Possible Involvement of Androgen in Increased Norepinephrine Synthesis in Blood Vessels of Spontaneously Hypertensive Rats

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ABSTRACT—We investigated the effects of castration and testosterone propionate on sympathetic nervous systems in spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY). Four-week-old male rats were castrated. For replacement of androgen, testosterone propionate (500 μ g/rat) was administered subcutaneously 2 times a week to castrated rats after their 14th week. The systolic blood pressure of the castrated SHR (44 weeks) was significantly lower than those of intact SHR and testosterone-replaced SHR. The norepinephrine (NE) levels and the tyrosine hydroxylase (TH) activities in the abdominal aorta and mesenteric artery of castrated SHR (45–50 weeks) were significantly lower than those of intact SHR. The NE levels and the TH activities in these blood vessels of testosterone-replaced SHR recovered to the levels obtained in those of intact SHR. As well as the systolic blood pressure, the NE levels and TH activities in blood vessels of WKY were significantly lower than those of intact SHR and showed no significant difference among the three groups. These results suggest that androgen may contribute to the development of hypertension in SHR via sustained enhancement of TH activity in blood vessels leading to increased NE level.

Keywords: Tyrosine hydroxylase, Norepinephrine, Androgen, Blood vessel, Spontaneously hypertensive rat

The sympathetic nervous system is thought to play an important role in pathogenesis of hypertension in spontaneously hypertensive rats (SHR) (1). Many studies suggested that norepinephrine (NE) in the plasma and heart is increased concomitantly with the increased catecholamine synthesizing enzyme activities in blood vessels of SHR (2–4). Judy et al. (5) reported that sympathetic nerve activity is increased in the nerves distributing to the abdominal regions in SHR. Iriuchijima (6) reported that the high vascular resistance in hindquarter and mesenteric arteries of SHR is maintained by an elevated sympathetic tone. Therefore, increased sympathetic tone or sympathetic nerve activity in arteries of these regions may contribute to hypertension in SHR.

There are epidemiological and experimental evidence suggesting that androgen contributes to hypertension (7, 8). We have reported that castration retards the development of hypertension in SHR (9). Cambotti et al. (10) demonstrated that neonatally-androgenized female SHR exhibited a pattern of increase in blood pressure similar to that of male SHR during maturation. Furthermore, Ganten et al. (11) demonstrated that chemical castration with cyproterone and flutamide, which are androgen

receptor antagonists, attenuated the development of hypertension in male SHR. Thus, androgen appears to be involved in the development and maintenance of hypertension in SHR, but its mechanisms are not yet clear. Recently, Hamill and Schroeder (12) reported that androgen facilitates neuronal activity. Therefore, it is expected that androgen might elevate blood pressure in SHR via potentiation of sympathetic nerve activities.

To evaluate this assumption, in the present study, we investigated the effects of castration and testosterone on NE level and tyrosine hydroxylase (TH) activity as indices of sympathetic nerve activities in the abdominal aorta and mesenteric artery of SHR.

MATERIALS AND METHODS

Animals

Four-week-old male SHR and Wistar Kyoto rats (WKY) were castrated under light ether anesthesia. The rats were housed in a semi-barrier system, in a room where temperature ($23 \pm 1^\circ\text{C}$), humidity ($55 \pm 5\%$) and lighting (06:00–18:00) were controlled.

Drug treatment

Testosterone propionate (500 µg/rat; Wako, Osaka) dissolved in sesame oil (500 µg/0.1 ml) was administered subcutaneously 2 times a week to castrated rats between their 14th week and the week of their sacrifice.

Blood pressure measurement

Systolic blood pressure was measured by the tail cuff method (Riken Kaihatsu PS-100, Tokyo) in conscious animals placed on a hot plate (37°C) at the age of 44 weeks. Six to seven blood pressure readings were obtained for each rat, and these were averaged.

Norepinephrine analysis

Rats were decapitated at the age of 45–50 weeks (24 hr after the final administration of testosterone propionate), and the abdominal aorta and mesenteric arteries were quickly removed. These tissues were defatted and rinsed with cold saline.

The tissues were homogenized in 1.05 ml of ice-cold 0.05 M perchloric acid with 5 ng dihydroxybenzylamine, an internal standard, in a glass tissue grinder. The homogenate was centrifuged at $15,000 \times g$ for 20 min at 4°C and the supernatant was used for the assay of NE. NE was extracted with aluminum oxide. The supernatant was mixed with 10 mg aluminum oxide and 100 µl of 2 M Tris-EDTA (pH 8.7) for 15 min. The precipitate was washed with 1 ml of 16.5 mM Tris-EDTA (pH 8.1). The precipitate was then dried and mixed with 200 µl of solvent medium (acetic acid : 10% sodium metabisulfite : 5% EDTA : water = 0.1 : 0.05 : 0.05 : 9.8) for 15 min, followed by centrifugation at $1,800 \times g$ for 1 min. The supernatant was passed through a 0.22-µm filter, and a 10-µl aliquot was injected into the HPLC (Waters, Milford, MA USA, 510) instrument, which was equipped with an electrochemical detector (Waters 460) and a Cosmosil 5C₁₈-AR packed column (4.6 × 150 mm; Nacalai Tesque Co., Kyoto). The mobile phase consisted of the following components: 50 mM sodium acetate, 20 mM citric acid, 3.75 mM sodium octyl sulfate, 1 mM di-*n*-butylamine, 0.134 mM EDTA and 5% (V/V) methanol. All separations were performed isocratically at a flow-rate of 0.9 ml/min at 35°C. The detector potential was maintained at +0.65 V.

TH activity analysis

TH activity was measured by a modification of the method of Nagatsu et al. (13). The tissues were homogenized with 0.25 M sucrose (7 volumes for the abdominal aorta and 15 volumes for the mesenteric artery) in a glass tissue grinder. The standard incubation medium consisted of the following components in a total volume of 250 µl: 100 µl of tissue homogenate, 40 µl of 1 M sodium acetate

buffer (pH 6.0), 40 µl of 1 mM L-tyrosine, 20 µl of 1 mM 6-methyl-5,6,7,8-tetrahydropterine in 1 M 2-mercaptoethanol, 20 µl of 20 mM catalase and 30 µl of water. For the blank incubation, D-tyrosine was used as the substrate instead of L-tyrosine. The medium was incubated at 37°C for 30 min, and the reaction was terminated by adding to it 200 µl of 1 M perchloric acid containing 0.1 µg/ml dihydroxybenzylamine as an internal standard and 50 µl of 0.2 M EDTA, in an ice bath. After 10 min, 150 µl of 1 M potassium carbonate and 1 ml of 0.2 M Tris-HCl (pH 8.5) containing 1% EDTA were added, and the mixture was centrifuged at $1,800 \times g$ for 5 min. To extract DOPA, the supernatant was mixed with 10 mg aluminum oxide and 100 µl of 2 M Tris-EDTA (pH 8.7) for 15 min. The precipitate was washed with 1 ml of 16.5 mM Tris-EDTA (pH 8.1), dried and then mixed with 200 µl of solvent medium (acetic acid : 10% sodium metabisulfite : 5% EDTA : water = 0.1 : 0.05 : 0.05 : 9.8) for 15 min. This medium was centrifuged at $1,800 \times g$ for 1 min. Forty microliters of supernatant was transferred to a new tube and mixed with 17 µl of 0.1 N NaOH and 10 mg of Amberlite CG-50 (Aldrich Chem., Co., Milwaukee, WI, USA) for 15 min. The supernatant was passed through a 0.22-µm filter, and a 20-µl aliquot was into the HPLC (Waters 510) instrument, which was equipped with an electrochemical detector (Waters 460) and a Cosmosil 5C₁₈-AR packed column (4.6 × 150 mm, Nacalai Tesque Co.). The mobile phase consisted of the following components: 50 mM sodium acetate, 20 mM citric acid, 12.5 mM sodium octyl sulfate, 1 mM di-*n*-butylamine and 0.134 mM EDTA. All separations were performed isocratically at a flow-rate of 0.6 ml/min at 28°C. The detector potential was maintained at +0.65 V. TH activity was calculated as the amount of DOPA formed from tyrosine per hour per g tissue.

Statistical analyses

Data are presented as the means ± S.E. Statistical difference between mean values were analyzed by Student's *t*-test; *P* values less than 0.05 were considered statistically significant.

RESULTS

The effects of castration and testosterone replacement on systolic blood pressure of SHR and WKY

Figure 1 shows the effects of castration and testosterone replacement on the systolic blood pressure of SHR and WKY. The systolic blood pressure of intact SHR (195.7 ± 3.0 mmHg) was significantly higher than that of intact WKY (144.7 ± 2.3 mmHg) ($P < 0.01$). The systolic blood pressure of castrated SHR (172.1 ± 2.6 mmHg) was significantly lower than those of intact SHR ($P < 0.01$).

Fig. 1. The effects of castration and testosterone replacement on systolic blood pressure in SHR and WKY. Values are means $\pm$ S.E. Int: intact, Cast: castrated, Cast+To: castrated and testosterone replaced. The number of rats measured is shown in parentheses. The significance of the difference was analyzed by Student's *t*-test.

and testosterone-replaced SHR (200.1 ± 3.8 mmHg) ($P < 0.01$). The systolic blood pressure of WKY showed no significant differences among the three groups: castrated WKY (139.6 ± 1.9 mmHg) and testosterone-replaced WKY (143.4 ± 2.6 mmHg).

The effects of castration and testosterone replacement on the NE levels in the abdominal aorta and mesenteric artery of SHR and WKY

Figure 2 shows the effects of castration and testosterone replacement on the NE levels in the abdominal aorta and mesenteric artery of SHR and WKY. The NE levels of intact SHR (abdominal aorta: 272.0 ± 14.3 ng/g tissue, mesenteric artery: 2275.6 ± 114.8 ng/g tissue) were significantly higher than those of intact WKY (abdominal aorta: 197.6 ± 11.5 ng/g tissue, mesenteric artery: 1294.6 ± 224.2 ng/g tissue) ($P < 0.01$). The NE levels of castrated SHR (abdominal aorta: 221.8 ± 10.6 ng/g tissue, mesenteric artery: 1554.3 ± 295.1 ng/g tissue) were significantly lower than those of intact SHR ($P < 0.05$). The NE levels of testosterone-replaced SHR (abdominal aorta: 269.9 ± 32.9 ng/g tissue, mesenteric artery: 2238.0 ± 144.2 ng/g tissue) recovered to the levels of intact SHR. The NE levels of WKY showed no significant differences among the three groups: castrated WKY (abdominal aorta: 174.5 ± 12.7 ng/g tissue, mesenteric artery: 1464.0 ± 177.3 ng/g tissue) and testosterone-

Fig. 2. The effects of castration and testosterone replacement on NE levels in the abdominal aorta and mesenteric artery of SHR and WKY. Values are means $\pm$ S.E. The number of rats measured is shown in parentheses. The significance of the difference was analyzed by Student's *t*-test. See the legend of Fig. 1 for details.

Fig. 3. The effects of castration and testosterone replacement on TH activities in the abdominal aorta and mesenteric artery of SHR and WKY. Values are means $\pm$ S.E. The number of rats measured is shown in parentheses. The significance of the difference was analyzed by Student's *t*-test. See the legend of Fig. 1 for details.

replaced WKY (abdominal aorta: 185.6 ± 11.0 ng/g tissue, mesenteric artery: 1247.8 ± 122.4 ng/g tissue).

The effects of castration and testosterone replacement on TH activities of the abdominal aorta and mesenteric artery of SHR and WKY

Figure 3 shows the effects of castration and testosterone replacement on TH activities in the abdominal aorta and mesenteric artery of SHR and WKY. The TH activities of intact SHR (abdominal aorta: 118.2 ± 23.0 ng/g tissue/hr, mesenteric artery: 833.0 ± 60.9 ng/g tissue/hr) were significantly higher than those of the intact WKY (abdominal aorta: 56.6 ± 17.4 ng/g tissue/hr, mesenteric artery: 450.3 ± 63.1 ng/g tissue/hr) ($P < 0.05$). The TH activities of castrated SHR (abdominal aorta: 45.2 ± 1.4 ng/g tissue/hr, mesenteric artery: 474.6 ± 126.4 ng/g tissue/hr) were significantly lower than those of intact SHR ($P < 0.05$). The TH activities of testosterone-replaced SHR (abdominal aorta: 122.8 ± 24.6 ng/g tissue/hr, mesenteric artery: 787.5 ± 191.8 ng/g tissue/hr) recovered to the levels of intact SHR. The TH activities of WKY showed no significant differences among the three groups: castrated WKY (abdominal aorta: 49.4 ± 7.8 ng/g tissue/hr, mesenteric artery: 331.8 ± 77.3 ng/g tissue/hr) and testosterone-replaced WKY (abdominal aorta: 84.6 ± 15.4 ng/g tissue/hr, mesenteric artery: 331.2 ± 78.6 ng/g tissue/hr).

DISCUSSION

In this study, we observed that the castration of male SHR retarded development of hypertension and that testosterone replacement reversed this retardation. However, neither castration nor testosterone replacement had any effects on the systolic blood pressure of WKY. Iam and Wexler (14) demonstrated that gonadectomy at an early age (30 days) retards the development of hypertension in SHR. We have previously reported that late gonadectomy of SHR (17 weeks) also significantly reduced blood pressure (8). Ganten et al. (11) demonstrated that chemical castration with cyproterone and flutamide, which are androgen receptor antagonists, attenuates the development of hypertension in male SHR. Lengsfeld et al. (15) and Chen and Meng (16) also suggested that androgen is important in producing the male pattern of hypertension in SHR. These findings suggested that androgen may be involved in the SHR's hypertension.

Vascular catecholamines are related to vascular resistance; and in particular, NE is reported to be important in the tonic response to nerve stimulation (17). The NE levels and the TH activities in the abdominal aorta and the mesenteric artery of SHR were significantly higher than those of WKY. Iriuchijima (6) reported that the elevation of the SHR's vascular resistance is not

uniformly observed over the whole body, and it is especially marked in the abdominal aorta and the mesenteric artery. The vascular resistance of these arteries of SHR was about 50% greater than that of normotensive rats (18). The present study suggests that the elevated NE level which is likely to result from the high activity of TH in the abdominal aorta and the mesenteric artery may be related to the SHR's increased vascular resistance.

The NE levels in the abdominal aorta and the mesenteric artery were reduced by castration of SHR, while testosterone replacement reversed the decrease in the SHR's NE level. Moreover, the alterations of TH activities by these treatments were consistent with those of the NE level of SHR. On the other hand, neither castration nor testosterone replacement had any effects on the NE level or TH activity in normotensive WKY. Kohler et al. (19) reported that gonadectomy of normotensive male and female Sprague-Dawley strain rats did not alter the TH activities in the mesenteric artery or the vein, and that testosterone had no effect on vascular TH activity. These findings suggest that the effects of testosterone on the NE synthetic pathway were specifically exhibited in the SHR. The underlying mechanisms by which testosterone affects NE level and TH activities of SHR are not yet understood.

There have been inconsistent data on plasma testosterone levels in SHR and WKY. We observed that the plasma testosterone concentration of SHR was significantly higher than that of WKY at the age of 14 weeks (unpublished data). However, Saito et al. (20) reported that the plasma testosterone of SHR was lower than WKY at the age of 8 months. These findings might show that age-related change of plasma testosterone levels in SHR is different from that in WKY. Further study will be needed to assess plasma androgen level and its influence on NE level and TH activity of SHR.

Changes in NE level and TH activity may result from the direct interaction of androgen with these arteries or sympathetic ganglion containing the TH gene. Notably, the ganglion contains cytosolic androgen receptors that specifically bind to androgen with high affinity in a saturable manner (21). Testosterone is known to play important roles in gene transcription (22). Regulation of specific gene transcription by steroid hormones is mediated by binding of hormone receptors to steroid responsive elements (23). Therefore, it is possible that the changes in NE level and TH activity by testosterone may be induced via alteration of the TH gene transcription in SHR. Turner et al. (24) have reported that the blood pressure increase shown by male SHR over WKY was related to the Y chromosome. Furthermore, Ganten et al. (11) reported that the blood pressure is higher in male hypertensives than female ones and that this sexual dimorphism is not

present in normotensive rats, and they suggested that this sexual dimorphism in SHR is linked to the "hypertensive genes". Therefore, the high blood pressure in male SHR may be related to the action of androgen, which interacts with "hypertensive genes" in the abdominal aorta and mesenteric artery or sympathetic ganglion.

In conclusion, our results suggested that androgen may contribute to the hypertension in male SHR, which increased NE levels due to the high TH activities of the abdominal aorta and the mesenteric artery.

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