Elevated Nerve Growth Factor Levels in Young Spontaneously Hypertensive Rats

Susan J. Donohue, Richard J. Head, and Robert E. Stitzel

It is generally agreed that sympathetic innervation of vascular tissues in spontaneously hypertensive rats (SHR) is greater than that existing in vascular tissues from normotensive Wistar-Kyoto (WKY) rats. One factor responsible for regulation of the growth of peripheral sympathetic nerves is the peptide nerve growth factor, which is released from effector cells. In the present study, an enzyme immunoassay was used to measure nerve growth factor levels in mesenteric arteries (densely innervated) and aortas (sparingly innervated) from both young (20-day-old) and mature (6-month-old) SHR and WKY rats. The nerve growth factor content of mesenteric arteries and aortas from 20-day-old SHR was significantly greater than that present in corresponding tissues from WKY rats. In contrast, the nerve growth factor content found in mesenteric arteries and aortas of adult SHR did not differ significantly from that found in the corresponding adult WKY rat tissues. Moreover, when the tissues were obtained from adult animals, nerve growth factor levels were substantially higher in mesenteric arteries compared with aortas, regardless of the rat strain. These results support the hypothesis that the greater nerve growth factor content of vascular tissues from young SHR is involved in the early increased sympathetic innervation of blood vessels in this animal model of hypertension.

(Hypertension 1989; 14:421–426)

It is now well established that the sympathetic innervation of selected vascular beds in spontaneously hypertensive rats (SHR) is greater than that prevailing in vessels from normotensive Wistar-Kyoto (WKY) rats.1–4 In the mesenteric vascular bed of the SHR the enhanced innervation occurs quite early during the time course of innervation of this vessel.5,6

There is general agreement that the growth of sympathetic nerves in the periphery is regulated by the trophic peptide nerve growth factor (NGF).7–10 Injection of antiserum against NGF (anti-NGF) into neonatal mice or rats results in the degeneration of sympathetic ganglia,11 as well as an irreversible decrease in the activity of the norepinephrine-synthesizing enzymes tyrosine hydroxylase and dopamine β-hydroxylase.12,13 In addition, NGF and NGF messenger RNA (mRNA) levels of target tissues have been shown to correlate with the density of sympathetic innervation to the tissue.14–16 It is, therefore, possible that the increased sympathetic innervation found in blood vessels in the SHR may be the result of an early exposure of these tissues to an elevated endogenous level of NGF.

To investigate this hypothesis, we have developed a sensitive and specific enzyme immunoassay for the measurement of NGF in vascular tissue. The procedure uses a monoclonal antibody and a galactosidase-conjugated form of the antibody against mouse NGF. Focus was directed toward measuring the levels of NGF in mesenteric arteries and aortas from both young (i.e., during the initial period of sympathetic innervation) and mature (i.e., when innervation is already established) SHR and normotensive WKY rats.

Materials and Methods

Animals and Tissue Removal

SHR and WKY rats were obtained from Taconic Farms (Germantown, New York). To verify that the SHR were indeed hypertensive, the blood pressures of adult animals were routinely measured with a Narco (Narco Bio-Systems, Houston, Texas) tail-cuff indirect blood pressure apparatus. Young (20-
day-old) and mature (6-month-old) SHR and WKY rats were decapitated and vascular tissues rapidly removed. Superior mesenteric arteries, including the primary arterial branches and entire aortas, were then excised and adhering tissue removed. Tissues were briefly rinsed in homogenization buffer (50 mM Tris, 400 mM NaCl, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 10 mM 6-aminohexanoic acid, and 1 mM N-ethylmaleimide, pH 7.4), blotted on dampened filter paper, and quickly frozen by placing the tissues on an aluminum foil-covered block of dry ice. The tissues were then stored at −70°C until assayed 1–5 days later. Approximately 2 volumes of homogenization buffer containing 1% bovine serum albumin (BSA) was added to tubes for mesenteric arteries. Approximately 10 volumes of buffer was added to tubes for aortas. After the tubes were weighed, the frozen tissues were placed in the buffer, and the weight of the tissues was determined.

**Tissue Homogenization**

Tissues were homogenized with a motorized (glass/glass) homogenizer. The homogenizer was then rinsed with the 1% BSA/homogenization buffer, and the rinse solution added to the homogenate. The final volumes of buffer for the mesenteric artery and aorta were 4 and 20 volumes, respectively. An aliquot of each homogenate was then added to tubes containing authentic 2.5S NGF (95% purity, Collaborative Res., Inc., Bedford, Massachusetts) in 1% BSA/homogenization buffer. The final concentration of CaCl₂ was 10 mM. The monoclonal antibody was allowed to coat the wells overnight at 4°C. The monoclonal antibody solution (60 μg/ml) diluted 1:499 with 5% BSA in Tris-saline. The nitrocellulose was then rinsed twice with Tris-saline and incubated for 1.5 hours at room temperature with peroxidase-conjugated goat antimouse immunoglobulin (Ig) diluted 1:499 with 5% BSA in Tris-saline. The nitrocellulose paper was subsequently washed four times (each wash 15 minutes) with Tris-saline. The peroxidase substrate was prepared immediately before use by addition of 2 ml of a 3 mg/ml solution of 4-chloro-1-naphthol in methanol and 5 μl of 30% H₂O₂ to 9 ml Tris-saline. The reaction was stopped by removal of the substrate and addition of 0.95 mM NaH₂PO₄, 8.1 mM Na₂HPO₄, 0.9% NaCl, and 0.05% azide. The migration of 7S NGF was compared with that of endogenous NGF isolated from vascular tissue.

To perform the enzyme immunoassay, the monoclonal antibody solution (60 μg/ml) was diluted to 0.5 μg/ml with 50 mM bicarbonate/carbonate buffer, pH 9.6, and 100 μl was added to wells in a 96-well Titertek plate (Flow Laboratories, McLean, Virginia). The monoclonal antibody was allowed to coat the wells overnight at 4°C. The samples and standards were prepared as described above. Two hours before sample addition, the antibody solution was removed and the wells rinsed five times with 150 μl of 50 mM Tris, 200 mM NaCl, pH 7.4 (TBS). Two hundred microliters of 1% BSA in TBS was then added to each well and the plate was placed in an oven for 2 hours at 37°C.

After the plate was washed five times with TBS, 100 μl of sample (assayed in duplicate) or NGF standard (assayed in triplicate) was added to each well. To determine the background interference in the assay, blank wells were also prepared. In blank wells, 1% BSA in homogenization buffer was added instead of a sample. The plate was then placed at 4°C overnight.

The galactosidase-conjugated monoclonal antibody solution was diluted 1:19 with 1% BSA in TBS, and 100 μl was added to each well after the plate was washed five times. The plate was then incubated for 4 hours at 37°C. A substrate solution for galactosidase was prepared by mixing 10 mg chlorophenol-red galactopyranoside with 5 ml of 100 mM HEPES, 150 mM NaCl, 2 mM MgCl₂, and 0.03% azide, pH 7.0, for 10 minutes. The plate was washed three times, and then 100 μl of substrate was added to each well at 10-second intervals. The plate was allowed to incubate for approximately 1.5 hours at 37°C before the absorbance at 570 nm (EL-308 EIA reader, Bio-Tek, Burlington, Vermont) was recorded for each well at 10-second intervals.

To further characterize the specificity of the antibody, the absorbance readings for 1 μg epidermal growth factor (EGF), 1 μg fibroblast growth factor (FGF), and 1 μg insulin were compared with the
absorbance value for 100 pg NGF. This comparison was performed before any samples were assayed.

Calculations and Statistical Analysis

The NGF content of the samples (A) was determined by plotting the absorbance values of each sample against an NGF standard curve. The amount of endogenous NGF in each recovery sample (B) was calculated from the NGF content of the corresponding sample. The concentration of exogenously added NGF was adjusted after taking into account the volume due to particulate matter in the homogenate. Percent recovery was then calculated as follows:

\[
\text{total NGF in recovery sample} - B
\text{pg of exogenously added NGF} = \% \text{ recovery}
\]

Final NGF content of the samples was calculated as follows:

\[
\frac{A \times \text{dilution factor}}{\text{mg of tissue} \times \% \text{ recovery}} = \text{ng/g tissue}
\]

Statistical analysis was performed using an unpaired t test.

Chemicals

IgG1 monoclonal antibody against NGF and its galactosidase conjugate, chlorophenol-red galactopyranoside and leupeptin were purchased from Boehringer Mannheim Biochems. FGF, EGF, 4-chloro-1-naphthol, and 6-aminohexanoic acid were purchased from Sigma Chemical Co. (St. Louis, Missouri). Insulin was purchased from Eli Lilly and Co. (Indianapolis, Indiana), goat anti-mouse Ig was purchased from Southern Biotechnology (Birmingham, Alabama), 7S NGF and 2.5S NGF were purchased from Collaborative Research (Bedford, Massachusetts), and nitrocellulose paper (BA83, 0.2 m) was purchased from Schleicher and Schuell (Keene, New Hampshire).

Results

Specificity of the Immunoassay for Nerve Growth Factor

Two methods were used to determine the specificity of the antibody. First, an enzyme immunoassay was performed with samples of NGF-related growth factors. The latter included insulin, FGF, and EGF. On completion of the assay, the absorbance readings generated by the growth factors were compared with the absorbance reading for the NGF standard (Table 1). The results indicate that the absorbance values for insulin and FGF are indistinguishable from the assay background. When the monoclonal antibody was incubated with 1 μg EGF, the absorbance reading was slightly less than that obtained for 100 pg NGF. In other words, the monoclonal antibody had no affinity for insulin or FGF and exhibited a 43,000-fold higher affinity for NGF than for EGF. It should be noted that the molecular weight of EGF is 6,045 Da, whereas the molecular weight of β-NGF is 26,000 Da.

Secondly, it was necessary to determine whether the monoclonal antibody bound nonspecifically to proteins present in supernatants obtained from mesenteric arteries and aortas from young and adult SHR and WKY rats. As shown in Figure 1, the monoclonal antibody appeared to recognize a triplet protein from 7S NGF. The most strongly labeled

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
<th>Absorbance units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank (BSA)</td>
<td>...</td>
<td>0.256</td>
</tr>
<tr>
<td>Insulin</td>
<td>1 μg</td>
<td>0.272</td>
</tr>
<tr>
<td>Fibroblast growth factor</td>
<td>1 μg</td>
<td>0.270</td>
</tr>
<tr>
<td>Epidermal growth factor</td>
<td>1 μg</td>
<td>2.678</td>
</tr>
<tr>
<td>Nerve growth factor</td>
<td>100 pg</td>
<td>2.780</td>
</tr>
</tbody>
</table>

The conditions for the analysis presented in this table are described in detail in the Materials and Methods section. BSA, bovine serum albumin.

FIGURE 1. Specificity of an anti-nerve growth factor (NGF) monoclonal antibody. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15% gel), the proteins were electroblotted onto nitrocellulose paper, and NGF was detected by immunolocation with a monoclonal antibody against NGF and peroxidase-conjugated goat anti-mouse immunoglobulin (Ig). Lanes A and H, prestained molecular weight standards; lane B, 5 μg 2.5S NGF; lane C, 10 μg 7S NGF; lane D, pooled mesenteric arteries from four male spontaneously hypertensive rats (SHR) and four male Wistar-Kyoto (WKY) rats at 11 weeks of age; lane E, pooled aortas from young and adult SHR rats; lane F, pooled mesenteric arteries from four male spontaneously hypertensive rats (SHR) and four male Wistar-Kyoto (WKY) rats at 15 days of age (male and female); lane G, pooled aortas (source of tissues same as lane D); and lane E, pooled aortas (source of tissues same as lane E).
protein from the tissue supernatants also appeared as a triplet, and the protein migrated the same distance through the polyacrylamide gel as did the labeled protein from 7S NGF. All of these proteins had an apparent molecular weight of approximately 26 kDa.

Although additional faint protein bands are also visible in the figure, it should be noted that these bands were not visible on the original nitrocellulose paper. Figure 1 also shows that the antibody did not react with 2.5S NGF. The reason for this will be discussed later. Collectively, the analysis indicates a high degree of specificity of the monoclonal antibody for NGF in the vascular tissues.

**Nerve Growth Factor Content in Vascular Tissues**

After it was established that the monoclonal antibody was specific for NGF in vascular tissues from the rat, NGF levels were measured in mesenteric arteries and aortas from SHR and WKY rats at 20 days or 6 months of age. The percent recovery of exogenously added NGF was used in the calculations to determine the amount of NGF present in the tissue supernatants. The average recovery values are shown in Table 2. Due to the limited amount of sample available for recovery determinations, these values were not obtained for aortas from 20-day-old rats.

The results from the enzyme immunoassay are illustrated in Table 3. The NGF content of the mesenteric artery and aorta in 20-day-old SHR was approximately 50% greater than the levels found in the corresponding tissues from age-matched WKY rats. These differences were not maintained, however, into adult life. In other words, the NGF content of mesenteric arteries and aortas from 6-month-old SHR did not differ significantly from the NGF content of their corresponding WKY rat tissues. In addition, the NGF content of mesenteric arteries from both adult rat strains was 12–15 times higher than the levels found in the aortas. The NGF content of adult SHR and WKY rat aortas was only about 10% of that in the 20-day-old rats.

**Discussion**

The stimulus for the present study came from the repeated observations that the extent of sympathetic innervation of vascular tissues in the SHR is increased. Because NGF plays a pivotal role in the survival and differentiation of sympathetic nerves in peripheral tissues (for review, see References 7, 9), it was viewed as important to examine the concentrations of the trophic peptide in blood vessel preparations from the SHR. To achieve this end, we established a sensitive two-site immunoassay for NGF by using a galactosidase-conjugated monoclonal antibody against NGF.

We demonstrated by immunolocalization that the monoclonal antibody reacted primarily with three proteins in the 7S NGF sample. The triplet had an apparent molecular weight of approximately 26 kDa. Importantly, an identical pattern was observed for the tissue supernatants. It is unlikely that the tissues contained three non-NGF proteins with identical molecular weights to β-NGF and possessed the specific epitopes for antibody recognition. Therefore, the triplet proteins recognized by the monoclonal antibody used in this study most likely represent intact and modified β-NGF. An octapeptide is cleaved at the NH₂-terminus of 50% of the β-NGF chains during the preparation of 2.5S NGF.18 Consequently, the β-NGF dimer can have three molecular weights [intact, des(1–8)-NGF, and bisdes(1–8)-NGF] depending on the extent of proteolysis.

It should be noted that on the original nitrocellulose paper only one or two faint protein bands were visible in addition to the "triplet." The film used to photograph the nitrocellulose is a high contrast

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**Table 2. Percent Recoveries of Exogenously Added Nerve Growth Factor to Vascular Tissue Supernatants**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Strain</th>
<th>20 days</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesenteric artery</td>
<td>WKY</td>
<td>41±2 (6)</td>
<td>76±7 (6)</td>
</tr>
<tr>
<td></td>
<td>STR</td>
<td>60±4 (6)</td>
<td>88±2 (5)</td>
</tr>
<tr>
<td>Aorta</td>
<td>WKY</td>
<td>ND</td>
<td>34±2 (6)</td>
</tr>
<tr>
<td></td>
<td>STR</td>
<td>ND</td>
<td>37±2 (6)</td>
</tr>
</tbody>
</table>

Mean percent recovery±SEM was calculated as described in the Materials and Methods section. The values in parentheses indicate the number of litters used (20 days) or the number of rats used (6 months). WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; ND, not determined.

**Table 3. Nerve Growth Factor Content of Vascular Tissues From Spontaneously Hypertensive Rats and Wistar-Kyoto Rats**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Strain</th>
<th>20 days</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesenteric artery</td>
<td>WKY</td>
<td>1.08±0.14 (6)</td>
<td>2.31±0.42 (6)</td>
</tr>
<tr>
<td></td>
<td>STR</td>
<td>1.72±0.04 (6)*</td>
<td>1.70±0.19 (6)</td>
</tr>
<tr>
<td>Aorta</td>
<td>WKY</td>
<td>1.23±0.07 (6)</td>
<td>0.15±0.02 (6)</td>
</tr>
<tr>
<td></td>
<td>STR</td>
<td>1.76±0.19 (6)*</td>
<td>0.14±0.02 (6)</td>
</tr>
</tbody>
</table>

Mean values±SEM are expressed as nanograms per gram of tissue and were calculated for 100% recovery. Average percent recovery obtained for aortas from 6-month-old Wistar-Kyoto rats (WKY) (34.7%) or spontaneously hypertensive rats (SHR) (37.1%) was used to calculate the values for aortas from 20-day-old rats. Values in parentheses indicate the number of litters used (20 days) or the number of rats used (6 months).

*Significantly different from corresponding tissue for WKY rats (p<0.05).
graphic arts film (Kodaline, Kodak, Rochester, New York) that is highly sensitive to the blue-green color spectrum and, therefore, was able to detect protein bands otherwise not visible. Consequently, our results show that although the monoclonal antibody did bind to several proteins in the tissue supernatants and the 7S NGF preparation, the cross-reactivity was negligible compared with the affinity for NGF.

An additional point is that the monoclonal antibody did not recognize 2.5S NGF or the NGF monomer (molecular weight 13 kDa). We have found that 2.5S NGF is completely dissociated after SDS-PAGE. In other words, there are no protein bands at 26 kDa; however, two bands are detected at 13 kDa after silver staining of the gel. The 7S preparation consists of several protein bands, including those at 26 kDa and a very prominent double band at 13 kDa. Greene et al. have shown that β-NGF slowly dissociates in the presence of SDS (t1/2 = 2 hours). Together, these results suggest that, under the conditions used for SDS-PAGE, the purified β-NGF dimer dissociated more rapidly than when the β subunit was initially part of the 7S complex. The monoclonal antibody probably recognizes an epitope formed by both chains because the antibody bound to the 26 kDa dimer but did not recognize the 13 kDa monomer. A recent report showed similar results with a polyclonal antibody against NGF purchased from Collaborative Res., Inc.

Further support for the specificity of the monoclonal antibody came from the results of studies in which the reactivity of the monoclonal antibody toward selected growth factors was determined. It was established that the antibody did not react with insulin and FGF. In addition, the antibody displayed at least a 40,000-fold greater affinity for NGF than it did for EGF. Finally, it should be noted that the antibody probably recognizes an epitope formed by both chains because the antibody bound to the 26 kDa dimer but did not recognize the 13 kDa monomer. A recent report showed similar results with a polyclonal antibody against NGF purchased from Collaborative Res., Inc.

The present study showed a greater concentration of NGF in aortas and mesenteric arteries from 20-day-old SHR compared with the concentrations present in blood vessels from normotensive WKY rats. In a preliminary report, we established that in young SHR the mRNA levels for NGF are also elevated. In another study, we showed that increased vascular innervation in SHR occurs during the normal time course of innervation of blood vessels (i.e., between 2 and 40 days of age). In addition, NGF has been shown to play a crucial role during the development of innervation. For example, sympathetically innervated organs become innervated with sympathetic nerves after the injection of NGF into newborn mice or rats. In contrast, injection of anti-NGF antibodies into neonatal mice or rats results in the degeneration of sympathetic ganglia. Together, these findings suggest that the increased innervation found in many vascular tissues from adult SHR may be a consequence of a greater early expression of NGF in these blood vessels.

It should be mentioned that there is a possibility NGF is less extractable from WKY rat tissues than from tissues of SHR, resulting in an apparently low level of NGF in WKY rat tissues. However, since recovery of exogenous NGF was lower for 20-day-old WKY rat mesenteric arteries than for tissues of SHR, the NGF value obtained from the enzyme immunoassay was divided by a smaller number (percent recovery) to determine NGF content at 100% recovery. This calculation would compensate for any lower recovery of endogenous NGF from WKY rat tissues. Another theoretical problem is that the recovery of exogenous NGF may not represent the recovery of endogenous NGF. Unfortunately, there is no better way to accurately determine endogenous NGF levels.

NGF content of mesenteric arteries and aortas obtained from adult rats correlated directly with their density of sympathetic innervation. Other investigators have shown that densely innervated tissues from adult rats contained higher amounts of NGF and NGF mRNA compared with sparsely innervated tissues. In addition, we found that NGF levels in mesenteric arteries were not significantly different between the two adult rat strains. Yet, previous studies have shown that sympathetic innervation of mesenteric arteries from adult SHR was increased compared with tissues from WKY rats.

The density of nerves to a target tissue is determined during the development of innervation, that is, during the process of nerve migration and synapse formation within a target tissue. As mentioned earlier, NGF has been shown to be an essential factor during this process. It is possible that after the completion of tissue innervation, nerve density is stable as long as NGF is present. Minor variations in NGF levels may no longer be critical at this point because NGF is now acting in a supportive role rather than a survival role. If this hypothesis is correct, then the dense innervation of mesenteric arteries is maintained by the presence of NGF, and the lack of difference in NGF levels between tissues of adult SHR and WKY rats is of little consequence. In contrast, the virtual disappearance of NGF in aortas from mature rats may be responsible for the sparse innervation of these tissues. Further studies are necessary to clarify the relationship between NGF levels and sympathetic innervation in vascular tissue. For example, a time course of NGF levels in aortas (compared with the time course of sympathetic innervation, see Reference 6) may shed light on the role of NGF in this tissue.

The increased levels of NGF in blood vessels from young SHR and the potential contribution of NGF to the development of hypertension deserve...
comment. It has been suggested that the early appearance of increased sympathetic innervation in the vasculature of the SHR may contribute to the development of hypertension in this model. Moreover, immunosympathectomy (using antibodies against NGF) is associated with a prevention of hyperplastic changes in the vascular smooth muscle of blood vessels from SHR. It is tempting to speculate that the early overproduction of NGF in the vasculature may play a central role in the pathophysiology of hypertension in the SHR model.

References

Key Words • norepinephrine • nerve growth factor • sympathetic nervous system • spontaneously hypertensive rats
Elevated nerve growth factor levels in young spontaneously hypertensive rats.
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Hypertension. 1989;14:421-426
doi: 10.1161/01.HYP.14.4.421

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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