Morphological Studies on the Autonomic Nervous System of Hypertensive Rats

IV. Fluorescence Microscopical Observation on the Superior Cervical Sympathetic Ganglia of Spontaneously Hypertensive Rats*

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ALTHOUGH the morphological basis of studies on the adrenergic mechanisms in sympathetic nervous system has been somewhat poor by reason of the lack of convincing methodology, the highly sensitive and specific fluorescence method of FALCK and HILLARP^{2,3,8,9} made it possible to demonstrate cellular catecholamines directly, and has contributed to the progress of the works in this field. In the present study, the superior cervical sympathetic ganglia of spontaneously hypertensive rats (OKAMOTO and AOKI)^{19,20} were examined applying this fluorescence method in order to understand the outlined feature of distribution of intraganglionic catecholamines and its relation to functional activity.

MATERIALS

A total of 10 male spontaneously hypertensive rats (abbreviated hereafter as S. H. rats) and 10 male normotensive rats (with blood pressure below 150 mmHg) of Wistar strain were used in this study. The S. H. rats were divided into two groups according to survival duration, i. e. (1) those sacrificed at 40 to 50 days after birth, and (2) those at 4 to 5 months after birth. The normotensive rats of almost the same age and body weight as the S. H. rats were

used as the controls. The production of the S. H. rats was performed by the usual method which was previously reported by our department^{19,20}. The normotensive rats were selected out of those obtained by matings of the parent rats which were supplied by the Animal Center Laboratory, Faculty of Medicine, Kyoto University.

The case numbers of the animals are as follows: The S. H. rats of 40 to 50 days of age (167001, 167004, 167006, 167007, 167008)** and their controls (905, 907, 908, 909, 911); the S. H. rats of 4 to 5 months of age (157012, 157013, 157014, 157015, 157016)** and their controls (835, 836, 837, 838, 839).

These animals were kept in the air-conditioned animal room (room temperature and humidity were set at a range of 22° to 24°C and 50 to 60 per cent, respectively) with artificial lighting, and fed with stock chow diets (CLEA CA-1 and CE-2, Japan Clea Co., Tokyo, Japan) and sometimes green vegetables. Tap water was given for drinking ad libitum.

METHODS

Blood pressure of animals was measured as a rule once a week by the tail-water-plethysmographic method (OKAMOTO and AOKI¹⁹), and body weight was checked simultaneously.

Fluorescence microscopical observations on the superior cervical sympathetic ganglia of rats were

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** The F₁₆ animals (167001 ♂, 167004 ♂, 167006 ♂, 167007 ♂, 167008 ♂) were obtained by matings of F₁₅ rats (157005 ♂, 157017 ♂, 157051 ♀, 157065 ♀), and the F₁₅ rats (157005 ♂, 157012 ♂, 157013 ♂, 157014 ♂, 157015 ♂, 157016 ♂, 157017 ♂, 157051 ♀, 157065 ♀) were bred from F₁₄ (140148 ♂, 140150 ♂, 140156 ♀, 140157 ♀). These F₁₄ animals were obtained by matings of F₁₃ rats (130036 ♂, 130038 ♀), which were bred from F₁₂ (120014 ♂, 120054 ♀). The source of these F₁₂ rats was previously reported^{19,20}.

performed by the method described originally by FALCK and OWMAN¹⁰, slightly modified in our laboratory. The details of this method were already mentioned by HAEBARA et al.¹¹ and HAEBARA¹², and only the outline will be presented here. Just after the sacrifice of animals, the superior cervical sympathetic ganglia were extirpated as rapidly as possible, and frozen immediately at -170°C in isopentane cooled indirectly with liquid nitrogen. The tissues were thereafter freeze-dried for 7 days. On that occasion, the frozen tissues were maintained in a bottle which was cooled with acetone/ethyl oxalate/dry ice mixture at -40°C to -35°C , and drying was performed with the apparatus having two-stage mechanical vacuum pumps (Kyowa RL 200 type, Kyowa Vacuum Co., Tokyo, Japan), which provided a vacuum of 1×10^{-5} to 5×10^{-5} mmHg. The fully dehydrated ganglia were treated with formaldehyde vapour at 80°C for 1 hour in a closed desiccator containing paraformaldehyde. Thereafter the specimens were embedded in paraffin in vacuo at 60°C . Sections were cut at 5 microns and placed on non-fluorescent glasses. Deparaffinization and mounting were made simultaneously with Entellan (Merck, Germany)-xylene mixture (1:3) with the aid of heating at 70°C for about 10 minutes.

The fluorescence was observed with Carl Zeiss fluorescence microscope (standard universal type, Carl Zeiss, Germany). An Osram HBO 200 high pressure mercury lamp served as a light source.

The light was passed through a heat-absorbing

filter and a Schott BG 12 filter, and focussed onto a dark field oil-immersion condenser. The emitted light was filtered through a Zeiss "50" filter. For microphotography, SAKURA R-100 positive color films (Konishiroku Photo Ind. Co., Ltd., Japan) were used.

Analysis of fluorescence microscopical findings was performed as follows. That the specific green or yellow-green fluorescence, which was observed in the sympathetic ganglia as mentioned later, was due to the presence of a primary catecholamine, probably noradrenaline, was ascertained by treatments with sodium borohydride, and thionyl chloride followed by sodium borohydride³. Intensity of such fluorescence of individual neurons in the ganglion varied considerably from cell to cell. Therefore, the number of cells in ganglion were differentially counted dividing them into three groups according to fluorescence intensity; i. e., intensely fluorescent cells were included in (+++) group, moderately fluorescent cells in (++) group, and slightly or non-fluorescent cells in (+) group. Thus the obtained frequency distribution of nerve cell populations in the S. H. rats was respectively compared with that in the corresponding controls by χ^2 -test for 95 per cent confidence limit. All these procedures were made under strictly identical conditions throughout with all materials.

RESULTS

Final blood pressure level and body weight

TABLE I MATERIALS SUBJECTED TO FLUORESCENCE MICROSCOPICAL STUDY ON THE SUPERIOR CERVICAL SYMPATHETIC GANGLION

Age	SHR ¹⁾			Normotensive Control Rats		
	Animal No.	Final Systolic Blood Pressure (mmHg)	Body Weight at Sacrifice (g)	Animal No.	Final Systolic Blood Pressure (mmHg)	Body Weight at Sacrifice (g)
40-50 Days	167001	144	125	905	122	100
	167004	134	100	907	116	110
	167005	136	110	908	112	118
	167007	142	120	910	120	114
	167008	136	118	911	116	132
	<i>MV</i> $\pm$ <i>SD</i> ²⁾	$138 \pm 4.4^*$	115 ± 9.8	<i>MV</i> $\pm$ <i>SD</i>	$117 \pm 3.9^*$	115 ± 11.7
4-5 Months	157012	172	382	835	106	392
	157013	200	415	836	116	420
	157014	190	384	837	114	486
	157015	178	378	838	102	340
	157016	170	350	839	120	388
	<i>MV</i> $\pm$ <i>SD</i>	$182 \pm 12.7^*$	382 ± 23.1	<i>MV</i> $\pm$ <i>SD</i>	$112 \pm 7.4^*$	405 ± 53.5

1) Spontaneously Hypertensive Rats

2) Mean Value $\pm$ Standard Deviation

* Statistically Significant ($0.01 > P$)

of the animals were as illustrated in Table I. The blood pressure level of the S. H. rats of 40 to 50 days of age was slightly but definitely higher than that of the controls, having still remained within a normotensive range, and that of the S. H. rats of 4 to 5 months of age was conspicuously high as compared with the controls. As for the body weight of the animals, no remarkable finding could be found.

Under fluorescence microscope the neurons in the superior cervical ganglia of normotensive rats exhibited some types of fluorescence. A relatively homogenous and faint dark green fluorescence was found in the cytoplasm. This background fluorescence was observed in most of them. At the same time, a small amount of orange fluorescence was seen in a form of tiny granules. Further, certain neurons showed a specific green or yellow-green fluorescence. It was observed diffusely, or sometimes more intensely at the peripheral portions, in the perikaryon of neurons, but not in the nucleus. This fluorescence disappeared by borohydride treatment and recovered again by formaldehyde. When treated beforehand with thionyl chloride, following treatment with sodium borohydride

could not decrease the fluorescence. These results, and the color and the conditions of development of fluorescence as well, indicated that this specific fluorescence was due to the presence of, in all probability, noradrenaline. The cell populations with such specific fluorescence were heterogeneous in regard to fluorescence intensity. In addition to the above, a few cells were found in the ganglion in clusters, which exhibited a much higher intensity of specific fluorescence.

These features of the ganglionic neurons were not so different between the younger and older normotensive animals. As to the S. H. rats, the fundamental pictures did not differ from those of the controls, while the mode of distribution of cell populations in respect to their fluorescence intensity varied to a certain extent. Namely, as seen in Table II, the ganglia of the S. H. rats of 40 to 50 days of age contained 14.6 to 18.0 per cent of the nerve cells of (+) group, 45.0 to 50.0 per cent (++) group, and 33.3 to 40.0 per cent (+++), whereas those of the controls contained 18.1 to 30.7 per cent of (+) group, 43.2 to 58.9 per cent of (++) group, and 20.8 to 32.5 per cent of (+++) group.

TABLE II FINDINGS OF THE FLUORESCENCE MICROSCOPICAL STUDY ON THE SYMPATHETIC GANGLIA OF SPONTANEOUSLY HYPERTENSIVE RATS AND NORMOTENSIVE CONTROLS.

Age	Spontaneously Hypertensive Rats					Findings**	Normotensive Control Rats				
	Animal No.	Differential Counts of Nerve Cells According to Fluorescence Intensity*					Animal No.	Differential Counts of Nerve Cells According to Fluorescence Intensity*			
		+	++	+++	Total			+	++	+++	Total
		(%)	(%)	(%)	(%)			(%)	(%)	(%)	(%)
40-50 Days	167001	24 (14.6)	78 (47.6)	62 (37.8)	164 (100.0)	↑	905	36 (24.3)	64 (43.2)	48 (32.5)	148 (100.0)
	167004	18 (16.7)	54 (50.0)	36 (33.3)	108 (100.0)	↑	907	24 (23.5)	55 (53.9)	27 (26.6)	102 (100.0)
	167005	32 (16.9)	89 (47.1)	68 (36.0)	189 (100.0)	←	908	37 (18.1)	114 (58.9)	53 (26.0)	204 (100.0)
	167007	18 (15.0)	54 (45.0)	48 (40.0)	120 (100.0)	↑↑	910	64 (26.8)	120 (50.4)	54 (22.8)	238 (100.0)
	167008	25 (18.0)	66 (47.5)	48 (34.5)	139 (100.0)	↑	911	40 (30.7)	63 (48.5)	27 (20.8)	130 (100.0)
4-5 Months	157012	36 (18.6)	90 (46.4)	68 (35.0)	194 (100.0)	↑	835	54 (28.1)	92 (47.9)	46 (24.0)	192 (100.0)
	157013	20 (16.5)	62 (51.2)	39 (32.3)	121 (100.0)	↑↑	836	54 (25.2)	138 (64.5)	22 (10.3)	214 (100.0)
	157014	25 (19.7)	68 (53.5)	34 (26.8)	127 (100.0)	←	837	35 (25.7)	72 (52.9)	29 (21.4)	136 (100.0)
	157015	50 (16.7)	140 (46.7)	110 (36.6)	300 (100.0)	↑↑	838	36 (24.7)	78 (53.4)	32 (21.9)	146 (100.0)
	157016	42 (21.4)	92 (46.9)	62 (31.7)	196 (100.0)	↑	839	38 (30.6)	62 (50.0)	24 (19.4)	124 (100.0)

* + : Not or Slightly Fluorescent Cells, ++ : Moderately Fluorescent Cells, +++ : Intensely Fluorescent Cells.

** (Findings in spontaneously hypertensive rats were obtained by comparing with those of corresponding controls and examining statistically by χ^2 -test.)

↑↑ : Increased Frequency of Intensely Fluorescent Cells in Spontaneously Hypertensive Rats ($0.01 > P$).

↑ : Increased Frequency of Intensely Fluorescent Cells in Spontaneously Hypertensive Rats ($0.05 > P > 0.01$).

→ : No Significant Difference.

Similarly, in the S. H. rats of 4 to 5 months of age, 16.5 to 21.4 per cent of cells were involved in (+) group, 46.4 to 53.5 per cent in (++) group, and 26.8 to 35.0 per cent in (+++) group, and in the controls, 24.7 to 30.6 per cent were involved in (+) group, 47.9 to 64.5 per cent in (++), and 10.3 to 24.0 per cent in (+++). By statistical examination, a significant increase of frequency of intensely fluorescent nerve cells was proved in 4 of 5 S. H. rats of 40 to 50 days of age, and the same result was obtained also in the S. H. rats of 4 to 5 months of age. To summarize, the intensely fluorescent nerve cells could be observed more frequently in the superior cervical sympathetic ganglia of the S. H. rats than in those of the control animals, irrespective of age.

DISCUSSION

The fact that the formaldehyde-induced specific green or yellow-green fluorescence, which could be observed in sympathetic ganglionic neurons, is due to the presence of a primary catecholamine, in all probability, nor-

adrenaline has been already recognized by certain experiments^{2,3,6,17,21}. Accordingly, the fluorescent ganglionic nerve cells may be regarded as adrenergic neurons, but their detailed functional significance has not been satisfactorily confirmed. Intraganglionic adrenergic mechanism has been believed to act on the ganglionic transmission with inhibitory effects, or to modulate primary cholinergic transmission^{5,18,22}. On that occasion, however, it has yet been unsolved what the physiological meaning of such inhibitory action of adrenergic mechanism is, or when the activity of sympathetic nervous system was accelerated, what kind of change may be induced in such adrenergic neurons. At the same time, it has been said that catecholamines are manufactured and stored in the ganglionic nerve cells and transported along the axons to the nerve terminals^{4,6,7,17}, and also the evidence that nerve terminals, postganglionic axons as well as cell bodies of the adrenergic neurons, have a specific uptake mechanism of noradrenaline has been presented^{1,13,17}. On the basis of these observations, the increase of in-

Fig. 1. Fluorescence microscopical feature of the superior cervical sympathetic ganglion of rat.

Left: A spontaneously hypertensive rat. No. 157013, male, final blood pressure 200 mmHg.

Right: A normotensive control rat, No. 836, male, final blood pressure 116 mm Hg.

The ganglionic nerve cells with intense specific fluorescence are observed more in the spontaneously hypertensive rat than in the control.

tensely fluorescent nerve cells, or, in consequence, that of catecholamine containing neurons, in the sympathetic ganglion may, at least in part, suggest the increased activity of intraganglionic adrenergic mechanism. This leads to the augmented inhibitory action in the sympathetic ganglion, but may not be necessarily connected with the hypoactivity of sympathetic nervous system as a whole, because such mechanism may serve, at most, as a modulator for the principal cholinergic mechanism; in other words, when the ganglionic activity was raised, the enhancement of adrenergic mechanism might possibly have increased together with that of cholinergic transmission. Next, if the catecholamines were synthesized or taken into the ganglionic neurons and brought to the peripheral effector sites along the axons, the increase of catecholamine content may reasonably be appreciated as indicating increased catecholamine synthesis and metabolism, and accordingly, augmented sympathetic postganglionic neuron activity.

From such viewpoints as mentioned above, the present results, that is, the increase of intensely fluorescent cells in the sympathetic ganglia of the S. H. rats may be interpreted as suggesting the possible augmented sympathetic activity in these animals, which has been presented already by other morphological approaches^{14, 15, 16}.

SUMMARY

1. Distribution of cellular catecholamine in the sympathetic neurons of superior cervical ganglia of the rats was studied by fluorescence method of FALCK and HILLARP. A total of 10 male spontaneously hypertensive rats (OKAMOTO and AOKI) (consisting of 5 animals of 40 to 50 days of age and 5 rats of 4 to 5 months of age) and 10 male normotensive control rats of Wistar strain were subjected to examination.

2. The sympathetic neurons in the superior cervical ganglia of the normotensive rats showed specific formaldehyde-induced green or yellow-green fluorescence, which is due to the presence of noradrenaline. The cell populations with such specific fluorescence were heterogeneous in respect of fluorescence intensity.

3. The fundamental fluorescence microscopical feature in the ganglionic neurons of the spontaneously hypertensive rats did not differ from that of the controls. However, by statistical analysis of distribution of cell populations in respect to fluorescence intensity, the significant increase of frequency of intensely fluorescent nerve cells was proved in 4 of 5 spontaneously hypertensive rats of 40 to 50 days of age and 4 of 5 spontaneously hypertensive rats of 4 to 5 months of age.

4. From the above results, possible augmentation of sympathetic neural activity in the spontaneously hypertensive rats was assumed and discussed in relation to the significance of catecholamine in the sympathetic ganglionic neurons.

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