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Noradrenergic hyperinnervation may inhibit necrosis of coronary arterial smooth muscle cells in stroke-prone spontaneously hypertensive rats

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Abstract Noradrenergic (NA) nerve fibre distribution and vascular smooth muscle morphology were investigated in the coronary artery of stroke-prone spontaneously hypertensive rats (SHRSP). Fluorescent NA nerve fibres of SHRSP aged 10, 30, 60, 90 and 180 days were examined by the glyoxylic acid method and compared with those of age-matched normotensive Wistar Kyoto (WKY) rats. The distribution densities of NA nerve fibres were measured by quantitative image analysis using the Interactive Bildanalyse System. The densities of NA nerve fibres of the left coronary artery of SHRSP were significantly higher than those of WKY rats at all ages examined. NA hyperinnervation in the coronary artery of SHRSP may be caused by the hyperfunction of the stellate ganglia which innervate the coronary arteries. Scanning electron microscopy observations showed that the surface of smooth muscle cells of the left coronary artery in SHRSP was smooth and similar to that of WKY rats at 120 days of age, but was slightly modified by more invaginations and projections than that in WKY rats at 180 days of age. No necrotic cells, however, were found in SHRSP. By transmission electron microscopy the smooth muscle cells in SHRSP were shown to be irregular in profile with deep indentations of the plasma membrane and surrounded by many layers of basal lamina-like material, but no necrotic cells were found. We suggest that NA hyperinnervation protects the vascular smooth muscle cells from necrosis in the coronary artery of SHRSP by a trophic effect mediated by NA nerve fibres.

Key words Hypertension · Stroke-prone spontaneously hypertensive rat · Coronary artery · Noradrenergic nerve fibre · Vascular smooth muscle cell

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Introduction

The superior cervical ganglia and stellate ganglia of spontaneously hypertensive rats (SHR) have been reported to exhibit hyperfunction when compared with those of Wistar Kyoto (WKY) rats [14–16]. Fluorescence microscopy has shown that the density of noradrenergic (NA) nerve fibres in the ophthalmic artery, which originate from the superior cervical ganglia, was increased in stroke-prone SHR (SHRSP) when compared with normotensive WKY rats [26]. It is unclear whether the coronary arteries, innervated by the stellate ganglia, are more densely innervated in SHRSP than those in WKY rats. A number of papers, using biochemical methods, have shown that NA levels in the heart in SHR and SHRSP were not higher than those in WKY rats [8, 20], but Head et al. [11] have reported that NA content in the heart in SHR was greater than that in WKY rats.

We investigated and compared the density of perivascular NA fibres and the structure of smooth muscle cells of the coronary arteries in SHRSP and WKY rats.

Materials and methods

All animals used in this study were maintained on a normal laboratory diet and tap water ad libitum in the Laboratory Animal Centre at Ehime University School of Medicine.

The animals were 10-, 30-, 60-, 90- and 180-day-old male SHRSP and WKY rats. The method of specimen preparation was similar to that previously reported [26]. Six animals of each age from both strains were anaesthetized with pentobarbital sodium (20 mg/kg body weight) and were then perfused with phosphate-buffered saline (PBS) followed by glyoxylic acid in phosphate buffer. After removal from the heart, the left coronary arteries, less than 500 μm in diameter and 6–7 mm in length in adult animals, were immersed in the same glyoxylic acid solution at room temperature for 40 min, during which the myocardium and connective tissues around the left coronary artery were removed. Subsequently the vessels were immersed in a drop of glyoxylic acid solution of nonfluorescent glass slides and glyoxylic acid solution was removed by filter paper. These preparations were dried at room temperature, 22–26° C, for 20 min, heated at 100° C for 7 min, and then mounted with paraffin oil. Four and five fields per specimen

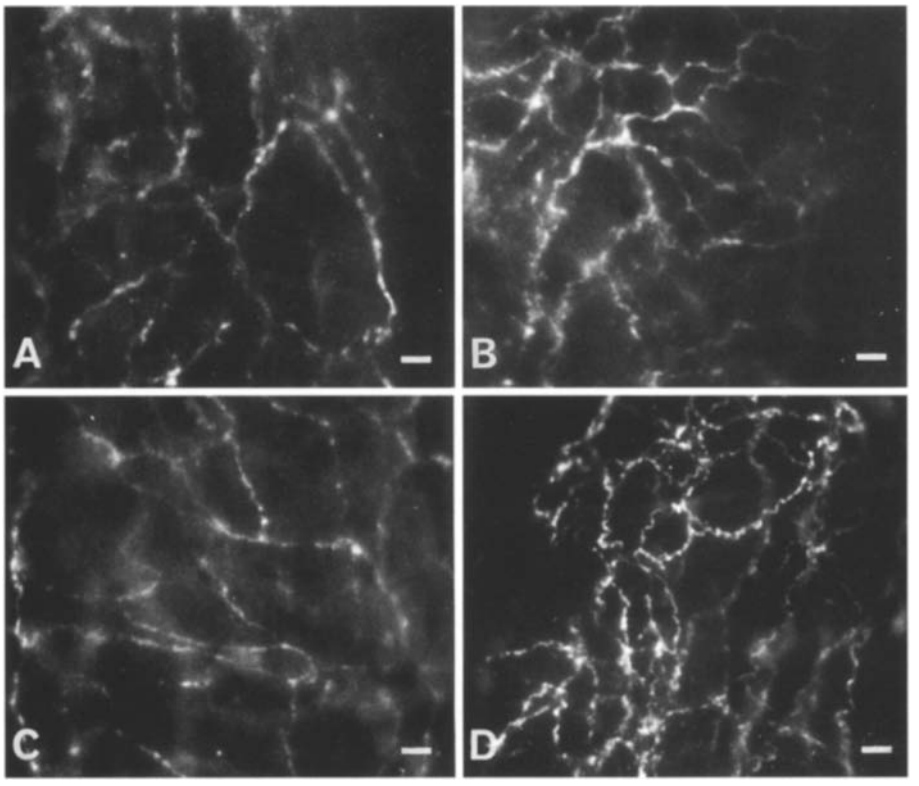

Fig. 1A–D Photomicrographs showing noradrenergic nerve fibres of the coronary arteries in Wistar Kyoto (WKY) rats (**A, C**) and stroke-prone spontaneously hypertensive rats (SHRSP; **B, D**) at 30 (**A, B**) and 60 (**C, D**) days after birth. More nerve fibres are observed in SHRSP compared with WKY rats at both ages. *Bars*: 30 μm

were photographed at a magnification of ×250 with a Zeiss photomicroscope.

The distribution densities of NA nerve fibres in each micrograph were evaluated by quantitative image analysis on an Interactive Bildanalyse System image analyzer (Kontron Bildanalyse). The density was expressed as a percentage of the area occupied by fluorescent nerve fibres per whole area of a blood vessel [1, 2].

The analyses were done in a blinded fashion. Student's *t*-test was used for comparison between SHRSP and WKY rats.

For scanning electron microscopy (SEM) the animals used were 120- and 180-day-old male SHRSP and WKY rats. The method of specimen preparation was similar to that previously reported [10]. Four-to-seven animals of both strains were anaesthetized with ether and perfused through the heart with, in succession, 120 ml of PBS, 150 ml of 2% glutaraldehyde fixative in 0.1 M phosphate buffer (pH 7.4) and 100 ml PBS at a constant pressure of 80% of blood pressure of each animal. These solutions were warmed to 37° C before perfusion. Immediately after perfusion, the rats were transfused with 30 ml of heparinized whole blood at the same pressure as above to prevent the blood vessels from collapsing during subsequent treament. The left coronary arteries were removed and fixed for an additional 2 h with the same glutaraldehyde fixative at room temperature. Specimens were washed in 0.1 M phosphate buffer solution and then postfixed with 2% osmium tetroxide (OsO_4) at 4° C for 2 h, followed by a brief rinse in distilled water. The specimens were treated with 8 N hydrochloric acid for 22–25 min at 60° C to remove connective tissue components [9]. The specimens were washed in distilled water, dehydrated in a graded series of ethanols, immersed in isoamyl acetate and critical-point dried with carbon dioxide. They were sputter-coated with platinum and examined in a Hitachi S-500A SEM.

For transmission electron microscopy (TEM) the animals used were 180-day-old male SHRSP. Three rats were anaesthetized with ether and perfused through the heart with 200 ml of 2% glutaraldehyde in 0.1 M phosphate buffer solution (pH 7.4) at a constant pressure of 80% of blood pressure for each animal. The left coronary arteries were removed and immersed in 3% glutaraldehyde at room temperature for 2 h, followed by a brief rinse in 0.1 M phosphate buffer solution. They were postfixed with 2% OsO_4 at 4° C for 2 h. After block-staining with saturated uranyl acetate in 50% methanol for 10 h, they were dehydrated in an ethanol series and embedded in epoxy resin. Thin sections were cut and stained with both uranyl acetate and lead citrate and observed in a Hitachi H-800 TEM.

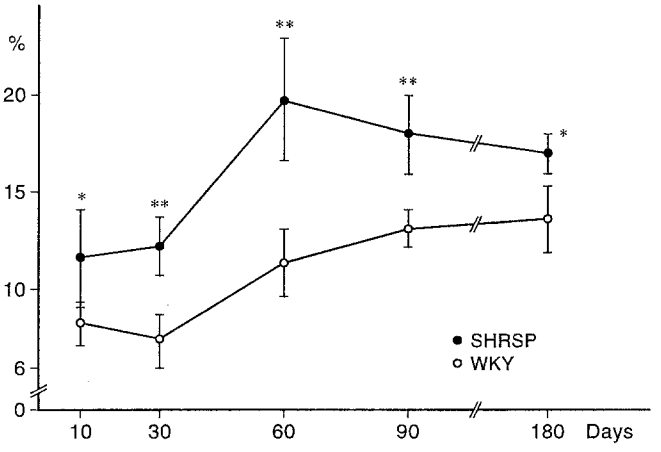

Fig. 2 Comparison of densities of noradrenergic nerve fibres in the coronary arteries between WKY rats and SHRSP at various ages. ** $P<0.01$ compared with WKY rats; * $P<0.05$ compared with WKY rats

Results

Perivascular NA nerve fibres exhibited the characteristic green fluorescence of noradrenaline [13]. Varicose NA nerve fibres showed a meshwork pattern at all ages examined in both strains (Fig. 1). In both strains, NA nerve

Table 1 Difference in the densities of nerve fibres between stroke-prone spontaneously hypertensive rats and Wistar Kyoto rats

[a] These figures are obtained by dividing the values in SHRSP by those in WKY rats

Days after birth	Difference[a]
10	1.4
30	1.6
60	1.7
90	1.4
180	1.2

fibres became thicker and more easily visible with age. The thickness of each nerve fibre did not differ between SHRSP and WKY rats. The distribution densities of NA nerve fibres in SHRSP increased markedly until 60 days of age and decreased gradually thereafter (Fig. 2). The increased NA fibre densities of SHRSP younger than 60 days of age were accompanied by gradual increase in the vessel diameter, and the decreased NA fibre densities of SHRSP older than 60 days of age were accompanied by rapid increase in the vessel diameter. In contrast, nerve densities in WKY rats continuously increased with age until 180 days after birth. The increase in nerve fibre density of WKY rats with age was accompanied by the gradual increase in the vessel diameter. The nerve densities in SHRSP were significantly higher than in age-matched WKY rats ($P<0.01$ and 0.05) at all ages examined. The difference in the nerve densities between SHRSP and WKY rats increased from 10 to 60 days after birth, reaching a peak at this time (×1.7) and then decreasing thereafter (Table 1).

By SEM, many pits and gutters were observed on the surface of smooth muscle cells of coronary artery in both WKY rats and SHRSP at 120 days after birth (Fig. 3A, B). The smooth muscle cells from 180-day-old WKY

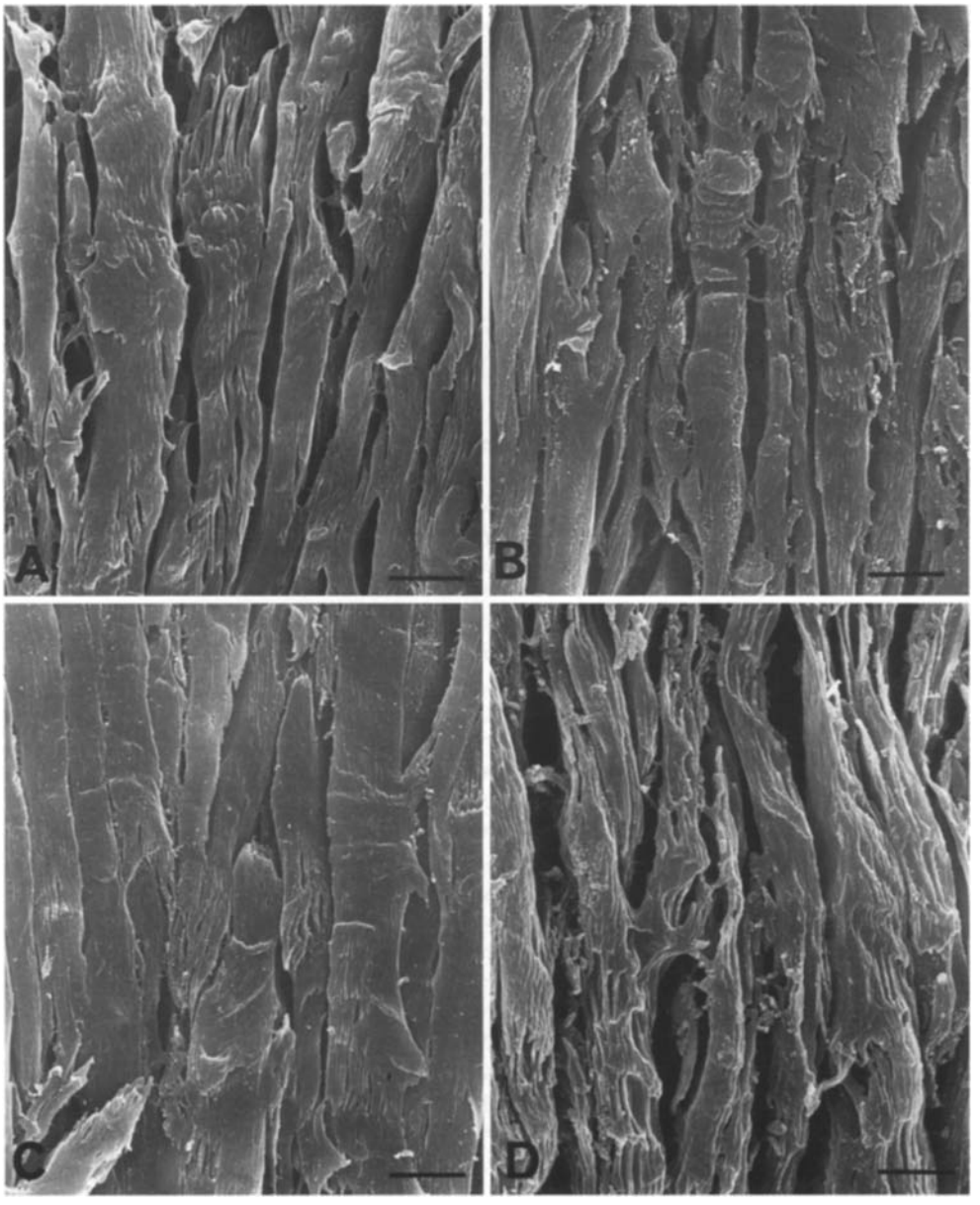

Fig. 3A–D Scanning electron micrographs of the adventitial surfaces of the outermost smooth muscle layer of the media in the coronary arteries from WKY rats (**A, C**) and SHRSP (**B, D**) at 120 (**A, B**) and 180 (**C, D**) days of age. The surface texture of smooth muscle cells is relatively smooth and similar to each other among 120-day-old SHRSP and WKY rats and 180-day-old WKY rat; but is quite rough in 180-day-old SHRSP. Intercellular spaces between smooth muscle cells from 180-day-old SHRSP increase in width. No necrotic cells are seen. *Bars*: 2 μm

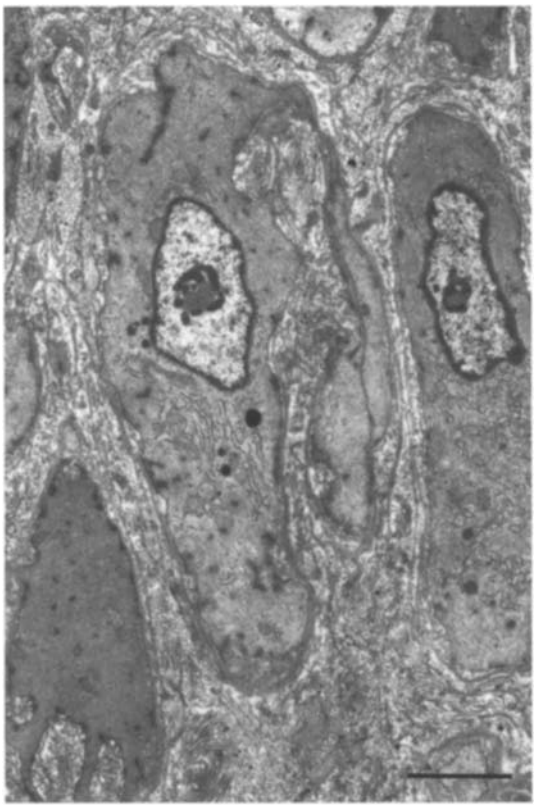

Fig. 4 Transmission electron micrograph of the media of the coronary artery sectioned parallel to the vessel long axis from a 180-day-old SHRSP. *Bar* 1 μm

rats showed rather similar surface texture to that of 120-day-old WKY rats (Fig. 3C). In contrast to WKY rats, smooth muscle cells of 180-day-old SHRSP exhibited a remarkably increased number of gutters and pits or splits on the surface (Fig. 3D). Intercellular spaces between smooth muscle cells from 180-day-old SHRSP increased in width compared with those of 120-day-old SHRSP and WKY rats and 180-day-old WKY rats. No necrotic cells, however, were found in the coronary artery of SHRSP and WKY rats at these ages.

By TEM, the smooth muscle cells in 180-day-old SHRSP were irregular in profile with deep indentations of the plasma membrane and were surrounded by many layers of basal lamina-like material (Fig. 4). Large amounts of collagen fibrils and fibrous intercellular matrix substances were observed between the smooth muscle cells.

Discussion

The density of NA nerve fibres in the coronary arteries was significantly higher in SHRSP when compared with that of WKY rats throughout the entire examination period. Increased NA innervation in the coronary artery of SHRSP may be caused by hyperfunction of the stellate ganglia [14–16] but apparently not by nerve growth factor activity in the heart, since this was reported to be slightly lower in SHRSP at 3 weeks of age when compared with age-matched WKY rats [21]. We suggest that hyperfunction of the stellate ganglia may promote the growth, elongation and branching of nerve terminals supplying the coronary artery in SHRSP.

Our results show that the innervation density of the coronary artery in SHRSP was 1.7, 1.6 and 1.4 times greater than that in WKY rats at 30, 60 and 90 days of age, respectively. In the coronary artery, the medial layer from SHR has been reported to become 1.4 times thicker than that of WKY rats by the age of 8 months [6]. It seems unlikely that the medial wall thickness of the coronary artery from SHRSP was 1.4–1.7 times as great as that of WKY rats less than three months after birth, because the medial wall of the various arteries of SHR or SHRSP takes some time to become thicker [3, 4, 21, 29] by smooth muscle hyperplasia [19]. Therefore, the degree of increase in innervation density appears to be greater than the increase in medial thickness in the coronary artery from SHRSP less than three months old, suggesting that innervation density per unit mass of smooth muscle is greater in SHRSP than WKY rats. Hyperinnervation of the coronary artery is present in SHRSP at the prehypertensive stage and during the developing stage of hypertension.

In the coronary artery, however, a remarkably large distance between nerve terminals and smooth muscle cells has been shown to account for the small range in their sympathetic control [18]. Furthermore, humoral and sympathetic NA activation produces heterogeneous vascular reaction in the coronary arterial vessels. Both constriction and dilation occur, depending on the diameter of the vessels; NA induces vasoconstriction in large coronary arteries [7, 12, 28, 30] and vasodilation in the coronary microvessels [5, 23]. It seems unlikely to us that sympathetic hyperinnervation of the coronary artery has a primary role in increasing blood pressure in SHRSP directly.

Sympathetic hyperinnervation of blood vessels in SHRSP has been suggested to protect against stroke by a trophic effect on vascular smooth muscle cells [24, 26]. We have reported that in the proximal portion of the middle cerebral artery which was innervated by NA nerve fibres of the same distribution density as that in WKY rats, all smooth muscle cells had become either modified or necrotic in SHRSP at 6 months of age [10, 17]. Similarly, many necrotic smooth muscle cells were observed in the distal portion of the anterior cerebral artery in 6-month-old SHRSP, which was innervated at the same density as that in the WKY rats (unpublished data). However, both proximal and distal portions in the ophthalmic artery were more heavily innervated in SHRSP than in WKY rats at 4 months of age and contained neither modified nor necrotic smooth muscle cells. In this context, the fact that no necrotic smooth muscle cells were observed in the coronary artery of SHRSP under 6 months of age may indicate a protective role for sympathetic hyperinnervation against the smooth muscle cell

necrosis caused by the tangential wall stress associated with chronic hypertension. A protective role for sympathetic nerves is of clinical importance, since primary rupture of cerebral arteries has been reported to be caused by "arteriosclerosis" accompanied by degeneration of the medial smooth muscle cells [25]. It will be worth comparing the innervation density of the coronary arteries with that of the cerebral arteries and the incidence of haemorrhage in the region which each artery supplies. Haemorrhage rarely occurs in the densely innervated coronary artery and occurs frequently in moderately innervated cerebral arteries [22]. Hyperinnervation of the coronary artery may protect the wall from any propensity to rupture in SHRSP, although other physical and haemodynamic factors may be involved.

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