Altered In Vitro Uptake of Norepinephrine by Cardiovascular Tissues of Spontaneously Hypertensive Rats

Part 1. Mesenteric Artery

JOON H. RHO, PH.D., BERTHA NEWMAN, PH.D., AND NATILIE ALEXANDER, PH.D.

SUMMARY The incorporation of tritiated norepinephrine (NE) by mesenteric arteries from spontaneously hypertensive rats (SHR) of the Okamoto strain and from age-matched Wistar Kyoto (WKY) controls was studied. The arteries were incubated with tritiated NE, and fractions were isolated by differential and sucrose density gradient centrifugations. The amount of radioactivity present in certain subfractions (P pellet) of the SHR arteries was significantly higher than that of WKY arteries. When the P subfraction was lysed and subjected to sucrose density gradient centrifugation, the tritiated NE was found to be associated with the 0.4-0.5 M interface. Electron micrographs of the P subfractions revealed a variety of vesicular structures which might represent storage sites for the tritiated NE. Although a number of factors could account for the finding of enhanced incorporation into mesenteric artery subfractions of hypertensive rats, the finding is compatible with the work of others who found increased ATPase activity in mesenteric arteries of SHRs, since ATPase is known to activate vesicular NE uptake. (Hypertension 3: 704-709, 1981)

KEY WORDS • mesenteric arteries • synaptosomal fraction • norepinephrine uptake • spontaneously hypertensive rats •

THE Okamoto strain of rats with genetic hypertension (SHR) has been the subject of many studies designed to examine possible mechanisms contributing to the maintenance of this hypertension. To account for this hypertensive state, the sympathetic nervous system has been strongly implicated and is a continuing focus of investigation. Current literature is conflicting regarding the level of activity and the role of the sympathetic nervous system in adult SHR. Approaches to assess sympathetic activity in SHR have included measurements of plasma catecholamines and other biochemical indices, estimates of catecholamine metabolism in central and peripheral tissues, evaluation of cardiovascular responses to pharmacologic interventions, and measurements of splanchnic action potentials. Most of these studies indicate that SHRs have enhanced sympathetic nervous system activity when compared to Wistar Kyoto (WKY) controls.

The purpose of our present study was to evaluate the sympathetic nervous system in yet another way, namely, by assessing the tritiated norepinephrine ("H-NE) incorporation by neuronal components of mesenteric arteries from SHR and WKY. To isolate these neuronal components (specifically, synaptosomes and/or neurotransmitter vesicles), we applied differential centrifugation and sucrose gradient procedures to homogenates of mesenteric arteries. Our procedures were similar to those used by other investigators to isolate synaptosomes and synaptic vesicles from brain, from cardiac tissue, and from the vas deferens and spleen. We examined the constituents of the subfractions from the mesenteric arteries by electron microscopy. Our results indicate that particular subfractions from SHR mesenteric arteries incorporate more "H-NE than comparable subfractions from WKY controls. Furthermore, as indicated by our studies of atria and veins, this enhanced incorporation was regionally specific.
Methods

Male SHR and age-matched WKY rats 7-38 weeks of age were used; body weights for SHR ranged from 200 to 360 g and for WKY from 190 to 640 g, (Charles River Breeding Company, Wilmington, Massachusetts). To confirm blood pressure (BP) differences between SHR and WKY controls, tail systolic blood pressures (SBP) were measured. The average of three readings for each rat was recorded. The SBP in WKY rats ranged from 113-130 mm Hg and in SHR from 140-200 mm Hg.

Preparation and Incubation of Tissues

Rats were decapitated, and the superior mesenteric artery with most of its branches and mesentery was placed in ice-cold incubation buffer. This buffer, prepared in double distilled H₂O, contained in grams per liter: NaCl, 8.06; KCl, 0.35; CaCl₂·2H₂O, 0.30; MgSO₄·7H₂O, 0.294; KH₂PO₄, 0.162; and dextrose 2.0. The medium was adjusted to pH 7.2-7.4 before each experiment. We used a pool of two tissues each for SHR and WKY in the first seven studies and one each for subsequent studies. Arteries with attached mesentery were preincubated for 15 minutes at 37°C in the buffer and then incubated 60 minutes in the same medium in the presence of [³H]-noradrenaline (Amersham Corporation). We used 1 nmole of [³H]-NE (specific activity 9.1 Ci/m mole) in 2 ml of buffer. During the incubation process, the medium was continually oxygenated with a gas mixture consisting of 95% O₂ and 5% CO₂.

Following incubation, tissues were washed five times in cold incubation medium, blotted, and, for convenience, frozen overnight. (Similar results were obtained whether or not the tissues were processed immediately after incubation or after freezing.) Tissues were then weighed and resuspended in cold 0.32 M sucrose containing 10⁻⁴ M EDTA, 0.1 M potassium phosphate, pH 7.4, and placed in a glass homogenizer with a loose-fitting pestle (0.004-0.006 inch clearance). The pestle was gently rotated by hand to remove fat. The remaining tissue was then minced with stainless-steel scissors on an ice-cold ceramic board. The minced tissue was homogenized in approximately 10 volumes of the sucrose medium. A cooled Teflon-in-glass homogenizer with a clearance of 0.004-0.006 inch was used (A. H. Thomas Company); 30 passes of the pestle were made at 10/minute. The pestle rotated at approximately 1500 rpm.

Differential Centrifugation and Cell Fractionation

Tissue homogenates were subjected to successive centrifugation, which led to separation of three pellets, P₁, P₂, and P₃, and the corresponding supernatants, S₁, S₂, and S₃, by a procedure slightly modified from that reported by others. The scheme of differential centrifugation is shown in figure 1. All steps were carried out at 4°C. The initial centrifugation was performed in an SS-34 rotor in a Sorvall RC-2 centrifuge. The last step was performed in a Beckman SW 60 Ti swing-out rotor on a Beckman L5-65 ultracentrifuge.

Discontinuous sucrose density gradients were prepared in 4.8 ml Beckman nitrocellulose tubes by layering eight successive concentrations of sucrose which varied from 0.4 M at the top of the gradient to 1.4 M at the bottom. The P₃ pellet was resuspended in 0.5 ml of glass-distilled water and washed in and out of a Pasteur pipette in an attempt to release neurotransmitter-containing vesicles. This 0.5 ml was transferred to the top of the discontinuous sucrose gradient and centrifuged in a Beckman SW 60 Ti swing-out rotor at 53,000 g for 90 minutes.

Ten drop (125 µl) fractions were collected from the bottom of the gradient through a small hole. The radioactivity associated with [³H]-NE in each fraction was counted by placing 100 µl aliquots from each fraction into 3 ml Beckman Sucro-Solv scintillation fluid and counted in a Beckman Model LS-335 liquid scintillation counter. The lowest counts obtained in any fraction exceeded background counts by at least 10 times.

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 tissues from each strain were processed identically and in parallel, thereby obtaining paired data from each study. The NE incorporation data were analyzed by the Wilcoxon two-tail signed rank test for paired observations.

Electron Microscopy

Pellets were fixed in 6% glutaraldehyde (0.1 M cacodylate buffer, pH 7.4) at 4°C for 12 minutes.
TABLE 1. Incorporation of Norepinephrine (\textsuperscript{3}H-NE) into P\textsubscript{3} Fraction from Mesenteric Arteries of SHR and WKY Rats

<table>
<thead>
<tr>
<th>Study</th>
<th>Age (wks)</th>
<th>Tissue weight (no. of tissues)</th>
<th>Total cpm P\textsubscript{3}</th>
<th>picomole X 10\textsuperscript{-3}NE/mg tissue</th>
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<tr>
<td></td>
<td></td>
<td>SHR</td>
<td>WKY</td>
<td>SHR</td>
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<tr>
<td></td>
<td></td>
<td>mg (no.)</td>
<td>mg (no.)</td>
<td></td>
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</table>

*Total cpm P\textsubscript{3} = sum of counts collected from sucrose density gradient subfractions.

Results

Distribution of Norepinephrine (\textsuperscript{3}H-NE) in P\textsubscript{3} Subfractions

Figure 2 shows the typical radioactivity data associated with \textsuperscript{3}H-NE in each fraction obtained from the sucrose gradients. The peak radioactivity was associated with a discrete region banded around the 0.4-0.5 M sucrose interface in both SHR and WKY samples. In 13 out of 14 experiments, however, the peaks of radioactivity were much higher in the SHR subfractions.

Incorporation of Norepinephrine (\textsuperscript{3}H-NE) into P\textsubscript{3} Fraction

Table 1 shows the total counts per minute (cpm) in P\textsubscript{3} fractions along with the wet weight of the tissues used in each of 14 experiments. The data also are expressed as NE incorporation per milligrams of tissue weight, as calculated from the specific activity of the \textsuperscript{3}H-NE. In 13 of the 14 experiments, the values were higher in SHR than in WKY tissues, and the differences between paired samples were statistically significant (p < 0.01). Figure 3 shows the incorporation of \textsuperscript{3}H-NE by SHR P\textsubscript{3} pellets expressed as a percent of that in the paired WKY pellet. This graphically reveals that incorporation was greater in SHR mesenteric artery P\textsubscript{3} pellets in all but one experiment. Additionally, in three of the 14 experiments we determined the total protein of the S\textsubscript{4} fraction from which the P\textsubscript{3} pellet was collected, and \textsuperscript{3}H-NE incor-
poration was expressed as cpm per µg of S₄ protein. Normalized in this manner, the data again showed that 3H-NE incorporation by SHR samples was 164%, 183%, and 200% of that incorporated by WKY pellets. (The total amount of protein in a P₄ pellet is in the order of 0.2-0.5 mg.) Concomitant protein analysis showed that SHR mesenteric arteries contain fewer intact synaptosomes and vesicular profiles of varying diameters and densities. In contrast, the arteries of WKY pellets than in the SHR pellets. In addition, free vesicles (with and without cores) were present as relatively isolated entities as well as clumped formations throughout the pellets. We believe (and are attempting to quantitate) that more synaptosome-like structures were found in the WKY pellets than in the SHR pellets. In contrast, the SHR pellets contained fewer intact synaptosomes and were composed predominantly of membranous, vesicular profiles of varying diameters and densities.

**Discussion**

Although Nedergaard and Bevan stated that incorporation of exogenous NE by a tissue reflects the density of its innervation, incorporation is the net result of uptake into neuronal terminals and by retention by storage vesicles. Vesicles, in turn, can vary in number and/or integrity and thereby affect overall incorporation.

The major finding in this study is that incorporation of 3H-NE by mesenteric arteries of SHR exceeds that of WKY arteries. It should be emphasized that this finding is due to differences in radioactivity of the P₄ pellet. Based on sucrose gradient determinations on the same tissues that found endogenous NE associated with the P₄ fraction in those particular tissues. We have now determined that 3H-NE was present mainly in the P₄ pellet of mesenteric arteries. Another interesting correlation is that we found the peak radioactivity present in a sucrose subfraction comparable to similar subfraction reported by others containing the maximum amount of endogenous NE in heart, spleen, and vas deferens tissues.

Our data are compatible with other studies on different tissues that found endogenous NE associated with the P₄ fraction in those particular tissues. We have now determined that 3H-NE was present mainly in the P₄ pellet of mesenteric arteries. Another interesting correlation is that we found the peak radioactivity present in a sucrose subfraction comparable to similar subfraction reported by others containing the maximum amount of endogenous NE in heart, spleen, and vas deferens tissues.

It is generally accepted that biogenic amine uptake into neuron terminals is, first, from synaptic cleft to neuronal cytoplasm and, second, from cytoplasm to the interior of storage vesicles. Uptake from the synaptic cleft utilizes a Na⁺-K⁺-activated ATPase and is partially responsible for the termination of the actions of the released neurotransmitter. In contrast, vesicular uptake is activated by Mg⁺⁺-activated ATPase and is responsible for maintenance and packaging of transmitters for release during neuronal stimulation. It is of interest that several investigators have observed that enzyme activities such as adenosine monophosphatase, alkaline phosphatase, Na⁺-K⁺-ATPase as well as Mg⁺⁺-activated ATPase are increased in mesenteric arteries of hypertensive animals. Since biogenic amine uptake into both
neuronal cytoplasm and vesicles is activated by Na+-K+- and Mg++-dependent ATPase, our finding of an enhanced NE incorporation into SHR mesenteric artery subfractions is totally compatible with the increased ATPase activity found in mesenteric arteries of hypertensive animals observed by the above investigators.

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References

J H Rho, B Newman and N Alexander

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