four-week-old male SHR, originally derived from National Institutes of Health stock were obtained from an inbred colony in the Biological Research Laboratories, Austin Hospital, Melbourne, Australia. The SHR colony was subjected to regular tests with biochemical polymorphic markers to ensure its inbred status, which was confirmed...
Selected Abbreviations and Acronyms

ACE = angiotensin-converting enzyme
Ang II = angiotensin II
BP = blood pressure
GFR = glomerular filtration rate
NGF = nerve growth factor
PCR = polymerase chain reaction
SHR = spontaneously hypertensive rat(s)
TBE = Tris-boric acid and EDTA

recently using DNA minisatellite fingerprint markers and isozyme analysis.27 All animals were housed in groups of 3 to 4 per box and fed standard laboratory rat chow containing 0.1 mmol/g NaCl (Norco Rat and Mouse Cubes) and had unlimited access to drinking water. A 12-hour light/dark regimen was maintained throughout in a temperature-controlled room (22°C to 25°C). All experimental protocols were approved by the Austin Hospital Animal Ethics Committee and the University of Melbourne Animal Experimentation Ethics Committee.

Treatments and BP Measurement

Short-term Studies to 10 Weeks of Age

Five groups of male SHR were treated from 6 to 10 weeks of age with one of the following: (1) water by gavage once per day (n=6), (2) perindopril dissolved in water (3 mg/kg per day) by gavage once per day (n=6), (3) Ang II (200 ng/kg per minute) dissolved in 150 mmol/L NaCl delivered subcutaneously by osmotic minipumps (Alzet) (n=6), (4) Hoe 140 (0.5 mg/kg per day; n=6) dissolved in saline delivered subcutaneously by osmotic minipumps, or (5) perindopril (3 mg/kg per day) in combination with Hoe 140 (0.5 mg/kg per day; n=6). The maximum volume of gavage was 0.5 mL. The efficacy, dose, and route of administration of perindopril, Ang II, and Hoe 140 were based on previously published experiments.27

Long-term Studies to 20 Weeks of Age

In the long-term experiment, four groups of male SHR were also treated from 6 to 10 weeks of age with one of the following: (1) water by gavage once per day (n=6), (2) perindopril (3 mg/kg per day) by gavage once per day (n=6), (3) Hoe 140 (0.5 mg/kg per day; n=6), or (4) perindopril (3 mg/kg per day) in combination with Hoe 140 (0.5 mg/kg per day; n=6). Tail BP (BP) was measured twice per week in conscious animals using a photoelectric tail-cuff pulse-detection system (IITC Inc). The sizes of the restrainer cylinders and cuffs were matched to those of the growing animals. Before the experimental period, the rats were conditioned to the restraining cylinders and the BP measurement. Body weight was measured weekly.

GFR

GFR was measured in all animals using the single-shot radiolabeled DTPA method.28 A calibrated dose of technetium reduced with stannous chloride complexed to DTPA (Sigma Chemical Co) was injected into the tail vein of conscious rats. After 43 minutes, a blood sample was taken from a different tail vein and centrifuged in a heparinized tube. Plasma radioactivity was counted in a gamma counter and compared with a reference prepared at the time of injection. GFR was calculated according to the following equation:

\[ \text{Clearance} = \frac{V \times \ln(P_{i}/P_{f})}{t} \]

where V is volume of distribution, P is theoretical plasma concentration at injection (ie, injected amount/volume of distribution), and P, is observed plasma concentration at t minutes after injection.28 GFR was measured at 10 weeks of age in short-term studies and at 20 weeks of age in long-term studies.

Isolation of RNA and Reverse Transcription

GFR and BP measurements were followed by an overdose of barbiturate (100 mg/kg Nembutal IP, Boehringer) and removal of both kidneys, which were weighed and then immediately frozen in liquid nitrogen and stored at −70°C until further use. Total RNA was isolated from 100 to 200 mg of kidney tissue using the acid phenol method of Chomczynski and Sacchi.27 Total RNA was quantified spectrophotometrically, and purity was assessed from the A260/A280 ratio (minimum 1.7). First-strand cDNA was synthesized from 5 μg of total RNA using a cDNA synthesis kit (Gibco BRL) by the random-primed avian myeloblastosis virus reverse transcriptase method.

PCR Amplification

Oligonucleotide primers were synthesized and purified (Gibco BRL), having been designed according to the nucleotide sequences encoding rat NGF and GAPDH. GAPDH amplification was carried out to provide internal standards for relative quantification of NGF transcripts in the isolated total RNA. The base sequences (5’ to 3’) of the oligonucleotides used for amplification of NGF (primer 1: AAG GATCCTGGACCCCAAGCTCACCTA; primer 2: GAGT GACGTGGATGACGCGTCTGCTCC) and GAPDH (primer 1: ATCACTGACCCTCAGAAGACT; primer 2: CATGGCAGT GAGCTTCGGGT) were chosen to amplify products that crossed exon-intron boundaries and thus preclude the amplification of potentially contaminating genomic DNA. cDNA equivalent to 0.25 μg of total RNA was amplified in a 25-μL reaction volume containing 1.5 mmol/L MgCl₂, 50 μmol/L dNTP, 5 U of Taq (Gibco BRL), and primers (0.5 μmol/L each) (Ultra Pure, Pharmacia). Taqstart antibody (Clonetech) was included to reduce nonspecific amplification. The cycling protocol (PE 480 thermal cycler, Perkin-Elmer) consisted of an initial denaturation (95°C, 5 minutes) followed by sequential cycles of denaturation (95°C, 1 minute), annealing (60°C, 1 minute), and extension (72°C, 1 minute) followed by a final extension stage (72°C, 7 minutes). The cycle numbers for NGF (30) and GAPDH (23) were chosen to ensure that both reactions were in the exponential phase. The two gene amplifications were carried out in separate reactions and repeated four times for each cDNA sample to limit the tube-to-tube variation of PCR amplification.

Controls included the omission of the reverse transcription step before PCR amplification and the inclusion of water blanks as negative controls for the detection of contamination. In parallel with each PCR amplification, a standard curve was constructed using increasing amounts of kidney cDNA to ensure that the amplification process was in linear relationship to the amount of input cDNA. The reproducibility of the quantitative amplifications was evaluated in replicate (n=10) PCR amplifications. The specificity of the PCR products was verified by Southern blot hybridization performed with end-labeled oligonucleotide probes internal to the primers.

Analysis of the cDNA-PCR-Amplified Products

A 10-μL aliquot from each PCR reaction was electrophoresed in 1.5% agarose gels at 10 V/cm and stained with ethidium bromide (0.25 μg/mL TBE buffer) for 20 minutes and destained for another 10 minutes. Control and treatment samples were run concomitantly and in different positions on the gel to reduce potential variation caused by electrophoretic and staining artifacts. Therefore, each sample was divided into four replicates that were measured on the same gel. Measurement of fluorescence was performed using a laser scanner (FluorImager 575, Molecular Dynamics) and was followed by quantification using the Genequant software. All results were expressed as ratios of the intensity of the band of the NGF product to the intensity of the band representing GAPDH after subtraction of background fluorescence. To account for intra-assay variation, each of the four replicates was included in the analysis of individual rats and treatment groups.

Statistical Analyses

Descriptive statistics are mean±SEM unless stated otherwise. ANOVA was used to test differences in tail BP, body weight, and GFR between the different treatment groups in the short-term and long-term studies separately. Between-group differences were tested using the Student-Newman-Keuls range test. NGF mRNA levels were analyzed initially by repeated measures ANOVA using each the
### Results

**Short-term Study**

The average weekly body weights for each of the five groups in the short-term study revealed that before and during treatment all animals grew normally and there were no significant differences \((P=.243)\). Average tail BP values are shown in Fig 1 for the short-term study. Control SHR showed a steady rise in BP, characteristic of the developmental phase of hypertension. Their average tail BP at 10 weeks of age was 193±5 mm Hg. Ang II–treated rats showed a significant increase in BP \((P<.001)\) at 10 weeks of age with an average of 240±3 mm Hg. Hoe 140 treatment alone produced no significant change in BP compared with the control rats \((182±4 \text{ mm Hg})\). Perindopril treatment decreased BP significantly below that of control rats \((145±2 \text{ mm Hg})\). The combination of Hoe 140 and perindopril treatment resulted in average BPs similar to those of the perindopril-treated rats \((148±2 \text{ mm Hg})\).

**NGF mRNA Levels**

Fig 2 shows representative images of NGF and GAPDH PCR products from kidney cDNA as scanned by the FluorImager. The sizes of PCR products were 440 bp for NGF and 220 bp for the GAPDH product. Southern blot analysis using oligonucleotide probe internal to the PCR primers (data not shown) confirmed the identity of these products. Relative abundance of renal NGF mRNA in the five treatment groups after correction for GAPDH is given in Table 1.

There were no significant differences between replicates for each animal as analyzed by repeated measures ANOVA \((P=.39)\). The NGF mRNA levels were highest in the Ang II–treated rats \((P<.01)\) and lowest for the perindopril-treated rats \((P<.01)\) at 10 weeks of age compared with untreated SHR. Hoe 140 treatment had no significant effect on NGF mRNA levels. However, treatment with both Hoe 140 and perindopril resulted in an intermediate level of NGF mRNA significantly different from both control \((P<.05)\) and perindopril–treated \((P<.05)\) animals (Table 1). No correlation was observed between NGF mRNA levels and BP at 10 weeks of age in any group.

### Renal Function

Table 2 shows the average GFR values, which were significantly different \((P<.0001, \text{ ANOVA})\) between the treatment groups at 10 weeks of age. Perindopril-treated SHR showed significantly higher GFR values than any other group. The GFRs of animals receiving both Hoe 140 and perindopril, Hoe 140 alone, or Ang II were significantly lower than those of controls. No correlation was observed between NGF mRNA levels and GFR at 10 weeks of age in any group.

### Long-term Study

The average weekly body weights of the four groups in the long-term study were not significantly different before, during, or after treatment. During treatment, the tail BPs showed changes similar to those seen in the short-term experiment (Fig 3). All rats showed an increase in BP in the 2 weeks after treatment was stopped, but the magnitude of increase varied between groups. Between 11 and 12 weeks of age, the tail BP of control animals rose on average by 7.5 mm Hg compared with an average rise of 39 mm Hg in the rats that had been treated with both Hoe 140 and perindopril. The pressure increases in the SHR treated with either perindopril or Hoe 140 were of a magnitude similar to that of the control group.

### Table 1. Average Abundance of Renal NGF mRNA at 10 and 20 Weeks of Age

<table>
<thead>
<tr>
<th>Age</th>
<th>Control</th>
<th>Hoe 140</th>
<th>Perindopril + Hoe 140</th>
<th>Perindopril</th>
<th>Ang II</th>
<th>(P) (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 Weeks</td>
<td>0.79±0.06</td>
<td>0.75±0.03†</td>
<td>0.66±0.06†</td>
<td>0.53±0.04*</td>
<td>0.90±0.04†</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>20 Weeks</td>
<td>0.60±0.08</td>
<td>0.61±0.07†</td>
<td>0.63±0.10†</td>
<td>0.46±0.03*</td>
<td>—</td>
<td>.005</td>
</tr>
</tbody>
</table>

Data are expressed as the ratio of fluorescence output NGF/fluorescence output GAPDH and corrected for background fluorescence.

* \(P<.05\) compared with control SHR; † \(P<.05\) compared with perindopril-treated SHR.
Between 13 and 20 weeks of age, the control SHR and Hoe 140–treated groups showed a steady rise in BP, although at a slower rate compared with that at 6 to 10 weeks of age. SHR treated with perindopril alone showed a very slow rise in BP between 13 and 20 weeks of age and at 20 weeks of age had a BP (average, 165±1.1 mm Hg) significantly lower than all other groups (P<.0001, ANOVA). At 20 weeks of age, the tail BP of rats that had been treated with perindopril plus Hoe 140 (average, 189±1.5 mm Hg) was significantly higher than that of the rats treated with perindopril alone but also significantly lower than that of the controls (average, 209±3.0 mm Hg) and SHR that received Hoe 140 alone (average, 208±1.6 mm Hg) (P<.0001, ANOVA).

NGF mRNA Levels

Compared with the levels seen at 10 weeks of age, the relative abundance of renal NGF mRNA in control SHR was significantly lower (P<.05 by independent t test) in the 20-week-old control SHR. The SHR that received perindopril between 6 and 10 weeks of age showed significantly lower levels of NGF mRNA expression (P=.005, ANOVA) than the other three treatment groups (Table 1). Hoe 140 and perindopril plus Hoe 140 treatments showed no significant difference in NGF mRNA levels compared with the control group. No correlation was observed between NGF mRNA levels and BP at 20 weeks of age in any group.

Renal Function

At 20 weeks of age, the perindopril-treated rats showed the highest average GFR, but ANOVA revealed marginal statistical significance for this result (P=.055, ANOVA). Renal NGF mRNA levels did not correlate with GFR at 20 weeks of age.

Discussion

This study reveals that the reduction in BP that occurs during and after perindopril treatment in young SHR is associated with a significant reduction in the relative abundance of renal NGF mRNA. The decrease in NGF gene expression may be an important component in the short-term and long-term effects of ACE inhibition in this strain.

Previous studies have emphasized the potential importance of NGF in the development and maintenance of high BP in the SHR. Both NGF mRNA and peptide levels are significantly higher in SHR than in normotensive strains in key tissues, including the kidneys and resistance vessels.30–34 Because NGF levels have been shown to correlate closely with the degree of sympathetic innervation,7,17,28–32 it seems likely that NGF in SHR contributes to the increased sympathetic innervation and activity that are evident in histological, electrophysiological, and biochemical experiments.2,17,28–32 The prevention of hypertension by administration of anti-NGF antibodies to young SHR is also consistent with an etiologic role for NGF in this genetic model of hypertension.33

Our results indicate that angiotensin exerts an important control over renal NGF expression. The relative abundance of NGF mRNA in young SHR was increased by angiotensin infusion and decreased by ACE inhibition. These findings extend previous results in other studies in which exogenous angiotensin increases NGF production,11,12 whereas AT1 angiotensin receptor antagonism decreases NGF levels.13

The most interesting observation was that brief treatment with perindopril was followed by a persistent and significant reduction in renal NGF mRNA. The findings imply that perindopril in some way resets renal NGF gene expression in young animals and that this effect continues into adulthood. The consequences of such downregulation are likely to affect sympathetic innervation in the kidney. NGF plays a central role as a trophic signal from tissues to the sympathetic nerves.34 It is important particularly in the perinatal period, when levels of NGF determine the degree of neuronal apoptosis34 and thereby set the degree of sympathetic innervation of the target tissues. NGF is also important in maintaining sympathetic innervation of target tissues into adult life.34 Although direct studies of innervation were not performed in these experiments, the correlation among NGF mRNA,15 NGF peptide,14 and sympathetic innervation in target tissues is well established.34 Therefore, it seems likely that brief ACE inhibition, by resetting renal NGF gene expression, may induce partial renal sympathectomy. This may explain the similarity in the BP effects of surgical renal denervation9 and brief ACE inhibition in young SHR.

Figure 3. Average biweekly tail blood pressures of four groups of male SHR between 6 and 20 weeks of age. Data are mean±SEM. Treatment between 6 and 20 weeks of age consisted of vehicle controls (C), perindopril (●), Hoe 140 (▲), or perindopril plus Hoe 140 (■).

### Table 2. Average GFR Values of Different Treatment Groups at 10 and 20 Weeks of Age

<table>
<thead>
<tr>
<th>Age</th>
<th>Control</th>
<th>Hoe 140</th>
<th>Perindopril+Hoe 140</th>
<th>Perindopril</th>
<th>Ang II</th>
<th>P (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 Weeks</td>
<td>12.0±0.5</td>
<td>9.2±0.4†</td>
<td>9.3±0.6†</td>
<td>13.6±0.2†</td>
<td>9.3±0.8†</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>20 Weeks</td>
<td>8.5±0.5</td>
<td>8.5±0.7</td>
<td>9.6±0.5</td>
<td>10.6±0.6</td>
<td>...</td>
<td>.055</td>
</tr>
</tbody>
</table>

Data are expressed as milliliter per minute per kilogram.

*P<.05 compared with control SHR; †P<.05 compared with perindopril-treated SHR.
Perindopril and Nerve Growth Factor in SHR

The timing of ACE inhibitor treatment in relation to NGF effects may also be important. The results from this experiment show that the relative abundance of NGF mRNA was highest in young animals and decreased with age in untreated SHR. Similar findings have been reported by other investigators. It seems also that the increase in NGF occurs only in certain tissues. In young SHR, the kidneys, spleen, blood vessels, and sympathetic nerves, but not the heart, show high NGF levels. This tissue-specific and developmental stage-specific increase of NGF in SHR may not only establish high levels of sympathetic innervation but also define a window during which ACE inhibitor treatment has an effect that is perpetuated into later life.

Our study raises interesting questions about the control of NGF gene expression in the kidney. The explanation for developmental stage-specific changes of NGF in SHR is not known, but altered transcriptional control of the NGF gene may be the result of genetic mutation in gene regulatory regions. We have identified linkage between the NGF gene and the inheritance of high BP in genetic crosses of SHR, although functional mutations have not yet been identified. It is possible that mutations in other genes have an impact on NGF gene expression. For example, increased renal renin gene expression may modulate tissue angiotensin and raise NGF mRNA in young SHR.

In addition to the influence of angiotensin, our findings also indicate that bradykinin may be important, at least in the pharmacological actions of perindopril. The accumulation of bradykinin during treatment may contribute to the reduction of renal NGF mRNA. Blockade of the bradykinin B2 receptor with Hoe 140 during perindopril treatment significantly attenuated the reduction of NGF mRNA observed with perindopril alone. However, the role of bradykinin may not be relevant to normal physiology because the administration of Hoe 140 alone had no significant effect on basal SHR NGF mRNA levels.

Interestingly, the effects of bradykinin accumulation on NGF mRNA appear relevant only to the treatment period and not to the long-term resetting of renal NGF mRNA. This is in contrast with the BP effects, in which bradykinin does not appear to contribute to lower pressure during perindopril treatment but is partially responsible for the long-term reduction in pressure after perindopril. Clearly, the mechanisms of BP reduction differ during and after treatment. Notably, the partial reduction in long-term BP in rats that had received perindopril and Hoe 140 was not associated with any change in renal NGF mRNA. Presumably, not all of the long-term effects of ACE inhibitors are related to changes in renal NGF. However, our findings suggest that the additional pressure reduction observed in the perindopril-treated rats is related to the lower NGF mRNA.

Given that Ang II increases and perindopril decreases both BP and NGF mRNA, it could be argued that changes in NGF mRNA are simply the result of changes in BP. However, our results argue against such a generality. First, at 10 weeks of age, SHR receiving perindopril plus Hoe 140 had the same BPs but significantly higher NGF mRNA levels than rats treated with perindopril alone. Second, at 20 weeks of age, SHR that had been treated with both perindopril and Hoe 140 had the same NGF mRNA levels but significantly lower BPs than control SHR. Third, there was no significant correlation in any individual group between renal NGF mRNA and BP. Finally, the increase in BP observed in untreated SHR was accompanied by a fall in renal NGF mRNA.

We have described previously that perindopril treatment is associated with an increase in GFR in young SHR. The present study demonstrated significantly elevated GFR at 10 weeks but no significant change in the long term, possibly because of a shorter ACE inhibitor treatment period. Although we observed opposite changes in NGF mRNA and GFR in SHR treated with perindopril and Ang II, there was no correlation between renal NGF mRNA and GFR. The significant changes in NGF mRNA levels in the absence of GFR alteration in the SHR receiving perindopril plus Hoe 140 also indicates that the link between these renal molecular and functional characteristics is not absolute.

In summary, these studies raise some interesting questions regarding ACE inhibition and the expression of the NGF gene in SHR. They also indicate the importance of treatment at an early age and its long-term effect on adult renal NGF gene expression and BP.

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References


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