Norepinephrine Release and Reuptake by Hypothalamic Synaptosomes of Spontaneously Hypertensive Rats

Takuzo Hano, Young Jeng, and Joon Rho

We compared the overflow of endogenous norepinephrine during electrical field stimulation, the norepinephrine content, and the rate of initial neuronal uptake of [3H]norepinephrine in synaptosomes isolated from hypothalamus and brainstem of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats at 7 and 13 weeks of age. The synaptosomes of two rats, a SHR and a WKY rat control, were simultaneously processed and subjected to the same electrical field stimulation. The overflow of endogenous norepinephrine during electrical stimulation (2 Hz, 2 minutes) in the hypothalamic synaptosomes of 7-week-old SHR was significantly greater, whereas the overflow of 13-week-old SHR was equivalent to the age-matched WKY rat. The norepinephrine content of synaptosomes was about the same in SHR and age-matched controls. There was also significantly enhanced [3H]norepinephrine uptake in the hypothalamic synaptosomes of young SHR, but neither the hypothalamic nor the brainstem samples of 13-week-old SHR showed any significant difference in their rate of [3H]norepinephrine uptake. These data are similar to those we observed (unpublished observations) in perfused mesenteric artery system in which norepinephrine release was significantly elevated during periartrial nerve stimulation only in young SHR. Thus, these results suggest that a parallel enhancement of norepinephrine release in hypothalamus with that of peripheral nervous system may play an important role during development of hypertension in young SHR. (Hypertension 1989;13:250-255)

Increased sympathetic nerve activity originating from central nervous system regions involved with cardiovascular regulation has been implicated in the development and maintenance of spontaneous hypertension.1-6 Several investigators have observed that lesions of the posterior hypothalamus or its projection can lower arterial pressure in spontaneously hypertensive rats (SHR).7,8 The development of hypertension in SHR was also inhibited by posterior hypothalamus lesions.9 We measured norepinephrine reuptake directly into synaptosomes isolated from the hypothalamus in SHR 4-15 weeks of age and also in SHR 40 weeks of age.10 We found significantly higher norepinephrine uptake in SHR than in the Wistar-Kyoto (WKY) control rats at the younger age.

The nucleus tractus solitarii (NTS) is the relay center containing the primary synapse of the baroreceptor afferents, which make contact with secondary neuronal pathways projecting to vasomotor and cardioinhibitory centers. Destruction of the NTS produces hypertension of varying severity,11-13 whereas microinjection of norepinephrine into the NTS area will cause a fall in blood pressure.14 Synaptosomes, or pinched-off nerve endings of presynaptic origin,15 reseal well enough after homogenization to maintain Na⁺ and K⁺ ionic gradients and a potential difference across the external membrane.16,17 The object of the present study was to measure the release of endogenous norepinephrine as well as the initial norepinephrine uptake by field stimulation in the same synaptosomes from both hypothalamus and brainstem of SHR and WKY rats and relate them to peripheral sympathetic activities in the perfused mesenteric artery system of the same age groups (unpublished observation).

Materials and Methods

Blood Pressure and Body Weight Measurements

Two age groups, 6 and 12 weeks old, of male SHR of the Okamoto-Aoki strain* and age-matched WKY rats were obtained from Charles River Breeding Laboratories, Wilmington, Massachusetts and were housed in groups of four or five in a 12-hour light/
dark cycle for 1 week. At 1 day before decapitation, after the rats were heated and restrained with Narco Rat Holder (Harvard Apparatus, South Natick, Massachusetts), systolic blood pressure was measured by tail-cuff plethysmography according to the procedure described by Bunag. 19

**Synaptosome Preparation**

Rats were decapitated and brain tissues were dissected on an ice-cold glass plate. The rhombencephalon was separated by a transverse section from the rest of the brain and dissected into the cerebellum and the brainstem. Brainstem consisted of the medulla oblongata andpons. Two brainstems were pooled and used for each experiment. A transverse section was then made at the level of the optic chiasma. Hypothalamus was dissected by taking the anterior commissure as a horizontal reference and the line between the posterior hypothalamus and the mamillary body as the caudal limit. Thus, one hypothalamus or two brainstems were suspended in 4 ml homogenization medium (0.32 sucrose, 10 mM Tris-HCl, 1 mM Na-EDTA, 1 mM KCl, pH 7.4), and homogenized by 15 up-and-down strokes (10 per minute) in a Teflon-in-glass homogenizer (clearance 0.004–0.006 in.) with a smooth-bottom pestle that rotated at 800 rpm. All procedures, including homogenization and centrifugation, were carried out at 0°C. The homogenate was processed by the ficoll method of Booth and Clark. 20 Our observations show this method demonstrates superior sodium and ouabain sensitivity compared with Whittaker et al’s method. 21 Our use of Booth’s method involved centrifuging the homogenate at 2,000g for 3 minutes and spinning the resultant supernatant at 26,500g for 10 minutes. This produced the crude mitochondrial/synaptosomal pellet, which was resuspended in 1 ml isolation medium (0.32 M sucrose, 1 mM K-EDTA, 10 mM Tris-HCl, pH 7.4), diluted with 6.5 ml 12% ficoll in 1.0 M sucrose containing 50 µM KCl and 50 µM Na-EDTA (pH 7.4), and gently homogenized by hand. This sample suspension was introduced into a centrifuge tube and 3 ml 7.5% ficoll in 0.32 M sucrose containing 50 µM Na-EDTA and 50 µM KCl (pH 7.4) was carefully layered above it. Finally, 1.5 ml isolation medium was layered on top. The tubes were then centrifuged at 100,000g for 30 minutes in a Beckman SW41 Ti rotor (Fullerton, California). Myelin and synaptosomes band at the first and second interfaces, respectively, from the top and the heavier subcellular debris was pelleted at the bottom. Synaptosomal fraction was gently sucked off from the interface, with about 1.5 ml recovered, diluted to 8 ml with isolation medium, and spun at 17,300g for 10 minutes to obtain the final pellet.

The final pellet was resuspended by 70 strokes of a p-200 pipetman in 1,200 µl incubation medium containing 0.32 M sucrose, 3 mM KCl, 3 mM MgCl2, 2 mM CaCl2, 20 mM Tris-HCl, 120 mM NaCl, pH 7.4. For measurement of the release, reuptake, and content of norepinephrine, a 100-µl sample containing about 100 µg protein was used.

**Release of Endogenous Norepinephrine**

Samples were preincubated in a water bath at 37°C for 3 minutes. The endogenous norepinephrine release was evaluated by electrical field stimulation at 2 Hz for a 2-minute period with rectangular pulses of 5 msec duration at 20 V. After the electrical stimulation, 300 µl ice-cold solution (0.32 M sucrose, 3 mM KCl, 3 mM MgCl2, 20 mM Tris-HCl, 120 mM NaCl, 10 mM EDTA, pH 7.4) was immediately added to the test tube to stop the reaction. The tubes were centrifuged at 17,300g for 10 minutes at 4°C. The released norepinephrine in the supernatant was first adsorbed on alumina, eluted with 0.1N perchloric acid, and then assayed by high-pressure liquid chromatography with an electrochemical detector (BAS LC-4, Bioanalytical Systems, Inc., West Lafayette, Indiana). 3,4-Dihydroxybenzylamine was used as an internal standard. The amount of endogenous norepinephrine released was expressed as the amount of norepinephrine released during electrical stimulation corrected for the baseline efflux of norepinephrine before nerve stimulation.

**Incubation of Synaptosomes for [3H]Norepinephrine Uptake**

The synaptosomes (100 µg protein) were incubated in 100 µl incubation buffer that contained 20 mM Tris-HCl, 3 mM KCl, 3 mM MgCl2, 2 mM CaCl2, 12 µM nialamide, 0.32 M sucrose, 120 mM NaCl, pH 7.4. 22 Samples were preincubated in a water bath at 37°C for 3 minutes and then incubated for 2 minutes with an addition of 35 pmol [3H]norepinephrine in 10 µl. Duplicate samples were kept on ice for the same time period to serve as blanks. Incubations were stopped by removing 100 µl from the sample tube and injecting it into 2 ml of 1,000-fold excess of unlabeled norepinephrine that was placed on top of a 0.45-µm Millipore (Bedford, Massachusetts) filter. The mixture was vacuum filtered promptly, trapping synaptosomes with incorporated [3H]norepinephrine on the filter surface. Any additional loosely associated radioactivity was washed away with the addition of 3-ml volume of the incubation buffer minus 0.32 M sucrose. The filter was dried, dissolved in Filtron-X scintillation cocktail, and counted in a Beckman Model LS-335 liquid scintillation counter.

**Measurement of Norepinephrine Content**

To determine the content of endogenous norepinephrine in the sample, 100 µl of the synaptosome suspension in incubation medium used for the norepinephrine release and norepinephrine uptake experiments was pelleted at 10,000 rpm for 10 minutes. The pellet was resuspended in 200 µl ice-cold 0.4N perchloric acid, sonicated for 1 minute, and centrifuged at 10,000 rpm for 10
minutes to obtain the supernatant. The supernatant solution was neutralized with 1.0 ml 0.1 M phosphate buffer to pH 7.4. Norepinephrine in the supernatant solution was first adsorbed on alumina, eluted with 0.1N perchloric acid, and then assayed with high-pressure liquid chromatography with electrochemical detector.

**Protein Assay**

The protein content of samples was determined by the method of Lowry et al,23 with bovine serum albumin as the standard.

**Data Analysis**

SHR and WKY rats were always decapitated on the same day and the hypothalamus and brainstem from each strain were processed identically and in parallel, thereby obtaining paired data from each study. Values are presented as mean±SEM. Statistical significance was determined by one-way analysis of variance. Difference of \( p < 0.05 \) was considered to be significant.

**Results**

Body weight, blood pressure, and tissue weight of SHR and WKY rats are shown in Table 1. There was no difference between the tissue weights of SHR and WKY rats.

**Content and Overflow of Endogenous Norepinephrine in Synaptosomes of Hypothalamus and Brainstem**

The hypothalamic synaptosomes of a SHR and a WKY rat control were simultaneously processed and subjected to the same electrical stimulation at 2 Hz for 2 minutes, using the same incubation solution. In both groups, electrical stimulation evoked a proportional frequency-dependent increase in the overflow of norepinephrine up to 5 Hz. Since we measured norepinephrine concentration in a diluted synaptosome solution, we have in essence measured the released amount of norepinephrine. This was referred to as overflow since there may undoubtedly be some norepinephrine uptake. There was no difference in basal norepinephrine overflow from isolated synaptosomes of hypothalamus and brainstem between SHR and WKY rats of both age groups (Table 2), and the evoked norepinephrine release induced by electrical stimulation (2 Hz) was two to four times higher than the basal release in each group.

As shown in Table 3, the overflow of endogenous norepinephrine during electrical field stimulation in the synaptosomes of 7-week-old rats was significantly greater (23%) in the SHR (\( p < 0.05 \)) when compared with that of the WKY rats. In contrast, the synaptosomes of 13-week-old SHR showed an equivalent extent of norepinephrine overflow on electrical stimulation compared with that of age-matched WKY rats. The norepinephrine concentration of hypothalamic synaptosomes from SHR and WKY rats were similar in the two rat strains at both ages (Table 3). The content and overflow of norepinephrine in brainstem regions of SHR were not different from those of age-matched WKY rats of both age groups (Table 4).

**Uptake of \( [\text{SH}] \) Norepinephrine**

To assess whether there is an imbalance between the exocytotic release of norepinephrine and its reuptake process at different ages of SHR, the initial uptake of \( [\text{3H}] \) norepinephrine was determined in the same hypothalamic and brainstem synaptosomes used for the norepinephrine overflow measurements. The uptake rate was linear with time, up to 5 minutes of the incubation period. As shown in Table 3, there was about 33% increase of \( [\text{3H}] \) norepinephrine uptake (\( p < 0.05 \)) in the hypothalamic synaptosomes of 7-week-old SHR.

**Table 1. Body Weight, Blood Pressure, and Tissue Weight in Spontaneously Hypertensive Rats and Wistar-Kyoto Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Blood pressure (mm Hg)</th>
<th>Tissue weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hypothalamus</td>
</tr>
<tr>
<td>7-week-old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>152±14</td>
<td>119.6±8.9</td>
<td>187.8±14.9</td>
</tr>
<tr>
<td>SHR</td>
<td>155±34</td>
<td>154.0±15.2</td>
<td>172.6±22.9</td>
</tr>
<tr>
<td>13-week-old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>257±20</td>
<td>115.1±2.5</td>
<td>174.8±30.5</td>
</tr>
<tr>
<td>SHR</td>
<td>275±18</td>
<td>173.0±11.92</td>
<td>180.8±22.1</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.

**Table 2. Basal Norepinephrine Release From Isolated Synaptosomes in Spontaneously Hypertensive Rats and Wistar-Kyoto Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Basal norepinephrine release (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hypothalamus (n=8)</td>
</tr>
<tr>
<td>7-week-old</td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>4.42±0.92</td>
</tr>
<tr>
<td>SHR</td>
<td>5.49±1.16</td>
</tr>
<tr>
<td>13-week-old</td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>5.45±0.78</td>
</tr>
<tr>
<td>SHR</td>
<td>5.42±0.24</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.
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Norepinephrine Release and Uptake by SHR Synaptosomes

TABLE 3. [3H]Norepinephrine in Synaptosomes of Hypothalamus From Spontaneously Hypertensive Rats and Wistar-Kyoto Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>NE uptake (pmol/mg protein)</th>
<th>NE overflow (pmol/mg protein)</th>
<th>NE content (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-week-old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY (n=8)</td>
<td>1.09±0.05</td>
<td>5.87±0.77</td>
<td>42.38±1.59</td>
</tr>
<tr>
<td>SHR (n=8)</td>
<td>1.45±0.08*</td>
<td>13.69±1.00*</td>
<td>41.07±1.41</td>
</tr>
<tr>
<td>13-week-old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY (n=8)</td>
<td>0.70±0.05</td>
<td>9.04±1.28</td>
<td>47.39±2.06</td>
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<tr>
<td>SHR (n=8)</td>
<td>0.75±0.05</td>
<td>8.06±1.04</td>
<td>42.58±3.55</td>
</tr>
</tbody>
</table>

Values are mean±SEM. NE, norepinephrine; WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.

*Significant difference between WKY rats and SHR in the same age group by analysis of variance.

Discussion

It has been suggested that maintenance of blood pressure within a given range is achieved by tonic inhibition of sympathetic outflow from the sites of cardiovascular regulation located in the brainstem. Major centers for cardiovascular control are the NTS and the locus ceruleus, which are rich in noradrenergic neurons. The NTS is the relay center that contains the primary synapse of the baroreceptor afferents, which make contact with interneurons that project to the hypothalamus, and vasomotor and cardioinhibitory centers of the brain.

The results of the present study indicate that the release of endogenous norepinephrine during electrical field stimulation in the hypothalamic synaptosomes of young SHR (7-week-old) but no difference was observable in the 13-week-old rat samples. The data on [3H]norepinephrine uptake measurements in the synaptosomes of brainstem regions indicated that there were no significant differences at either age (Table 4).

Table 4. [3H]Norepinephrine in Synaptosomes of Two Pooled Brainstems Each From Spontaneously Hypertensive Rats and Wistar-Kyoto Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>NE uptake (pmol/mg protein)</th>
<th>NE overflow (pmol/mg protein)</th>
<th>NE content (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-week-old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY (n=4)</td>
<td>0.95±0.05</td>
<td>8.18±1.38</td>
<td>33.44±2.99</td>
</tr>
<tr>
<td>SHR (n=4)</td>
<td>1.04±0.12</td>
<td>11.45±2.51</td>
<td>27.78±2.73</td>
</tr>
<tr>
<td>13-week-old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY (n=4)</td>
<td>0.86±0.19</td>
<td>15.64±1.15</td>
<td>27.57±2.45</td>
</tr>
<tr>
<td>SHR (n=4)</td>
<td>0.98±0.11</td>
<td>14.15±1.88</td>
<td>31.51±5.05</td>
</tr>
</tbody>
</table>

Values are mean±SEM. NE, norepinephrine; WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.

Abnormalities of the monovalent cation transport and membrane calcium handling have been demonstrated in vascular smooth muscle, adipose tissue, and red blood cells of SHR. The structural alterations in cell membranes are known to be diffusely spread in the body of SHR. Moreover, the erythrocyte membrane differences were observed in SHR at a prehypertensive stage, which indicates that membrane alterations seem to precede hypertension and are not secondary to it. Thus, the increased release of norepinephrine from young SHR found in the present study may be the manifestation of membrane alteration at a prehypertensive stage and appears to be an important process associated with the development of hypertension.

The use of the synaptosomal model for a study of norepinephrine efflux and uptake processes is advantageous in that rat brain synaptosomes retain certain functional characteristics of nerve tissue in vivo. The model does provide a useful in vitro preparation for the study of transmembrane release and uptake of norepinephrine in isolation from the rest of the central nervous system. A decrease in sympathetic activity and an increase of vagal nerve activity are known to occur during electrical stimulation of the anterior hypothalamus, of the depressor areas of the anterior cingulate.
gyrus, and of certain parts of the medulla and afferent baroreceptor nerves.41 42

An increased release of norepinephrine in synaptosomes from the hypothalamus of 7-week-old SHR, which we observed in the present study, may reflect an enhanced activity of noradrenergic neurons opposing the increase in peripheral sympathetic activity as a compensatory mechanism. This compensatory activity may not persist in adult rats as the cell membrane defects are corrected and the peripheral norepinephrine release is brought down to a normal level, although the blood pressure is still higher than WKY rats due partly to postsynaptic and structural adaptation. It may be that the opposing effect of the hypothalamic neuronal activity is not adequate enough to completely overcome an enhanced vasoconstrictive force generated by highly elevated norepinephrine overflow in the peripheral system of young SHR (unpublished observations).

In the present study we observed that the initial neuronal uptake of [3H]norepinephrine in hypothalamic synaptosomes was about 33% higher in young SHR, but it was equivalent to that of the age-matched WKY control rats in the 13-week-old animals. In our preliminary study an addition of a neuronal uptake inhibitor, cocaine (5 x 10^-5 M), to the incubation medium produced maximal inhibition of uptake (85-90%) in both SHR and WKY rat synaptosomes (unpublished observation); the remainder reflects non-neuronal uptake. This indicates that the [3H]norepinephrine uptake we measured during the 2-minute period is essentially a neuronal uptake process.

Since the released amount of norepinephrine during electrical field stimulation in the synaptosomes of young rats was significantly greater (223%) than that of the WKY control rats, the net norepinephrine concentration in the extrasynaptosomal medium would be much higher in the young SHR when compared with that of WKY rats. The norepinephrine uptake system inactivates synaptically released transmitter; the efficacy of this process is dependent on the number of available uptake sites and the concentration of released norepinephrine.44 These dynamic modulations in the norepinephrine uptake process appear to regulate synaptic function homeostatically, which provides an increased reuptake when norepinephrine release is high in the young SHR and less reuptake when the synaptic concentration of the transmitter is low in the 13-week-old rats.

There was no significant difference in either [3H]norepinephrine uptake values or in overflow of endogenous norepinephrine during electrical stimulation of brainstem synaptosomes in both age groups. Saavedra and associates45-47 reported that an epinephrine-forming enzyme, phenylethanolamine-N-methyltransferase, was increased in the A1 and A2 areas of brainstem only in young SHR and that neither in young nor in older SHR was norepinephrine activity abnormal in medullary regions associated with regulation of blood pressure. Furthermore, the changes of enzymatic activity in their study reflect the function of total nerve cells since A1 and A2 of the rat brain contain mostly the cell bodies, whereas our present study represents the noradrenergic neuronal activities at the level of nerve terminals that were resealed into isolated synaptosomes.

References


21. Whittaker VP, Michaelson IA, Kirkland RJA: The separation of synaptic vesicles from nerve ending particles ('synaptosomes'). *Biochem J* 1964;90:293–303


KEY WORDS • norepinephrine • hypothalamus • brainstem • synaptosomes
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