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Characteristics of Voltage-Dependent Calcium Uptake and Norepinephrine Release in Hypothalamus of SHR

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The characteristics of voltage-dependent ^{45}Calcium uptake and norepinephrine release as factors controlling neural activities in the hypothalamus which is an important regulatory site for cardiovascular function were studied. Two groups of animals: male spontaneously hypertensive rat (SHR) and age-matched normotensive wistar rat (NW) were used in this study. Animals at 4, 6 and 16 weeks of age were sacrificed by decapitation and the hypothalamus was dissected out. Voltage-dependent calcium uptake and norepinephrine release were determined from hypothalamic synaptosomes either in low potassium (5 mM) or high potassium (41 mM) stimulatory conditions by using ^{45}Ca isotope and HPLC-ECD techniques. Degrees of voltage-dependent ^{45}calcium uptake and norepinephrine release evoked by calcium uptake in the hypothalamus of prehypertensive phase (4 weeks old) of SHR were significantly smaller than those in NW of the same age. However, in the developmental phase (6 weeks old) and the established phase (16 weeks old) of hypertension in SHR, degrees of voltage-dependent ^{45}calcium uptake and norepinephrine release were similar to those of age-matched normotensive wistar rats. These data imply that the deficit in hypothalamic norepinephrine release might be an important underlying factor for the development of hypertension in SHR.

Key words: Spontaneously hypertensive rat, Hypothalamus, Calcium, Norepinephrine

INTRODUCTION

The central noradrenergic nervous system has been implicated in the development and maintenance of hypertension in spontaneously hypertensive rats (SHR) (Mannelli *et al.*, 1990; Brody *et al.*, 1980). Preteatment with 6-hydroxydopamine prevents the development of hypertension in SHR. Altered catecholaminergic input to cardioregulatory centers as well as decreased norepinephrine levels in the brainstem and hypothalamus in SHR have been reported (Hano *et al.*, 1989; Yao *et al.*, 1989; Fujino, 1984).

Among brain structures involved in the regulation of blood pressure the hypothalamus seems to be of prime importance (Shonis and Waldrop, 1993; Li *et al.*, 1992; Sasaki *et al.*, 1986; Gordon *et al.*, 1985). Electrical stimulation of the hypothalamus leads to a pressor response which is more pronounced in SHR than in normotensive wistar rats (Juskevich *et al.*, 1978). The activities of norepinephrine synthesizing enzymes tyrosine hydroxylase and dopamine beta-hydroxylase in the hypothalamus of SHR were greater than those of normotensive wistar rats (Saavedra *et al.*, 1978; Nagaoka and Lovenberg, 1977). Also, increased uptake of norepinephrine and an increase in the number of alpha-adrenoceptors in the hypothalamus of SHR have been reported (Yamada *et al.*, 1985; Cantor and Abraham, 1981). However, it is not clear yet whether noradrenergic neural activity in the hypothalamus of SHR is a regulatory factor for blood pressure or not.

The purpose of the present study was to address this question: which alterations in the noradrenergic neural activity of the hypothalamus may be related to the development and maintenance of hypertension in SHR. We have examined two parameters; (1) Release of norepinephrine as an index of noradrenergic neural activity was determined from the synaptosome of the hypothalamus when animals were in either the

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normotensive or hypertensive stage depending on their ages. This was done to examine whether noradrenergic neural activity in the hypothalamus indexed by the release of norepinephrine is related to a particular stage of hypertension such as stage of development or stage of maintenance of hypertension in SHR. If so, is it a genetically-determined characteristics or not. (2) Voltage-dependent ^{45}calcium uptake was also determined from the synaptosome of the hypothalamus everytime when release of norepinephrine was measured as an additional index of neural activity in the hypothalamus.

MATERIALS AND METHODS

Animals

Male SHR and wistar rats at 4, 6 and 16 weeks of age were used in this study. SHR was obtained from Charles River Breeding Laboratories, and wistar rats from the Seoul National University animal breeding center. All animals were fed *ad libitum* on a standard laboratory diet throughout this study.

Measurement of Blood Pressure

The systolic blood pressure was measured using tail cuff plethysmography (Narco Bio-Systems, Texas, U.S.A.) according to the procedure described by Pfeffer *et al.* (1971).

Synaptosomal Preparation

Animals were decapitated and hypothalami were removed to prepare crude synaptosome. Hypothalami were placed in 20 vol. of a solution consisting of 0.32 M sucrose at pH 7.5, 1 mM EDTA and 2 mM HEPES. This procedure was carried out at 4℃, as were all subsequent procedures. Hypothalami were homogenized using a Thomas C homogenizer with a Teflon pestle. The homogenate was then centrifuged at 3,200 rpm for 5 min. The supernatant was centrifuged at 12,000 rpm for 12 min. The pellet was washed once with 20 vol. of the 0.32 M Sucrose-EDTA-HEPES solution and recentrifuged at 12,000 rpm for 12 min. The pellet was resuspended in 7 vol. of the 0.32 M Sucrose-EDTA-HEPES solution, diluted slowly with 35 vol. of a low calcium buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 0.02 mM $CaCl_2$ and 10 mM HEPES, pH adjusted to 7.5 with Tris base and centrifuged at 9,000 rpm for 5 min. The resulting pellet was suspended in a low calcium buffer to make a final protein concentration of approximately 0.8-1 mg/ml. Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Measurement of Calcium Uptake

For ^{45}Ca uptake, 0.2 ml of a synaptosomal suspension were preincubated for 10 min at 30℃. Uptake of ^{45}Ca was initiated by rapid addition of an equal volume of a high K^+ buffer containing $^{45}Ca^{++}$ (final concentration of 1.25 mM, 1 μCi/ml), 73 mM NaCl, 77 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 0.06 mM $CaCl_2$ and 10 mM HEPES, pH 7.5 with Tris base. ^{45}Ca uptake was also carried out using a low K^+ buffer containing 5 mM KCl. At 2, 5, 10, 20, 60 and 120 seconds after initiation, uptake of ^{45}Ca was stopped by the addition of 5 ml of the ice-cold stopping solution containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, and 3 mM EGTA, pH 7.5 with Tris base. Each sample was immediately filtered on Whatman GF/B filters on a Hoefer vacuum filtration manifold. The filters were quickly washed with two 5 ml aliquots of the ice-cold stopping solution and placed in vials for determination of radioactivity by the liquid scintillation counter.

Measurement of Norepinephrine Release

After a 0.2 ml portion of resuspended membrane was preincubated for 10 min at 30℃, norepinephrine release was initiated by rapid addition of an equal volume of low K^+ or high K^+ buffer. At 2, 5, 10 and 20 seconds after the initiation, norepinephrine release was stopped by the addition of 1 ml of the ice-cold stopping solution containing 3 mM EGTA. Each sample was immediately filtered on Whatman GF/B filters. The filtrates were put in screw-capped glass test tubes containing 100 μl of 1 M perchloric acid and 50 μl of dihydroxyl benzyl amine (DHBA, 6.25 ng/50 μl) as internal standard for HPLC. The filtrates were assayed for norepinephrine and DHBA after alumina extraction. 70 mg of acidified alumina and 0.5 ml of 1.5 M Tris (pH adjusted to 8.6 with HCl) was added to each tube. After mechanical shaking for 10 min, the supernatant in each tube was aspirated and 3 ml of wash buffer (1 ml of 1.5 M Tris for every 500 ml H_2O, pH 8.6) was added. The resulting supernatant was aspirated and this procedure was repeated. The tubes were then centrifuged at 1,000 g for 1 min and the remaining supernatant was aspirated. To elute catecholamines, 200 μl of 0.3 N H_3PO_4 was added to each tube which was then immediately vortexed. The usual injection volume for HPLC analysis was 5 μl. The HPLC system consisted of an LC-4B amperometric detector with glassy carbon electrode (Bioanalytical System, IN, U.S.A.), a BAS PM-600 pump, and a Biophase cartridge 3 μm ODS, 3.2 mm ID, 10 cm length column (Bioanalytical System, IN, U.S.A.). The mobile phase consisted of 0.1 M citric acid, 0.225 mM octyl sodium sulfate, 0.06% diethylamine, 0.05 mM Na_2EDTA and 9% aceto-

Table I. Systolic blood pressure (mmHg) of wistar rats and SHR at 6, 16 weeks of age

Age	6-week-age	16-week-age
Wistar rats	95.00±1.54	106.46±2.87
SHR	118.64±1.35	180.42±2.57

The blood pressure of rats was determined by indirect method using tail-cuff plethysmography. The blood pressure from 4 week old rats could not be detected due to the size of tail. Each value represents the mean±S.E.M. of the data from 10 animals. (*p<0.01, **p<0.001)

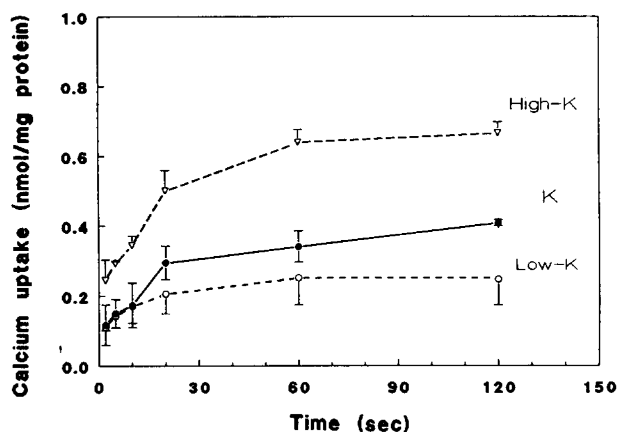

Fig. 1. The time course of calcium uptake into hypothalamic synaptosomes of 16 week old wistar rats. Ca uptake was measured in Low-K (5 mM) and High-K (41 mM) conditions respectively. ΔK: amount of voltage-dependent Ca uptake. These values were calculated by subtracting the amount of uptake in Low-K condition from that in High-K condition.

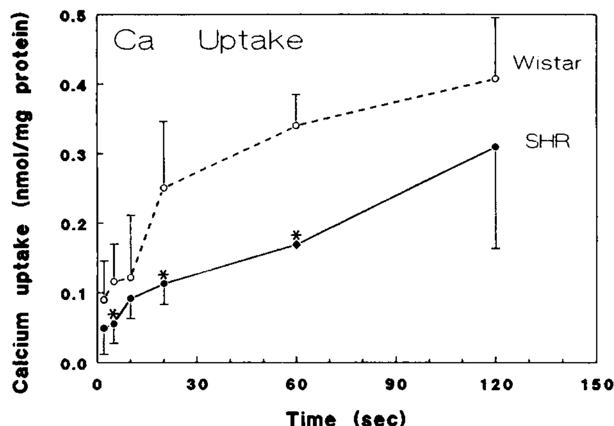

Fig. 2. The time course of voltage dependent calcium uptake into hypothalamic synaptosomes of 4 week old wistar rats and SHR. Each value represents the mean±S.E.M. of the data from 5 animals. (*p<0.05)

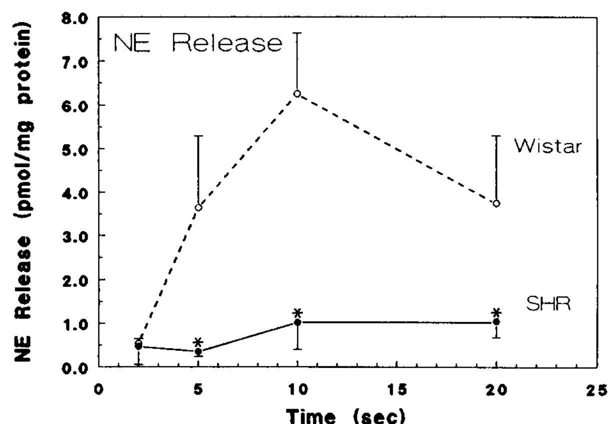

Fig. 3. The time course of voltage dependent norepinephrine release from hypothalamic synaptosomes of 4 week old wistar rats and SHR. Each value represents the mean±S.E.M. of the data from 5 animals. (*p<0.05)

nitrile (pH adjusted to 2.55 with solid NaOH). The flow rate was maintained at 0.7 ml/min. The electrode potential was set at +0.7 V and the sensitivity range was 1 nA-2 nA/V.

Calculation of Data

Voltage-dependent calcium uptake and norepinephrine release were calculated by subtraction of values obtained in low K^+ conditions (5 mM KCl) from values obtained in high K^+ conditions (41 mM KCl).

Statistics

The data were presented as mean±standard error mean. One way analysis of variance followed by Newman-Keuls post hoc test ($p<0.05$) was used to test the significance of differences.

RESULTS

Blood Pressure of SHR and Wistar Rats

The systolic blood pressure of SHR and wistar rats are shown in Table I. At 6 weeks of age, the sytolic pressure of SHR was significantly higher than that of wistar rats (106.46 vs 95.00 mmHg). At 16 weeks of age, hypertension was established in SHR (180.42 mmHg). The blood pressure of rats at 4 weeks of age could not be detected due to the size of tail.

Time Course of Calcium Uptake

Time course of resting and high K^+-stimulated Ca uptake is shown in Fig. 1. The uptake of calcium was markedly increased when the K^+ concentration in the depolarizing medium was raised from 5 mM to 41 mM. The uptake was faster during the initial seconds than that at 120 sec. Calcium uptake into the hypothalamus of SHR and wistar rats is summarized in Table II. Values of calcium uptake into the hypothalamic synaptosome were markedly decreased in 4 week old

Table II. Voltage dependent calcium uptake (nmol/mg protein) into the hypothalamic synaptosome of SHR and wistar rats

		2 sec.	5 sec.	10 sec.	20 sec.	60 sec.	120 sec.
4 week	Wistar rats	0.090± 0.055	0.116± 0.054	0.112± 0.090	0.250± 0.096	0.339± 0.096	0.407± 0.045
	SHR	0.050± 0.037	0.056± 0.028	0.092± 0.028	0.113± 0.029	0.169± 0.000	0.308± 0.145
6 week	Wistar rats	0.072± 0.070	0.095± 0.071	0.185± 0.066	0.185± 0.075	0.332± 0.132	0.416± 0.279
	SHR	0.024± 0.008	0.074± 0.002	0.108± 0.062	0.112± 0.012	0.145± 0.013	0.193± 0.011
16 week	Wistar rats	0.074± 0.010	0.144± 0.011	0.135± 0.035	0.242± 0.097	0.271± 0.095	0.453± 0.056
	SHR	0.036± 0.016	0.080± 0.063	0.113± 0.069	0.158± 0.105	0.253± 0.224	0.306± 0.257

Each value represents the mean± S.E.M. of the data from 5 animals. (*p<0.05)

Table III. Voltage dependent norepinephrine release (pmol/mg protein) into the hypothalamic synaptosome of SHR and wistar rats

		2 sec.	5 sec.	10 sec.	20 sec.
4 week	Wistar rats	0.536± 0.112	3.640± 0.164	6.254± 1.385	3.739± 1.552
	SHR	0.469± 0.412	0.353± 0.114	1.019± 0.618	1.045± 0.374
6 week	Wistar rats	0.538± 0.589	0.609± 0.369	1.561± 0.199	2.417± 0.866
	SHR	0.475± 0.119	0.189± 0.088	1.310± 0.444	1.584± 0.783
16 week	Wistar rats	0.889± 0.484	1.174± 0.572	1.901± 1.137	2.071± 1.045
	SHR	0.608± 0.467	0.146± 0.038	0.633± 0.329	0.429± 0.169

Each value represents the mean± S.E.M. of the data from 5 animals. (*p<0.05)

SHR as compared with normotensive wistar rats at the same age (Fig. 2). However, no significant difference was observed in 6 and 16 week old SHR as compared with age-matched normotensive wistar rats.

Norepinephrine Release from Hypothalamus of SHR and Wistar Rats

Results of norepinephrine release are summarized in Table III. Similarly to the case of calcium uptake, the values of norepinephrine release in 4 week old SHR were significantly smaller than those of normotensive wistar rats at the same age (Fig. 3). No significant difference was observed in 6 and 16 week old SHR as compared with age- matched normotensive wistar rats.

DISCUSSION

The purpose of the present study was to determine whether noradrenergic neural activity in the hypothalamus of SHR is related to the development and/or the maintenance of hypertension. The voltage-dependent norepinephrine release for 20 sec from the hypothalamus was significantly lower in SHR at the prehypertensive phase (4 weeks of age). On the other hand, at the developmental phase (6 weeks of age) and the established phase (16 weeks of age) of hypertension, there were no significant differences between SHR and normotensive wistar rats. These were consistent with the previous reports. Fujino *et al.* (1984) reported that the content of norepinephrine was lower in young SHR than normotensive wistar rats but there was no significant difference between adult SHR and normotensive wistar rats. It was also reported that uptake of norepinephrine in young SHR was greater than that in young normotensive wistar rats, and that in adult SHR was not different from that the norepinephine uptake was the same for both adult SHR and adult normotensive wistar rats (Hano *et al.*, 1989).

The voltage- dependent calcium uptake was determined because calcium is known to enter the nerve terminal as a consquence of a depolarization- induced increase in calcium conductance of the presynaptic membrane, resulting in release of neurotransmitters at critical sites on the inner face of presynaptic membrane (Daniell and Lesli, 1986) and because the measurement of calcium entry into presynaptics membrane gives important information on the activity of the nervous system (Kamisaki *et al.*, 1993; Keith *et al.*, 1993). In the present study, the voltage-dependent calcium uptake for 20 sec into the hypothalamus was decreased at only 4 week old, prehypertensive SHR. At 6 and 16 weeks of age, there were no significant differences between SHR and normotensive wistar rats.

It is interesting to note that an abnormally low release of norepinephrine from the hypothalamus was accompanied by an abnormally deficient voltage-dependent calcium uptake only in the 4 week old, prehypertensive SHR.

It may be that the deficiency of voltage-dependent calcium uptake observed in the prehypertensive phase of young SHR might induce the decrease in intracellular calcium which is essential to evoke neurotransmitter

release. From these observations one can speculate that a change in noradrenergic activity in the hypothalamus may be related to the pathogenesis of hypertension.

In the present study, the deficiency of norepinephrine release observed at the prehypertensive phase, resulting from a deficiency of calcium uptake, seems to act as a triggering factor to evoke hypertension in SHR. This deficiency has not been observed at 6 weeks old, which is equivalent to the developmental phase of hypertension, nor at 16 weeks old age, which is equivalent to the established phase of hypertension. There is no clear answer to the reason why these deficiencies norepinephrine release and calcium uptake exist only at the prehypertensive stage of hypertension and then disappear. However, it may be plausible to suggest that the deficiency might disappear through some compensatory mechanism at the developmental and estabilished phases of hypertension and behave as an assistant factor which might help some major genetic factors triggering precipitation of hypertension.

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