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Impairment of Endothelium-dependent Relaxation and Changes in Levels of cyclic GMP in Carotid Arteries from Stroke-prone Spontaneously Hypertensive Rats

NORIYUKI MIYATA, KATSUHARU TSUCHIDA, MAKOTO TANAKA AND SUSUMU OTOMO

Research Center, Taisho Pharmaceutical Co. Ltd, 1-403 Yoshino-cho, Ohmiya, Saitama 330, Japan

Abstract—Endothelium-dependent relaxation of carotid arteries and changes in levels of cyclic (c)GMP between stroke-prone spontaneously hypertensive (SHRSP) and Wistar-Kyoto (WKY) rats have been compared. The concentration-response curve for acetylcholine (ACh)-induced relaxation was shifted to the right in carotid arteries from SHRSP. Relaxation responses produced by calcimycin (A 23187) and melittin, both endothelium-dependent agents, were depressed in carotid arteries from SHRSP. Relaxation responses produced by sodium nitroprusside and 8-Br-cGMP were similar to those in strips from WKY. ACh-induced production of cGMP was significantly decreased in carotid arteries from SHRSP when compared with the level for similarly treated strips from WKY. These results suggest that functional changes in endothelium, but not guanylate cyclase activity or cGMP sensitivity in the carotid arteries, may occur in hypertension. Thus, impaired endothelium-dependent relaxation in SHRSP may play an important role in hypertensive vascular diseases such as stroke.

Hypertension has been characterized by increased peripheral vascular resistance as a consequence of morphological or functional changes in the arterial walls (Mulvany & Halpern 1977; Folkow 1982).

The endothelium plays a role in the relaxation of isolated arteries in response to agents such as acetylcholine (ACh), bradykinin, and ATP (Furchgott & Zawadzki 1980; Furchgott et al 1981; Cherry et al 1982). Decreased endothelium-dependent relaxations to ACh have been reported in aorta from spontaneously hypertensive rats (SHR) (Konishi & Su 1983; Luscher & Vanhoutte 1986; Shirasaki et al 1988), and from mineral corticosterone hypertensive rats (Van de Voorde & Leusen 1986; Otsuka et al 1988). Morphological changes also occur in endothelial cells (Huttner & Gabbiani 1983).

It has been suggested that endothelium-dependent relaxants, such as ACh and calcimycin (A 23187), and endothelium-independent vasodilators such as nitric oxide and sodium nitroprusside induce vascular relaxation by increasing cyclic (c)GMP (Grutter et al 1981; Rapoport & Murad 1983; Ignarro et al 1984).

However, no data are available regarding the cerebral circulation, and until now, there have been no data on changes in levels of cGMP in small arteries from stroke-prone spontaneously hypertensive rats (SHRSP) in response to ACh. These factors were the subject of this investigation.

Materials and Methods

Male SHRSP, 13–15 weeks old, and age-matched Wistar-Kyoto rats (WKY) were used. Blood pressure was recorded in unanaesthetized rats by the tail cuff plethysmographic method (model KN-210-1, Natume Seisakusho Co Ltd, Tokyo, Japan). The blood pressure was measured in a quiet environment, and an average of three successive readings was recorded. The rats were decapitated and the common carotid arteries removed and placed in oxygenated modified Krebs-Henseleit solution (KHS). The solution consisted of (mM): NaCl, 118·0; KCl, 4·7; $NaHCO_3$, 25·0; $CaCl_2$, 1·8; NaH_2PO_4, 1·2; $MgSO_4$, 1·2; and dextrose, 11·0. Each carotid artery was cleaned of loosely adhering fat and connective tissue and cut into rings (2·5 mm long). The tissues were placed in a well-oxygenated (95% O_2, 5% CO_2) bath of 10 mL KHS at 37°C with one end connected to a tissue holder and the other to a force displacement transducer (Nihon Kohden TB 612T). The tissue was equilibrated for 60–90 min under a resting tension of 1·0 g; the KHS in the tissue bath was replaced every 20 min. After equilibration, the carotid artery was contracted by treatment with 10^{-7} M noradrenaline (NA), to ensure stabilization of the smooth muscles, and then ACh at a final concentration of 10^{-5} M was added to the bath. The presence of functional endothelial cells was confirmed by the fact that 10^{-5} M ACh caused the carotid arterial strips to relax by more than 75% of the contractile force induced by 10^{-7} M NA.

The effects of the drugs were then tested. For the contraction studies, NA (10^{-10}–10^{-6} M) was added cumulatively to the bath until maximal response was achieved. Because the maximal contraction of carotid arteries from SHRSP in response to NA was similar to that in WKY (Fig. 1), for relaxation studies, the carotid arteries were precontracted with 10^{-7} M NA. This contraction produced 80–90% of the maximal response. To examine the effect of indomethacin, an inhibitor of cyclo-oxygenase, carotid arteries from SHRSP were treated with 5×10^{-6} M indomethacin for 15 min and then strips were precontracted with 10^{-7} M NA. When the NA-induced contraction reached a plateau, ACh (10^{-8}–10^{-5} M), sodium nitroprusside (10^{-10}–10^{-6} M) and 8-Br-cGMP (10^{-6}–10^{-4} M) were added cumulatively. When the NA-induced contraction reached the plateau level, single concentrations of calcimycin (3×10^{-7} M), and melittin

Correspondence to: N. Miyata, Research Center, Taisho Pharmaceutical Co. Ltd, 1-403 Yoshino-cho, Ohmiya, Saitama 330, Japan.

($10\ \mu g\ mL^{-1}$) were added to the bath. Each carotid artery was exposed to only one relaxant.

Measurement of cGMP

Basal concentrations of, or ACh-induced changes in levels of cGMP were measured in a separate series of experiments. Endothelium-intact carotid arteries were allowed to equilibrate in tubes that contained KHS, gassed with 95% O_2, 5% CO_2, at 37°C for 60 min. After equilibration, the carotid arteries were exposed to 10^{-7} M NA for 10 min and a single concentration of ACh was added to the tubes. One minute after the addition of the drugs, the tissues were frozen in liquid N_2, and stored at −80°C until assayed for cGMP. The tissues were homogenized in 1 mL 6% trichloroacetic acid, and centrifuged at 3000 *g* for 10 min. The supernatants were extracted three times in three volumes of water-saturated ether. Following succinylation, the levels of cGMP were determined by radioimmunoassay (Kamata et al 1989; Tanaka et al 1989).

Statistical analysis

Contractile force was expressed as mg tension developed mg^{-1} wet weight of tissue, to take account of any difference in the cross-sectional area of the preparations. The relaxation in response to each agent was expressed as the percentage of decreased tension of contractile force induced by 10^{-7} M NA. EC50 values were determined by the method of Fleming et al (1972). When appropriate, statistical differences were compared by use of analysis of variance and Student's *t*-test for unpaired observations. Differences between mean values of cGMP were evaluated by analysis of variance. If analysis of variance demonstrated a significant difference among means, Tukey's test was then used to determine which strips from SHRSP were significantly different from the mean control value. $P<0.05$ was considered significant.

Drugs

NA bitartrate and sodium nitroprusside were purchased from Wako Chemical Co. (Tokyo, Japan). 8-Br-cGMP, indomethacin and melittin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). ACh chloride was purchased from Daiichi Pharmaceutical Co. (Tokyo, Japan). Calcimycin (A 23187) was purchased from Calbiochem. 8-Br-cGMP and calcimycin were dissolved in dimethyl sulphoxide (DMSO). The final concentration of DMSO in the bath was less than 0·01%, and did not affect contraction or relaxation. Indomethacin was dissolved in distilled water containing 5×10^{-3} M Na_2CO_3 and was sonicated before use. Other drugs were dissolved in distilled water. All concentrations are expressed as final concentrations of the base in the organ bath. Radioimmunoassay kits for cGMP were purchased from Yamasa Shoyu Co. (Choshi, Japan).

Results

Mean systolic blood pressure of WKY and SHRSP was 131·6 ± 1·4 mm Hg and 256·6 ± 3·4 mm Hg at 13–15 weeks of age, respectively (n = 15, $P<0.01$).

Contractile response of carotid arteries induced by NA

NA contracted the carotid arteries with endothelium from WKY and SHRSP in a concentration-dependent manner. However, contractile responses to NA in carotid arteries from SHRSP were similar to those in strips from WKY (Fig. 1). The maximal contractile force for 10^{-6} M NA was 186·7 ± 24·5 mg (mg tissue)$^{-1}$ and 209·4 ± 25·0 mg (mg tissue)$^{-1}$ in WKY and SHRSP, respectively (n = 6). The EC50 values for NA were $1.28\pm0.2\times10^{-8}$ M and $1.46\pm0.17\times10^{-8}$ M in WKY and SHRSP, respectively (n = 6).

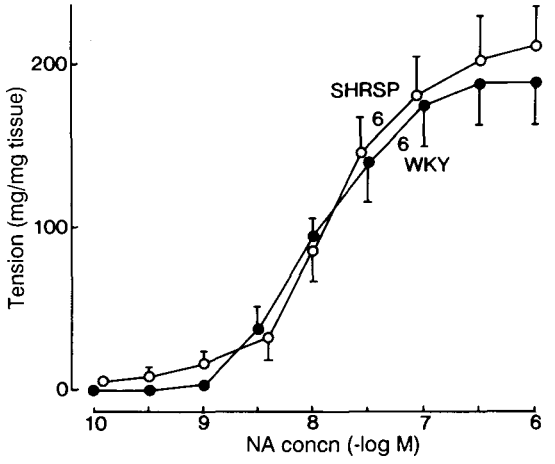

FIG. 1. Cumulative-concentration response curves for noradrenaline (NA) in carotid arteries with endothelium from WKY (●) and SHRSP (○). Values represent the mean from 6 experiments, respectively, with the s.e. values indicated by vertical lines.

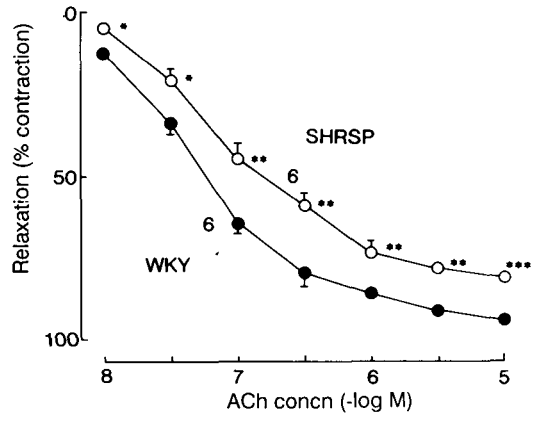

FIG. 2. Concentration–response curves for the relaxation responses to acetylcholine of carotid arteries with endothelium from WKY (●) and SHRSP (○). The carotid arteries were precontracted with 10^{-7} M NA. Values represent the means of 6 experiments, respectively with the s.e. values indicated by vertical lines. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Relaxation response of carotid arteries induced by ACh

When carotid arteries were precontracted with 10^{-7} M NA, ACh relaxed the carotid arteries with endothelium in a concentration-dependent manner. However, concentration-response curves for ACh were shifted to the right for carotid arteries from SHRSP (Fig. 2).

The IC50 value for ACh in carotid arteries with endothelium from SHRSP was significantly greater than that in carotid arteries from WKY (Table 1).

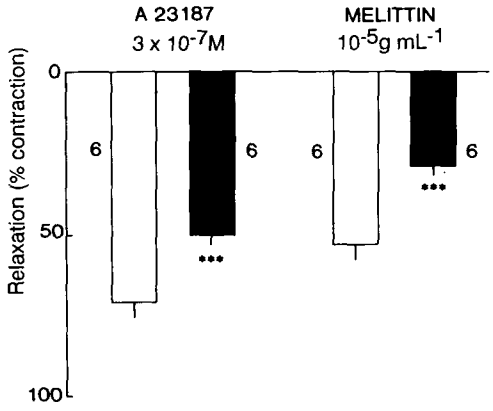

FIG. 3. Relaxation responses to calcimycin of carotid arteries with endothelium from WKY (□) and SHRSP (■) (left). Relaxation responses to melittin of carotid arteries with endothelium from WKY (□) and SHRSP (■) (right). The carotid arteries were precontracted with 10^{-7} M NA. Values represent the means of 6 experiments, respectively, with the s.e. values indicated by vertical lines. *** $P < 0.001$.

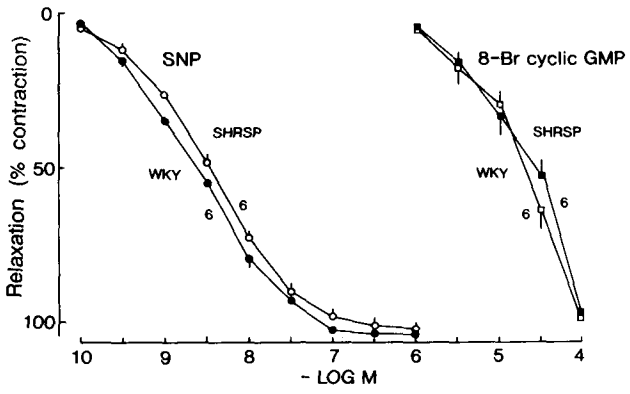

FIG. 4. Concentration–response curves for the relaxation responses to sodium nitroprusside (SNP) of carotid arteries with endothelium from WKY (●) and SHRSP (○) (left). Concentration–response curves for the relaxation responses to 8-Br-cGMP of carotid arteries with endothelium from WKY (■) and SHRSP (□) (right). Values represent the means of 6 experiments, respectively, with the s.e. values indicated by vertical lines.

In carotid arteries from SHRSP with endothelium incubated for 15 min with 5×10^{-6} M indomethacin, the relaxation produced by ACh did not differ statistically from that in strips from SHRSP in all the concentrations (data not shown, $P > 0.05$, $n = 6$).

Relaxation response of carotid arteries induced by calcimycin and melittin

The relaxations produced by 3×10^{-7} M calcimycin and 10 μg mL^{-1} melittin, both endothelium-dependent agents, were lower in carotid arteries from SHRSP when compared with those in strips from WKY (Fig. 3).

Relaxation response of carotid arteries induced by sodium nitroprusside and 8-Br-cGMP

In contrast to the effects of endothelium-dependent agents, concentration-dependent relaxations produced by sodium nitroprusside and 8-Br-cGMP in carotid arteries from SHRSP were similar to those in strips from WKY (Fig. 4). The IC50 values for sodium nitroprusside and 8-Br-cGMP in carotid arteries with endothelium from SHRSP did not differ from the value for WKY vessels (Table 1).

Table 1. IC50 values for acetylcholine (ACh)-, sodium nitroprusside- and 8-Br-cGMP-induced relaxation of carotid arteries from WKY and SHRSP.

Drugs	WKY (M) (n = 6)	SHRSP (M) (n = 6)
ACh	$5.11 \pm 0.54 \times 10^{-8}$	$1.40 \pm 0.19 \times 10^{-7}$**
Sodium nitroprusside	$2.35 \pm 0.37 \times 10^{-9}$	$3.63 \pm 0.61 \times 10^{-9}$
8-Br-cGMP	$1.82 \pm 0.30 \times 10^{-5}$	$1.54 \pm 0.26 \times 10^{-5}$

Values are means ± s.e.; n = number of animals.
* $P < 0.001$ compared with age-matched controls.

Table 2. Basal, noradrenaline (NA)-treated and acetylcholine (ACh)-induced production of cGMP in carotid arteries from WKY and SHRSP.

Agents (M)	WKY cGMP (pmol (g tissue)$^{-1}$)	SHRSP cGMP (pmol (g tissue)$^{-1}$)
None	536.0 ± 110.0 (5)	502.4 ± 22.9 (5)
NA (10^{-7})	284.3 ± 49.3 (5)	249.9 ± 30.4 (5)
NA (10^{-7}) + ACh (3×10^{-7})	2246.5 ± 221.4 (12)	1002.4 ± 259.3** (12)

** $P < 0.001$ compared with WKY.

Alterations in cGMP concentrations

Basal concentrations of cGMP and NA-treated levels of cGMP in carotid arteries from SHRSP were similar to those in WKY vessels (Table 2).

In both WKY and SHRSP, ACh increased levels of cGMP in isolated carotid arteries with endothelium. The increase in levels of cGMP (measured 1 min after the addition of ACh) was significantly lower in carotid arteries from SHRSP compared with those from WKY (Table 2).

Discussion

In the present study, we found that the relaxation response of carotid arteries from SHRSP was significantly depressed in response to the endothelium-dependent agents, ACh and calcimycin. The relaxation response of aortic strips to ACh from a variety of hypertensive animals is reduced when compared with that in strips from normotensive animals (Konishi & Su 1983; Winquist et al 1984; Luscher & Vanhoutte 1986; Van de Voorde & Leusen 1986; Luscher et al 1987; Otsuka et al 1988). However, there are few reports of impairment of endothelium-dependent relaxation of small arteries from hypertensive animals in response to ACh. In this respect, our results are consistent with the findings of Mayhan et al (1987) and Tesfamariam & Halpern (1988).

Melittin, a polypeptide found in bee venom (Habermann 1972), which is known to activate phospholipase A_2 and release arachidonic acid (Mollay et al 1976; Shier 1979), induced endothelium-dependent relaxation of rat (Thomas et al 1986) and rabbit (Forstermann & Neufang 1985; Forstermann et al 1987). Recently, it was reported that

melittin-induced relaxation is associated with elevated intracellular levels of cGMP (Rapoport et al 1989). To investigate the effects of melittin as an endothelium-dependent relaxant, we examined the melittin-induced relaxation responses. Relaxations produced by 10 $\mu g\ mL^{-1}$ melittin were lower in carotid arteries from SHRSP when compared with those in strips from WKY.

The concentration-response curves for the relaxant effects of sodium nitroprusside and 8-Br-cGMP, both endothelium-independent agents (Rapoport & Murad 1983), in carotid arteries from SHRSP, showed no shift and no depression of the maximal response. The decrease in the relaxation response of the carotid arteries with endothelium to endothelium-dependent agents may be due to an impairment of endothelial cells; it is unlikely to be due to guanylate cyclase activity or cGMP sensitivity in vascular smooth muscle.

In the aortic strips from SHR, ACh may induce endothelium-dependent contractions mediated by a vasoconstrictor prostanoid (Luscher & Vanhoutte 1986). In the present study, indomethacin did not affect the endothelium-dependent relaxations to ACh in the carotid arteries from SHRSP, suggesting that ACh does not release major amounts of prostanoid-like endothelium-derived constrictor substances in this vessel.

It has been suggested that endothelium-dependent relaxants, such as ACh and calcimycin, and endothelium-independent vasodilators such as nitric oxide and sodium nitroprusside induce vascular relaxation by increasing the levels of cGMP (Grutter et al 1981; Rapoport & Murad 1983; Ignarro et al 1984). In the present study, we found that ACh-induced production of cGMP was significantly lower in carotid arteries from SHRSP when compared to that in strips from WKY.

A possible explanation for the impairment of endothelium-dependent relaxation in carotid arteries from SHRSP may be reduced production or release of endothelium-derived relaxing factor(s) only when stimulated by the endothelium-dependent agents.

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