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Charateristics of Voltage Dependent Calcium Uptake and Norepinephrine Release in Hypothalamus of DOCA-salt Hypertensive Rats

Jean Young Lee[1], Hae Jung Kim[1], Eun Young Jung[1] Hye Joo Chung[2] and Kwang Ho Ko[1,3,*]

Department of Pharmacology; [1]College of Pharmacy, Seoul National University, Seoul; [2]National Institute of Safety Research, Seoul and [3]Center for Biofunctional Molecules, Postech, Pohang, Korea

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Abstract—Purpose of the present study was to clarify the role of noradrenergic neural activities in hypothalamus for either triggering or maintaining hypertension in deoxycorticosterone (DOCA)-salt hypertensive rats. Two groups of animals were prepared: 1) normotensive Wistar rats and 2) DOCA-salt induced hypertensive rats. Voltage dependent $^{45}Ca^{++}$ uptake, endogenous norepinephrine release, and the catecholamine content in the hypothalamus of DOCA-salt hypertensive and normotensive Wistar rats were compared. Animals at 4, 6 and 16 week-old of two groups were sacrificed by decapitation and hypothalamus was dissected out. Voltage dependent calcium uptake and norepinephrine release were determined from hypothalamic synaptosomes either in low potassium or high potassium stimulatory condition by using $^{45}Ca^{++}$ isotope and HPLC-ECD technique. Degrees of voltage dependent $^{45}Ca^{++}$ uptake and norepinephrine release in hypothalamic synaptosomes of 16-week-old DOCA-salt hypertensive rats were significantly greater than those of age matched normotensive control rats. The norepinephrine and dopamine contents of hypothalamus were about the same in two groups of animals. These results suggest that the alteration of evoked norepinephrine release related to calcium uptake in hypothalamus may play a role in the maintenance of hypertension in DOCA-salt hypertensive rats.

Keywords □ hypertension, hypothalamus, norepinephrine, calcium, DOCA, rat.

Catecholaminergic neural activities play an important role in hypertension. Evidence exists for the participation of central neural factors in several types of hypertension as well as peripheral factors (Shonis and Waldrop, 1993; Li *et al.*, 1992). Increased peripheral sympathetic activity has been recorded directly (Judy *et al.*, 1976) and the development of hypertension can be attenuated by peripheral sympathectomy (Folkow and Hallback, 1977). Central catecholaminergic neurons play an important role in the regulation of blood pressure and in the expression of some forms of hypertension (Luque *et al.*, 1991; Yao *et al.*, 1989). Pretreatment with 6-hydroxydopamine prevents the development of some forms of hypertension, including those of spontaneously hypertensive rats (SHR) and deoxycorticosterone (DOCA)-salt hypertensive rats. In various brain areas, the changes were reported in the synthesis, release, content, and turnover rate of central catecholamines in SHR. Such examples include elevated norepinephrine levels in the pons, cerebellum and spinal cord, increased dopamine release from superfused hypothalamus and increased norepinephrine turnover rate in dorsomedial hypothalamic nucleus, etc.. However, there were some conflicting results and the role of individual brain areas in regulation of blood pressure and the relationship or interconnection of these regions are not clear yet. Thererfore, hypertension-associated alterations in various brain regions need to be further investigated.

Several brain regions are involved in the regulation of blood pressure, but of the most importance is the hypothalamus among those as a higher center. The hypothalamus has been shown to play a major role in the integration of baroreceptor and chemoreceptor reflexes. Hypothalamus seems to be of prime importance in the regulation of sympathetic activity. Electrical

* To whom correspondence should be addressed.

stimulation of posterior hypothalamus elicited a rise in the mean arterial blood pressure (Philippu *et al.*, 1973) and electrical stimulation of the posterior area of hypothalamus led to pressor response which was more pronounced in SHR than in normotensive rat (Juskevich *et al.*, 1978). In addition, it was found that the activities of norepinephrine synthesizing enzymes tyrosine hydroxylase and dopamine β-hydroxylase in the hypothalamus of SHR were greater than those of normotensive rats. Further, there was a significant correlation between hypothalamic DBH activities and blood pressure in SHR (Nagaoka and Lovenberg, 1977). These data indicate that hypothalamus plays a major role in the control of blood pressure through central catecholaminergic nervous system.

The purpose of the present study was to clarify the role of noradrenergic neural activities in hypothalamus in hypertension of DOCA-salt hypertensive rats. To investigate the pathophysiological mechanism for the manifestation of hypertension related to noradrenergic neural activities in hypothalamus, the following factors were examined from DOCA-salt hypertensive rats: First, voltage dependent calcium uptake was determined as a criterion of neural activities in hypothalamus. Second, norepinephrine content and voltage dependent norepinephrine release were determined to define parameters to the noradrenergic activities. In order to find whether these factors play a role either in development or in maintenance of hypertension, the experimental animal groups were subdivided according to ages and treatment periods, whose blood pressures were maintained within certain ranges. We expect that the findings from the present study may also be a clue to differentiate the noradrenergic role in essential hypertension and DOCA-salt hypertension.

Materials and Methods

Materials

Sucrose, EDTA, EGTA, HEPES, sodium chloride, potassium chloride, magnesium chloride, calcium chloride, glucose, POPOP, PPO, Trizma base and Triton X-100 were purchased from Sigma Chemical Company (St. Louis, U.S.A.). 45-calcium chloride (1 mCi/mmol) was purchased from Amersham International plac (Amersham, UK) and aluminum oxide from Merck (E. Merck, Darmstadt, Germany). All other reagents were reagent grade. Deionized double distilled water was used in the preparation of reagents and all the experimental procedures and especially filtered for HPLC.

Animals

Male Wistar rats from SNU animal breeding center were used in this experiment. Animals were maintained in air-conditioned and temperature-controlled room (23± 1℃) under controlled light (14 hr light and 10 hr dark), with rat chow and water *ad libitum*. Experimental animals were divided into two groups: 1) DOCA-salt induced hypertensive rats and 2) Normotensive control rats. Experiments were performed at the age of 4-, 6- and 16-weeks in two groups of animals.

DOCA-salt rats received every three days S. C. injections of 12.5 mg/kg of body weight of DOCA dissolved in cotton seed oil and 1% NaCl as drinking water *ad libitum* during certain period. 4-week-old rats had been administered with DOCA for a week, 6-week-old rats for three weeks and 16-week-old rats for four weeks before each reached the age of experiment. A day after last treatment the systolic blood pressure was measured using tail cuff plethysmography (Narcotrace 40, NBS, Houston, Taxas, U.S.A.) according to the procedure described by Pfeffer *et al.* (1971).

Animals were decapitated and brains were rapidly removed from the skull between 10 : 00 and 11 : 00 a.m.. Hypothalamic tissues were dissected on the ice plate including Preoptic area-Anterior hypothalamus-Mediobasal hypothalamus, frozen on dry ice and stored at −70℃ until assayed.

Preparation of Crude Synaptosomal Fraction

Hypothalamic tissues from about 20 rats were homogenized in 20 volumes (v/w) of ice-cold sucrose-EDTA-HEPES solution (0.32 M sucrose, 1 mM EDTA, 2 mM HEPES, pH 7.4) with teflon glass homogenizer. And the homogenate was centrifuged at 1,200 g for 5 min at 4℃. The supernatant was recentrifuged at 17,300 g for 5 min at 4℃. The pellet was suspended in 20 volumes of fresh cold buffer and centrifuged at 17,300 g for 12 min at 4℃. The sediment was resuspended in 7 volumes of ice-cold sucrose-EDTA-HEPES solution and diluted with 30 volumes of low potassium buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 0.06 mM $CaCl_2$, 10 mM HEPES, pH 7.5 with Tris). This was centrifuged at 1,200 g for 5 min at 4℃. The final pellet was resuspended in 15 volumes of low potassium buffer (incubation medium) to make a final protein concentration of approximately 0.8~1.2 mg/ml.

Determination of Voltage Dependent Calcium Uptake

The experiment of voltage dependent calcium uptake was performed by the method of Harris (1985) with modification. 200 μl portion of synaptosomal suspension was preincubated for 12 min at 32℃. Depolarization was carried out by rapid addition of 200μl of

depolarizing solution (73 mM NaCl, 77 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 0.06 mM $CaCl_2$, 10 mM HEPES, pH 7.5 with Tris) containing $^{45}Ca^{2+}$ (4.5×10^5 dpm). At the appropriate time (2, 5, 10, 20, 60 and 120 sec), the uptake of Ca^{2+} was terminated by the prompt addition of 5 m*l* of ice-cold EGTA 'stopping solution' (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, 3 mM EGTA, pH 7.5 with Tris). Each sample was immediately filtered through a pre-soaked Whatman GF-B filter using a Hoefer vacuum filtration manifold (vacuum at 25 mmHg). Each filter was washed with two 5 m*l* portion of ice-cold EGTA buffer within 10 sec and placed in a scintillation vial to which 10 m*l* of toluene based cocktail was added and counted in a liquid scintillation counter. To calculate the net uptake of Ca^{2+} into synaptosomes, the uptake in 5 mM KCl condition where depolarization is absent was subtracted from the uptake 77 mM KCl condition where depolarization is present. This value represents net KCl-induced calcium uptake and was expressed in nmol Ca/mg protein.

Determination of Voltage Dependent Norepinephrine Release

The experiment of voltage dependent norepinephrine release was performed by the method of Daniel and Leslie (1986) with modification. As was done in calcium uptake experiment, synaptosomal suspensions were preincubated and norepinephrine release from synaptosomes was initiated by addition of the same aliquot of either depolarizing or non-depolarizing solution. At the time of 2, 5, 10 or 20 sec, the release of norepinephrine was terminated by addition of 1 m*l* of ice-cold EGTA buffer. Each sample was immediately filtered through a pre-soaked Whatman GF-B filter, which was washed with two 1 m*l* portions of ice-cold EGTA buffer within 10 sec. Filtrates (containing released norepinephrine) were collected in test tubes containing 100 μl H_3PO_4, 2.5 ng dihydroxybenzylamine as an internal standard, and 2 mg sodium bisulfite. Catecholamine was extracted by the method of Hamlet *et al.*(1981) with slight modification. Recoveries of internal standard were 60~70%. The usual injection volume for HPLC analysis was 5 μl. The HPLC system (Bioanalytical system 400) consisted of a pump (BAS LC-22A), electrochemical detector (BAS LC-4B) and injection valve (BAS model 7175). The analytical column used for chromatographic separation of catecholamines was Biophase cartridge column (length 10 cm; particle size 3 μm). The mobile phase consisted of 0.1 M citric acid, 0.225 mM octyl sodium sulfate, 0.06% triethylamine, 0.05 mM Na_2EDTA and 9% acetonitrile (volume basis) and adjusted to pH 2.55 with solid NaOH. The working potential of electrochemical detector was set at +0.7 V relative to the Ag/AgCl reference electrode, and the detector sensitivity was set at 0.5 nA/volt. The flow rate was maintained at approximately 0.7 m*l*/min. Quantitization of norepinephrine in samples was performed by comparison of peak heights with those of known concentrations of standard curves. Data obtained as ng norepinephrine released per 5 μl were converted to pmol norepinephrine released/mg protein. The net KCl-induced release of norepinephrine was calculated by subtracting the release in absence of depolarization from the release in presence of depolarization.

Measurement of Catecholamines

Norepinephrine and dopamine in hypothalamic tissues were analyzed by HPLC-ECD system. Each hypothalamus was homogenized with 10 volumes (v/w) of 0.1 N perchloric acid containing 4 mM sodium metabisulfite as antioxidant using glass to glass homogenizer (Radnoti Glass Technology, Inc.). The homogenates were centrifuged at 12,000 g for 10 min at 4℃ and then the supernatant was used for assay. The analytical column used for chromatographic separation of catecholamines was Lichrosorb RP-18 column (length 25 cm; particle size 10 μm; Merck) and was protected by using Hibar precolumn (length 3 cm; particle size 25~40 μm; Merck). The mobile phase used was the same as described above. The working potential of electrochemical detector was set at +0.7 V relative to Ag/AgCl reference electrode, and detector sensitivity was at 20 nA full scale. The flow rate was 1.5 m*l*/min. Catecholamine contents were expressed in ng/mg hypothalamus.

Protein Assay

Protein concentration was determined by the method of Lowry *et al.* (1951).

Analysis of Data

Data were expressed as mean± S.E.M.. For statistical evaluation of data, unpaired t-test was used. Differences were considered statistically significant when $p<0.05$ was obtained.

Results

Blood Pressure

The data on systolic blood pressure of DOCA-salt hypertensive and normotensive control rats are shown in Table 1. There was no significant difference in systolic blood pressure between the groups of animals at 4 weeks of age (88± 3 vs. 89± 3). At the age of

6 weeks, the systolic pressure of DOCA-salt rats was significantly higher than that of control rats (127± 6 vs. 93± 5). Systolic blood pressure rose progressively during DOCA-salt treatment reaching 167± 4 (vs. 99± 4) when it had been given for 4 weeks at the age of 16 weeks.

Voltage Dependent Calcium Uptake

At the age of 4 weeks, voltage dependent calcium uptake into hypothalamic synaptosomes did not differ between DOCA-salt and control rats. At the age of 6 weeks, voltage dependent calcium uptake into hypothalamic synaptosomes of DOCA-salt rats was slightly greater than that of controls but there was no statistical significance. At the age of 16 weeks, voltage dependent calcium uptake of DOCA-salt rat was significantly greater than that of controls. At 2, 5, 10, 20 and 60 sec there were statistical significances between DOCA-salt and control rats (Fig. 1). The results of voltage dependent calcium uptake were summarized in Table II.

Voltage Dependent Norepinephrine Release

At the age of 4 weeks, voltage dependent norepinephrine release did not differ between DOCA-salt and control rats as the results of calcium uptake of age matched groups of animals. At the age of 6 weeks, voltage dependent norepinephrine release of DOCA-salt rat was slightly greater than that of controls but there was no statistical significance as the results of calcium uptake. At the age of 16 weeks, voltage dependent norepinephrine release of DOCA-salt rats was significantly greater than that of age matched control

Table I. Systolic blood pressure (mmHg) of DOCA-salt Hypertensive and normotensive control rats at 4, 6 and 16 weeks of age

Age	Control	DOCA-salt
4-week-old	88± 3	88± 3
6-week-old	93± 5	127± 6**
16-week-old	99± 4	167± 4**

The values are expressed as the mean± S.E.M.
**: $p<0.01$.

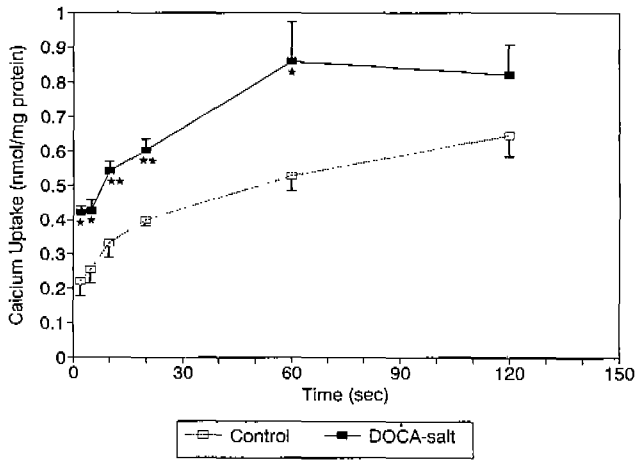

Fig. 1. The Time course of voltage dependent calcium uptake into hypothalamic synaptosomes of 16-week-old DOCA-salt and normotensive control rats (*: $p<0.05$,**: $p<0.01$).

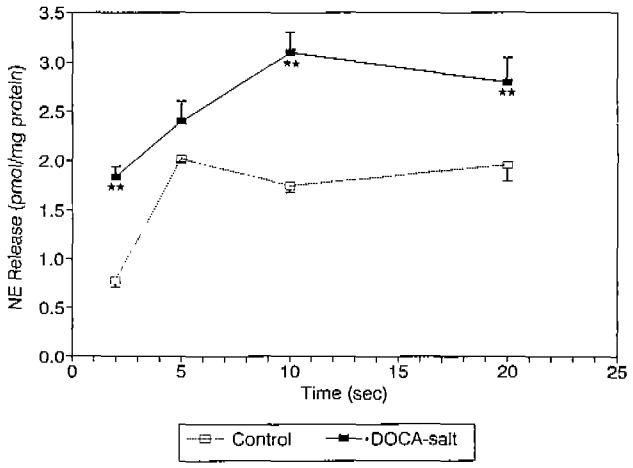

Fig. 2. The Time course of voltage dependent norepinephrine release from hypothalamic synaptosomes of 16-week-old DOCA-salt and normotensive control rats (*: $p<0.05$,**: $p<0.01$).

Table II. Voltage dependent Calcium uptake (nmole/mg protein) into hypothalamic synaptosome of DOCA-salt hypertensive and normotensive control rats

	Age	2 sec	5 sec	10 sec	20 sec	60 sec	120 sec
Control	4 weeks	0.136± 0.035	0.145± 0.041	0.235± 0.009	0.344± 0.075	0.503± 0.107	0.605± 0.114
	6 weeks	0.140± 0.016	0.155± 0.020	0.307± 0.040	0.414± 0.060	0.667± 0.087	0.643± 0.075
	16 weeks	0.220± 0.041	0.254± 0.040	0.331± 0.042	0.399± 0.017	0.529± 0.045	0.644± 0.059
DOCA-salt	4 weeks	0.101± 0.029	0.183± 0.032	0.203± 0.060	0.391± 0.017	0.526± 0.130	0.588± 0.109
	6 weeks	0.148± 0.017	0.220± 0.037*	0.305± 0.031	0.559± 0.039	0.727± 0.095	0.820± 0.093
	16 weeks	0.422± 0.015*	0.425± 0.032*	0.543± 0.028*	0.603± 0.032**	0.861± 0.111*	0.821± 0.087

The value are expressed as the mean± S.E.M.
*: $p<0.05$, **: $p<0.01$.

Table III. Voltage dependent norepinephrine release (pmol /mg protein) from hypothalamic synaptosome of DOCA-salt hypertensive and normotensive control rats

	Age	2 sec	5 sec	10 sec	20 sec
	4 weeks	0.915	0.119	0.1002	0.787
		± 0.092	± 0.105	± 0.086	± 0.133
Control	6 weeks	0.598	1.126	0.103	1.095
		± 0.081	± 0.109	± 0.067	± 0.119
	16 weeks	0.773	2,020	1.740	1.957
		± 0.075	± 0.053	± 0.069	± 0.017
	4 weeks	1.066	1,225	0.979	0.974
		± 0.072	± 0.071	± 0.147	± 0.080
DOCA-salt	6 weeks	0.826	1.338	1,250	1,254
		± 0.222	± 0.130	± 0.109	± 0.019
	16 weeks	1.831**	2,400	3.101**	1.794**
		± 0.110	± 0.205	± 0.204	± 0.250

The value are expressed as the mean± S.E.M.
*: $p<0.05$, **: $p<0.01$.

Table IV. Content of norepinephrine (NE) and dopamine (DA) in the hypothalamus of DOCA-salt hypertensive and normotensive control rats

	Age	NE (pmol/mg hypothalamus)	DA
	4 weeks	1.916± 0.286	0.935± 0.108
Control	6 weeks	2.974± 0.479	2.177± 0.654
	16 weeks	4.150± 0.663	1.514± 0.218
	4 weeks	1.720± 0.220	0.929± 0.173
DOCA-salt	6 weeks	3.239± 0.694	1.855± 0.421
	16 weeks	3.360± 0.710	1.259± 0.140

The value represents the mean± S.E.M. of the data from seven hypothalami.

rats as the results of calcium uptake. At 2, 10 and 20 sec there were statistical significances between DOCA-salt and control rats (Fig. 2). The results of voltage dependent norepinephrine release were summarized in Table III.

Catecholamine Content

The concentrations of norepinephrine and dopamine did not differ between DOCA-salt and control rats in hypothalamus at 4, 6 and 16 weeks of age. The values of contents of noreinephrine and dopamine were summarized in Table IV.

Discussion

The present study was designed to investigate the differences in the noradrenergic neural activities in hypothalamus between DOCA-salt hypertensive rats and normotensive control rats. Degrees of voltage dependent calcium uptake and norepinephrine release in hypothalamic synaptosomes of 16-week-old DOCA-salt hypertensive rats were significantly greater than those of age matched normotensive control rats. But, at the age of 4 and 6 weeks, there was no statistical significance between two groups of animals, even though degrees of voltage dependent calcium uptake and norepinephrine release in hypothalamic synaptosomes of 6-week-old DOCA-salt rats had a tendency to be slightly greater than those of age matched control rats. The norepinephrine and dopamine contents of hypothalamus were about the same in two groups of animals, and these findings are consistent with the observations of Fujino (1984). At this point it should be kept in mind that an increased rate of release of neurotransmitters does not need to be accompanied by alteration in their tissue concentrations. Therefore, in order to investigate neural activities, we should examine dynamic factor as well as static factor. In present study, we measured voltage dependent calcium uptake and norepinephrine release as dynamic factors and 16 week-old DOCA-salt rats showed increased noradrenergic neural activities in hypothalamus.

Enhancement of voltage dependent norepinephrine release from hypothalamic synaptosomes was found only when voltage dependent calcium uptake was increased in the group of DOCA-salt hypertensive rats at the age of 16 weeks. This result from the present study was consistent with 'Calcium hypothesis' (Katz, 1969). Calcium entry triggers the release of transmitter substance which is norepinephrine in this situation. Synaptosomes prepared from rat hypothalamus are heterogeneous and include dopaminergic ones. Since dopamine release from nerve terminals is also calcium dependent, it is not surprising that increased $[K]_o$ also stimulates calcium dependent dopamine release from rat hypothalamic synaptosomes. But Danielle and Leslie (1986) already showed the good correlation of rates of calcium entry and release of endogenous norepinephrine in hypothalamic synaptosome, and our data were consistent with the previous report, though rate coefficient of calcium uptake and norepinephrine release were not same exactly. Thus these data indicate that voltage dependent calcium uptake may be a useful factor for investigating presynaptic neural transmission in noradrenergic system.

There have been some reports demonstrating that noradrenergic nervous systems in hypothalamus are involved in the regulation of cardiovascular functions and in the development of certain forms of hypertension (Hano *et al.* 1989; Qualy and Westfall, 1988; Meldrum and Westfall, 1986). In the present study, the voltage dependent calcium uptake and norepinephrine re-

lease in hypothalamus were equal to those of control group when blood pressure was within normal range in the group of DOCA-salt rats. The voltage dependent calcium uptake and norepinephrine release in hypothalamus were significantly increased when blood pressure was fully elevated after DOCA-salt treatment. Because the enhancement of noradrenergic neural activity in hypothalamus was found in 16-week-old DOCA-salt rat of which the blood pressure was fully elevated, this change may play a role in maintenance of hypertension. Calaresu and Cirello (1980) suggested that paraventricular nucleus in hypothalamus be a part of a feedback reflex loop involved in the integration of baroreceptor reflexes and in the maintenance of tonic inhibition on cardiovascular reflexes probably at the level of the medullar and spinal cord. This report supports that an increased release of endogenous NE in hypothalamus might lead to a rise in blood pressure. However, it is also possible that the increased calcium uptake and norepinephrine release were secondary to the hypertension. It is necessary to perform further experiment in order to elucidate the causal relationship between these changes.

Finally, the results of the present study support the importance of noradrenergic nervous system in hypothalamus in regulation of blood pressure and pathophysiology of DOCA-salt induced hypertension.

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