SUMMARY The in vitro uptake of $^3$H-NE by storage vesicles from the hypothalamus of age-matched spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats has been studied using a new reliable procedure for the isolation of biochemically active storage vesicles. In each of 13 paired studies, done in triplicate, storage vesicles of SHR took up more $^3$H-NE than those of WKY. (The mean difference was 37% more uptake by SHR.) Electron-microscopic examination of normotensive samples showed a concentration of intact synaptic vesicles; whereas SHR subfractions were composed of fragmented membranes that resembled swollen, distorted vesicles. These findings in the brain tissues of SHR parallel our previous results found in SHR peripheral tissues. Taken together, we interpret the results to indicate that the membranes of synaptic vesicles of SHR are altered structurally and biochemically. (Hypertension 5: 3-7, 1983)

KEY WORDS • norepinephrine uptake • isolated hypothalamic storage vesicle • spontaneously hypertensive rats

NUMEROUS studies have implicated the hypothalamus with cardiovascular control mechanisms. Some investigators focused on responses of single units of the hypothalamus to stimulation of baroreceptor afferent input while others studied cardiovascular and plasma catecholamine responses to stimulation of different areas of the hypothalamus.

To investigate the role of the hypothalamus in hypertension, we elected to study the Okamoto strain of rats with spontaneous hypertension (SHR), since their hypertension is associated with elevated indices of sympathetic activity. Hypothalamic lesions, or separation of connections between hypothalamus and mesencephalon, cause a significantly greater decrease of blood pressure in SHR than in Wistar-Kyoto (WKY) control rats. Conversely, electrical stimulation of the posterior hypothalamus elicits greater blood pressure increases in the SHR than in control WKY. Furthermore, SHR show changes in hypothalamic catecholamine metabolism in those areas which affect blood pressure. Thus, Nagaoka and Lovenberg reported that during the early development of hypertension in the SHR, the activities of both tyrosine hydroxylase and dopamine-B-hydroxylase (DBH) in the hypothalamus were significantly higher than in the control WKY rats. Nevertheless, a lower NE content in the hypothalamus of SHR was reported as early as 1970 by Yamori et al. and recently by Saavedra et al. and Tsobe et al. The concentration of epinephrine was elevated in the hypothalamus of SHR as reported by Gianutsos and Moore. Other approaches to studies of the hypothalamus in SHR have focused on adrenoceptor activity and, at present, the findings are conflicting.

We recently reported results of in vitro $^3$H-NE uptake by peripheral tissues in SHR and WKY. In the present study, we examined the in vitro uptake of $^3$H-NE by isolated hypothalamic storage vesicles from age-matched SHR and WKY rats. Results show that the Mg$^{2+}$-activated ATPase dependent amine uptake of SHR hypothalamic storage vesicles is significantly greater than that of WKY controls.
Methods

Male SHR and age-matched WKY animals, 8-13 weeks of age, were used (Charles River Breeding Company, Wilmington, Massachusetts). Blood pressure differences between SHR and WKY controls were confirmed by measurement of tail systolic blood pressures. The average of three readings for each rat was recorded; pressures of the WKY rats ranged from 85 to 140 mm Hg and of SHR from 150 to 185 mm Hg.

Preparation and Incubation of Hypothalamic Storage Vesicles

A subcellular fraction from rat hypothalamus was prepared by our modified differential centrifugation procedure, which was designed to concentrate storage vesicles. Rats were decapitated, and the hypothalamic region, weighing from 0.25 to 0.30 grams was removed. A 5% homogenate (w/v) of the hypothalamus was made in ice-cold 0.32 M sucrose in 10 mM tris-HCl (pH 7.4) with the aid of a Teflon-in-glass homogenizer (clearance, 0.004-0.006 inches). Fifteen passes of the pestle were made at 10 per minute and the pestle rotated at approximately 800 rpm. All procedures, including homogenization and centrifugation, were carried out on ice at 0°C. The homogenate was centrifuged at 1500 \( \times g \) for 10 minutes and the pellet (P1) resuspended in an additional homogenization buffer equal in volume to the original sample and centrifuged at the same speed for another 10 minutes. The pooled supernatant (S1) was then spun at 20,000 \( \times g \) for 20 minutes to sediment the crude synaptosomes (P2). Hypotonic rupture of this synaptosome fraction was accomplished by resuspending it in 2.4 ml of 0.06 M sucrose and mixing vigorously with 70 up-and-down strokes with a Pasteur pipet without bubbling. The suspension was left on ice for 15 minutes, and then 0.6 ml of 1 M sucrose, 50 mM Tris-HCl (pH 7.4), and 4 mM MgCl2 were added to the 2.4 ml; thus, the final concentration of the suspension medium was 0.24 M sucrose, 1 mM MgCl2, and 12 mM Tris. After the hypotonic shock, the suspensions were recentrifuged at 20,000 \( \times g \) for 10 minutes to remove the debris (P3). The concentration of the suspension medium was 0.24 M sucrose, 1 mM MgCl2, and 12 mM Tris. After the hypotonic shock, the suspensions were recentrifuged at 20,000 \( \times g \) for 10 minutes to remove the debris (P3). The supernatant (S3) was then spun at 164,000 \( \times g \) for 20 minutes to sediment the crude synaptosomes (P4). Hypoosmotic rupture of this synaptosome fraction was accomplished by resuspending it in 2.4 ml of 0.06 M sucrose and mixing vigorously with 70 up-and-down strokes with a Pasteur pipet without bubbling. The suspension was left on ice for 15 minutes, and then 0.6 ml of 1 M sucrose, 50 mM Tris-HCl (pH 7.4), and 4 mM MgCl2 were added to the 2.4 ml; thus, the final concentration of the suspension medium was 0.24 M sucrose, 1 mM MgCl2, and 12 mM Tris. After the hypotonic shock, the suspensions were recentrifuged at 20,000 \( \times g \) for 30 minutes to remove the debris (P3), and the supernatant (S3) was centrifuged at 164,000 \( \times g \) for 30 minutes to obtain a pellet (P4) rich in storage vesicles.

By using 150 strokes of P-1000 Pipetman, the storage vesicle pellet (P4) was resuspended in 900 \( \mu l \) of incubation buffer containing 20 mM KH2PO4 (pH 7.4) and 1 mM MgCl2. This suspension was used for subsequent assay. Each sample contained 100 \( \mu l \) of vesicle preparation (20 to 30 \( \mu g \) protein), 5 mM Tris-ATP, and incubated in the presence or absence of N,N-dicyclohexylcarbodiimide (DCCD), an inhibitor of Ca2+/Mg2+-ATPase. The samples were equilibrated in a water bath at 30°C for 3 minutes and then incubated for 5 minutes with the addition of 35 picomoles of \( ^{3} \)H-1-NE in 10 \( \mu l \) (Amersham/Searle Corporation, Chicago, Illinois). Duplicate samples were kept on ice for the same period of time to serve as blanks. Incubations were stopped by removing 100 \( \mu l \) from the sample tube and injecting it into 2 ml of a 1000-fold excess of unlabeled NE that was placed on top of a 0.45 \( \mu m \) Millipore filter. The mixture was filtered promptly, trapping the vesicles with incorporated \( ^{3} \)H-NE on the filter surface. Any additional loosely associated radioactivity was washed away with the addition of 3 ml volume of the incubation buffer. The filter was dried, then dissolved in Filtron-X liquid scintillation cocktail, and counted in a Beckman Model LS-335 liquid scintillation counter.

Protein Assay

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

Data Analysis

SHR and WKY rats were always sacrificed on the same day, and hypothalami from each strain were processed identically and in parallel, thereby obtaining paired data from each study. The NE uptake data were analyzed by the two-tailed t test.

Electron Microscopy

The pellets (P4) were fixed in 6% glutaraldehyde (0.1 M cacodylate buffer, pH 7.4) at 4°C for 45 minutes and block-stained with 0.5% uranyl acetate in 0.9% NaCl at 4°C overnight. The specimens were dehydrated and embedded in Epon 812. Thin sections were cut with a LKB 8800 ultratome III, stained with uranyl acetate and lead citrate, and examined with a Jeol 100 C electron microscope to evaluate the components of the P4 pellets.

Results

Uptake of \( ^{3} \)H-NE by Storage Vesicles of SHR and WKY Rats

The mean values of \( ^{3} \)H-NE uptake by the storage vesicles of SHR and WKY rats in 13 paired experiments ranged from 39 to 68 picomoles per milligram of protein in SHR samples and from 33 to 38 picomoles in WKY samples. Thus, in all 13 experiments, the uptake values were higher in SHR than in WKY samples. The difference between mean uptake values was 37% and statistically highly significant as shown in table 1.

<table>
<thead>
<tr>
<th>SHR</th>
<th>WKY</th>
<th>Difference (%)</th>
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<tr>
<td>48.4</td>
<td>35.2</td>
<td>37</td>
</tr>
<tr>
<td>±8.9</td>
<td>±1.8</td>
<td></td>
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<tr>
<td>p&lt;0.001</td>
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<td>n = 13 paired incubations and each was done in triplicate.</td>
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3H-NE Uptake Kinetics by SHR and WKY Storage Vesicles

To test whether the enhanced uptake by SHR vesicles resulted from a time-dependent difference in uptake kinetics, vesicles were prepared and incubated with 3H-NE for different time periods. As shown in figure 1, the uptake by vesicles from both SHR and WKY appeared to be linear and parallel for the entire time period studied, with SHR again showing consistently enhanced uptake. This indicates that the difference between SHR and WKY vesicular uptake of NE in the 5-minute studies shown in table 1 was due to a difference in uptake kinetics independent of time.

Electron Microscopy of Storage Vesicle Fraction

The P4 hypothalamic fractions from SHR and WKY animals showed differences when examined by electron microscopy. The WKY fractions were quite pure in their content of well-defined vesicles, many of which were dense-cored and of the size that has been linked to biogenic amines (fig. 2 left). SHR fractions differed in that they were composed of membranes that resembled swollen, distorted vesicles (fig. 2 right).
Discussion

To the best of our knowledge, this is the first biochemical and electron microscopic investigation of isolated hypothalamic storage vesicles from hypertensive and normotensive rats. Utilizing our new technique for isolation of storage vesicles, we have determined that the P, vesicle fraction obtained from SHR takes up an average of 37% more 3H-NE than comparable fractions from WKY controls. The difference appeared to result from a strain difference in NE uptake kinetics.

Other investigations of the hypothalami of SHR and WKY rats have reported that catecholamine levels in SHR are higher or lower than those of controls. Further, catecholamine synthesizing enzyme activity has been reported to be increased or decreased in hypothalamic areas. The present results, however, are independent of NE metabolism and turnover processes. Rather it focuses on a high affinity uptake system for NE. We interpret the enhanced NE uptake by hypothalamic storage vesicles of SHR as a reflection of altered membrane structure revealed in the present study by electron microscopic examination.

Thus, P, fractions from hypertensive rats appeared to contain few intact dense-cored vesicles and, in general, the vesicles appeared swollen, distorted, and fragmented. In contrast, P, pellets from normotensive rats were composed of well delineated vesicles, a number of which were the dense-cored type thought to be associated with biogenic amines. Furthermore, our previous electron-microscopic studies revealed that vesicles of cardiovascular tissue of SHR also have altered cellular membrane structure similar to the changes found here in hypothalamic vesicles. Most recently, other investigators reported that membranes of both erythrocytes and smooth muscle cells of SHR have altered structure as well as functional characteristics.

The uptake of 3H-1-NE into storage vesicles isolated from rat hypothalamus in the present study showed an absolute requirement for ATP and a complete inhibition by addition of 60mM DCCD, which is an inhibitor of mitochondrial Ca2+/Mg2+-ATPase. To date, experiments working with chromaffin granules, chromaffin ghosts, or synaptic vesicles have presented evidence that strongly suggests that amine accumulation into these subcellular organelles is dependent upon the magnitude of the proton gradient. The proton gradient can be established by ATP hydrolysis catalyzed by a Ca2+/Mg2+-ATPase associated with the synaptic vesicle membranes. It was suggested that ATP stimulates catecholamine uptake by serving as an effector substrate for the ATPase, establishing a transmembrane proton gradient. The enhanced NE uptake by hypothalamic storage vesicles of SHR which we have observed in the present study may reflect either an increased amount of Ca2+/Mg2+-ATPase or a higher activity of the enzyme in the vesicular membrane of SHR. It seems possible that a higher content of this enzyme would have altered structural as well as functional characteristics of the SHR membranes.

Whether or not these functional and structural alterations in hypothalamic storage vesicles are the basis for maintenance of hypertension in adult SHR is unknown. Higher uptake of NE would reduce the amount of neurotransmitter concentration in cytoplasm and subsequent degradation. This could increase the amount of NE packaged in vesicles for subsequent release. Thus, one can envisage a greater quantal release within the hypothalamus which in turn could enhance sympathetic activity. Whether such functional and structural alteration reported here are genetically transmitted and thereby contribute to the initiation of hypertension in SHR is currently under investigation in our laboratory.

Acknowledgment

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