2) Cellular Mechanisms of Cardiovascular Hypertrophy in Spontaneously Hypertensive Rats (SHR) and Their Modification. Yukio Yamori, Takehiro Igawa, Toshimi Kanbe, Motoki Tagami*, Masahiro Kihara, Yasuo Nara, Kengo Fujimara, and Ryoichi Horie. Department of Pathology, Shimane Medical University, Shimane 693, *Sanraku Hospital, Tokyo 101.

Summary
Major cellular abnormalities in the cardiovascular system of SHR detected up to the present "in vitro" under tissue culture condition were accelerated proliferation and deviated ionic transport of vascular smooth muscle cells (SMC). The experimental modification of these abnormalities 'in vitro' and 'in vivo' indicated that neural innervation and/or catecholamines as well as sodium and nutritional conditions might substantially affect the basic process of hypertension-induced vascular changes, hypertrophy and/or hyperplasia of medial SMC which further raised the peripheral vascular resistance in hypertension.

Introduction
Peripheral vascular resistance is mostly elevated both in genetic hypertension in rats and in primary (essential) hypertension in humans (1). Studies in SHR up to the present disclosed that this elevation was functional due to increased sympathetic tone in the initial stage but was later on caused partially by nonfunctional structural changes of resistance vessels (2-4). In order to clarify the cellular mechanisms involved in the functional and structural elevation of peripheral vascular resistance, vascular smooth muscle cells (SMC) were obtained by an explant method from the thoracic aorta of SHR, stroke-prone SHR and Wistar-Kyoto rats (WKY) (5) and their characteristics related to proliferation and ionic transport were examined under various tissue culture conditions with altered sodium (6) and serum concentration (7) or modified by adding catecholamines or calcium blockers. Since these 'in vitro' experiments indicated that catecholamines, especially β agonistic effect affected the protein synthesis of SMC (5), the effect of life long treatment of a β blocker on blood pressure and heart size was observed in SHRSP and compared with the effect of trichloromethiazide.

Materials and Methods
Experiment 'in vitro': Medial segments of the thoracic aorta from age-matched SHR, SHRSP and WKY were placed on a surface of plastic culture flasks and incubated in modified medium 199 (8) with 10% fetal calf serum (FCS) at 37°C in humidified air with 5% CO₂. SMC outgrowth around these explants was numerically observed and transferred into subculture after trypsinization.

The growth rate of these SMC under subculture was obtained from the slope of growth curves and expressed as mean doubling times (MDT). DNA synthesis was estimated by an isotopic method of ³H-thymidine (9), and Na⁺ requirement for the stimulation of DNA synthesis in SMC from SHRSP, SHR and WKY was observed in the culture media with different concentration of Na⁺ (0-150 mmol/l) for 20 hours. The effect of various concentrations of diltiazem, a Ca-blocker, on DNA synthesis was also observed.

For measuring ornithine decarboxylase (ODC) activity (10), 10% FCS or catecholamines—norepinephrine or epinephrine (2 μg/ml) with or without DL-propranolol (5 μg/ml) was added to the culture media after SMC were kept in FCS-free media for 16-18 hours. SMC were washed with phosphate buffered saline at selected time intervals and suspended in a solution containing 5 mm dithiothreitol, 0.2 mM pyridoxal phosphate, 4 mM EDTA and 50 mM tris-HCl (pH 7.4). After sonication and centrifugation the supernatant fraction was assayed for ODC activity by determining the generated ¹⁴CO₂ from 0.05 μCi L(¹⁴C)-ornithine, which was trapped with Hyamine hydroxide and counted by a liquid scintillation spectrometer.

Total cell Na⁺ and K⁺ were measured with a flame photometer. 1 mM ouabain
with or without WKY serum was added to the cultured SMC. At selected time intervals, SMC were washed rapidly six times with 0.1 M MgCl$_2$ at 4°C. After the removal of the last MgCl$_2$ wash, 1.0 ml of 5% trichloroacetic acid (TCA) was added to extract Na$^+$ and K$^+$. SMC were solved in 0.5N NaOH for analyzing protein content. Na$^+$ and K$^+$ contents of SMC were expressed in µmol/mg of cell protein. $^2$Na$^+$ uptake was assayed in the medium containing 140 µM choline chloride, 2.7 mM Cl, 10 mM Tris-HCl (pH 7.5) and 1 mM ouabain. After one hour incubation at 37°C 1 µCi/ml $^2$Na$^+$ was added. Uptake was stopped 5 min later by placing the dish on ice and washing six times with 0.1 M MgCl$_2$. $^2$Na$^+$ in SMC was extracted into 1.0 ml of 5% TCA and counted in a gamma counter (Packard, model 5360).

For electron microscopy, SMC grown on films in the medium with or without 10 or 15% of FCS for 10 to 15 days were fixed by 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature. After osmication by 2% osmium tetroxide for 2 hrs, the cells were stained with 2% uranyl acetate in 50% ethanol for 1 hr at 4°C, dehydrated in graded ethanol and embedded in Epon 812. Thin sections were cut on a Sorvall Porter-Blum, MT-2B ultratome, and stained with lead citrate. These sections were examined by a transmission electron microscope (Hitachi HS-9).

Experiment 'in vivo': Six pregnant litter mates of female SHRSP were grouped into 3 of equal number. One group was given pindolol (125 mg/l) and the other trichloromethiozide (500 mg/l) in drinking water. The remainder served as the controls. After delivery, the offspring was given these drugs throughout nursing and after weaning. The same rats were continuously given drugs in drinking water until senescence. As a result, 12 rats were obtained for the control group, 7 for pindolol and 9 for trichloromethiazide at entry. Body weight and blood pressure were checked at the age of 13 and 15 months. Blood pressure (by an indirect tail-pulse pick up method) was measured several times and the average was used for analysis. Organ weights were checked after sacrifice by decapitation over the age of 15 months.

Results

1) Cultured SMC from SHR and SHRSP at the age of 1 to 11 months migrated faster than those from WKY. The ratios of positive explants with migrating SMC observed 2 weeks after explantation were 29, 54 and 68% in 1-month-old WKY, SHR and SHRSP, respectively, and 30, 45 and 47% in 3-month-old WKY, SHR and SHRSP, respectively.

2) Growth rates of subcultured SMC were obviously greater in SHR and SHRSP than in WKY. The mean doubling times were 51.2 ± 9.1, 31.1 ± 4.4 and 23.8 ± 2.3 hours in the SMC obtained from 12-week-old WKY, SHR and SHRSP, respectively and 46.6 ± 10.8, 30.0 ± 3.6, 26.2 ± 1.2 hours in the SMC obtained from 24-week-old WKY, SHR and SHRSP, respectively.

3) DNA synthesis measured by $^3$H-thymidine incorporation was greater in SMC from SHR and SHRSP than in those from the age-matched WKY.

4) The growth of SMC from WKY, SHR and SHRSP as estimated by DNA synthesis was accelerated similarly with the elevation of Na$^+$ concentration in the medium, in the absence of 10% FCS. The growth of SMC from SHR and SHRSP was much more stimulated by the lower concentration of Na$^+$ than that of WKY in the presence of 10% FCS: Na$^+$ concentration required for half maximum stimulation of DNA synthesis was 42 and 90 mmol/l, in SMC from SHR and WKY, respectively. The intracellular Na$^+$/K$^+$ ratio tended to be higher in SMC from SHR than in those from WKY at the lower extracellular Na$^+$ concentration (50 mmol/l) in the medium with 10% FCS.

5) Enhanced DNA synthesis in SMC from SHR and SHRSP estimated by $^3$H-thymidine incorporation was more sensitively suppressed by 10$^{-4}$M diltiazem down to the level in SMC from the age-matched 1-month old WKY.

6) When Na$^+$ -K$^+$ ATPase was inhibited by 1 mM ouabain, Na$^+$ content of SMC increased as K$^+$ content fell. Na$^+$ influx and K$^+$ efflux were greater in SMC from
SHRSP and SHR than in those from WKY. The addition of 10^{-4}M diltiazem did not affect these results.

7) ODC activity of cultured SMC was greatly activated 30-40 times above the basal level within 5 hours by the addition of 10% FCS. These stimulated ODC activities in SMC from SHRSP and SHR were 1.7 and 1.5 times greater than that of SMC from the age-matched WKY.

8) Isoprenaline but not norepinephrine markedly stimulated ODC activity of cultured SMC and this activation was completely blocked when β blocker, propranolol was added to the medium 30 min. in advance. Propranolol itself did not affect ODC activity.

9) When 10% or 20% FCS was added to the medium, cultured SMC were activated and transformed into proliferative form with more frequent mitosis and with lesser intracellular myofilaments. Intercellular space became narrow with little amount of collagen. In contrast, when 10% FCS was deprived for 7-10 days cultured SMC became multi-layered and changed into mature form with numerous myofilaments. Intercellular space became widened with much collagen. Intracellular degenerative changes and intercellular abundant collagen were noted, when the deprivation of 10% FCS was extended for over 15 days.

10) After the life-long treatment of commonly-used antihypertensive agent, β blocker (pindol) and thiazide diuretics (trichloromethiazide), blood pressure at the age of 13 and 15 monts were significantly decreased in both groups of SHRSP compared with nontreated control group, and heart weight even in the absolute value was significantly decreased only in pindol group in spite of their greater body weight. No significant reduction in heart weight was noted in trichloromethiazide group although their blood pressure reduction was greater than that of pindol group.

<table>
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<th>Table I. Effect of Life-long Treatment of β-blocker and Diuretics on Blood Pressure, Body Weight and Heart Weight in SHRSP</th>
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<td>Blood Pressure (mmHg)</td>
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<td>control</td>
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<td>trichloromethiazide</td>
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*, **, ***: Significant difference from control (p<0.05, 0.01, 0.001)

Discussion

Although cardiovascular hypertrophy is the most common finding noted in hypertension, both in SHR (3) and humans (2) and has mostly been regarded as the secondary phenomenon due to hypertension, early development of cardiac hypertrophy (12) as well as early acceleration of cardiovascular protein synthesis (13) which do not always parallel with blood pressure level indicate the involvement of some other factors in the development of cardiovascular structural changes in hypertension (4, 7). In order to analyze such factors under a condition free from blood pressure and also free from complicated humoral influence, vascular SMC from these strains of rats, SHRSP, SHR and WKY were obtained under tissue culture condition. The present ‘in vitro’ studies have indicated that accelerated growth activity of SMC appears to be intrinsic in genetic hypertension but also is affected by the β effect of catecholamine, extracellular Na+ concentration and local nutritional conditions such as the presence or absence of serum.

Recent studies on the heart (14) and mesenteric arteries of SHR (15) showed Na+, K+-ATPase activity was rather reduced after the establishment of hypertension and also suggested that Na+-Ca2+ exchange existed or rather enhanced in the membrane fraction of mesenteric arteries. Since ouabain insensitive Na+
and \( K^+ \) permeability or leakiness was increased in SMC from SHR and SHRSP, intracellular Na\(^+\) balance might be more easily affected in these SMC, when they were exposed to different concentration of extracellular Na\(^+\). Such intracellular ionic imbalance not only alters sensitivity to vasoactive substances through a tendency to membrane depolarization but also stimulates the contraction of SMC probably through Ca\(^{2+}\)-related mechanisms. Since diltiazem, a Ca-blocker, suppressed the enhanced DNA synthesis in SMC of SHR, intracellular Ca\(^{2+}\) may be involved in the activation of growth activity in these SMC.

The activation of ODC activity in SMC by a \( \beta \) agonist and its suppression by a \( \beta \) blocker further indicated the involvement of \( \beta \) mechanisms in the neural or circulating catecholamine-induced cardiovascular structural changes. This was partly supported by the significant reduction of heart sizes after the life-long treatment of a \( \beta \) blocker, which could not be observed in thiazide diuretics-treated group even with significantly lower blood pressure.

Finally, the present 'in vitro' observation on the transformation of SMC under different medium with or without serum suggests the importance of local nutritional factors in the development of hypertension-related vascular lesions. Medial hypertrophy and myointimal cell proliferation are caused not only by physical stress of hypertension but also may be stimulated by more serum component penetrating into vascular wall in hypertension. This may also explain the mechanisms for SMC degeneration and necrosis without hypertrophic responses in intracerebral arteries in hypertension, since these SMC not directly innervated are neither exposed to serum nor to circulating catecholamines due to the tight blood-brain barrier. This 'in vitro' observation may be also applied to the explanation for preventive effect of high protein diet on stroke caused by arterionecrosis in SHRSP.

References