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Effects of cGMP on the Contractility and Ca Movement in the Aorta of Normotensive Wistar-Kyoto Rats and Spontaneously Hypertensive Rats

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=ABSTRACT=

Endothelium-derived relaxing factor (EDRF) activates guanylate cyclase which mediates the formation of cGMP from GTP in vascular smooth muscle. It is well known that endothelium-dependent relaxation is impaired in spontaneously hypertensive rats (SHR). However, it is still unknown whether the impaired endothelium-dependent relaxation in SHR results from the reduced release of EDRF or from the decrease of vascular response to EDRF.

We investigated the effects of cGMP on the contractility and Ca movement in the aorta of SHR and Wistar-Kyoto rats (WKY). The amplitude of the endothelium-dependent relaxation to actylcholine (ACh) was significantly less in SHR than in WKY. L-arginine (10^{-3}M) did not increase endothelium-dependent relaxation in both strains. Sodium nitroprusside (SNP), an activator of guanylate cyclase, relaxed the 40 mM K^+-induced contraction in a dose-dependent manner (10^{-10} $\sim 10^{-6}$ M) in the endothelium-rubbed aortic strips of both strains. However, there was no significant difference in these relaxations between WKY and SHR.

8-bromo-cyclic guanosine monophosphate (8-Br-cGMP), a cell membrane-permeable derivative of cGMP, relaxed the 40 mM K^+-induced contraction in a dose-dependent manner ($10^{-6} \sim 10^{-4}$ M) in the endothelium-rubbed aortic strips of both strains. Also norepinephrine (10^{-6} M)-induced contractions in normal and Ca-free Tyrode's solution were suppressed by the pretreatment with 8-Br-cGMP (10^{-4} M) in either strain. However, the amplitudes of suppression induced by 8-Br-cGMP were greater in SHR than that in WKY.

Basal ^{45}Ca uptake and 40mM K^+-stimulated ^{45}Ca uptake were not suppressed by pretreatment with 8-Br-cGMP (10^{-4} M) in single aortic smooth muscle cells of both SHR and WKY.

From the above results, it is suggested that cGMP decreases Ca sensitivity in vascular smooth muscle cells and that the impaired endothelium-dependent relaxation in the aortic strips of SHR is not the result of a reduced vascular response to EDRF.

Key Words: Cyclic GMP, Sodium nitroprusside, Endothelium-dependent relaxation, Acetylcholine, Spontaneously hypertensive rats

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INTRODUCTION

The endothelium may contribute in several ways to the local regulation of vascular function. Endothelial cells produce endothelium-derived relaxing factors (EDRF), endothelium-derived contracting factors (EDCF) and prostaglandin I_2 (prostacyclin) and also contain enzymes that can activate or degrade vasoactive hormones (Moncada & Vane, 1978; Furchgott & Zawadzki, 1980; Dzau, 1984; Lüscher & Vanhoutte, 1986).

Endothelium-derived relaxing factor i.e. nitric oxide, is synthesized from the amino acid L-arginine in endothelial cells (Palmer et al, 1988). Its synthesis is catalyzed by nitric oxide synthase (Palmer & Moncada, 1989) and is Ca-dependent (Long & Stone, 1985; Edwards et al, 1985). Nitric oxide is a potent activator of soluble guanylate cyclase and increases intracellular levels of guanosine 3', 5'-cyclic monophosphate (cGMP) in target cells (Ganz et al, 1986). The synthesis of nitric oxide is inhibited by structural analoges of L-arginine such as N^G-nitro L-arginine methyl ester(L-NAME) and is stimulated by the addition of L-arginine(Rees et al, 1989; Moore et al, 1990).

Depending on the vascular bed studied, the vasodilator response to various humoral and pharmacological agents may be classified as either endothelium-dependent or endothelium-independent (De Mey et al, 1982 ; Furchgott, 1983; Peach et al, 1985). Whereas endothelium-dependent vasodilators, such as acetylcholine (ACh) and A23187 (Rapoport & Murad, 1983) induce endothelium-dependent relaxation, endothelium-independent nitrovasodilators, such as nitric oxide, sodium nitroprusside (SNP), and nitroglycerine(NTG), induce vasodilatation by increasing cGMP (Axelsson et al, 1979 ; Clark & Linden, 1986 ; Clyman et al, 1978 ; Fiscus & Dyer, 1981; Fiscus et al, 1984 ; Gruetter et al, 1981; Ignarro et al, 1984). Actually cGMP concentration has been reported to rise in response to many vasodilators, and the rise in cGMP concentration noted in isolated blood vessels parallels the magnitude of the relaxation response of these blood vessels to the vasodilator (Gruetter et al, 1981; Fiscus & Dyer, 1981; Ignarro et al, 1984; Clark & Linden, 1986).

Alterations in the relaxation of vascular smooth muscle have been reported in various models of hypertension. A decrease in endothelium-dependent relaxation in response to the endothelium-dependent vasodilator, ACh and the calcium ionophore A23187 has been previously reported in various models of experimental hypertension (Konishi & Su, 1983 ; Winquist et al, 1984 ; Lockette et al, 1986 ; Lüscher & Vanhoutte, 1986). These could be related 1) to a decreased release of EDRF, 2) to a reduced vascular responsiveness to EDRF, and/or 3) to the concomitant release of an endothelium-derived contracting factor in the hypertensive strain.

There are reports that the release of EDRF is not reduced but that the sensitivity of vascular smooth muscle to EDRF is decreased in hypertensive animals(Van de Voorde & Leusen, 1986). However, it was reported that relaxation induced by nitric oxide was similar in mesenteric artery of both normal and hypertensive strains (Diederich et al, 1990), indicating that the vascular smooth muscle responsiveness to EDRF in SHR is normal. Therefore, it is still unclear whether impaired endothelium-dependent relaxation in SHR results from a reduced release or synthesis of EDRF, or from a decrease of vascular response to EDRF.

We undertook this study to investigate the hypertensive mechanism by comparing the effects of cGMP on contractility and Ca movement in the aorta between SHR and WKY.

METHODS

Animals

All experiments were performed on 12~16 week-old SHR (Okamoto, 1969) and aged-matched normotensive WKY. The systolic blood pressure was measured in conscious restrained rats by the tail-cuff plethysmographic method (PE-300 , Narco-Biosystems, Houston, Texas). Systolic blood pressure was 204±5 mmHg in SHR (n=20) and 136±8 mmHg in WKY (n=20).

Measurement of isometric tension

The rats were stunned and exsanguinated. The aorta was quickly dissected and adhering adventitia and remaining fat were removed under a stereoscopic microscope. The aorta was allowed to recover for 2 hours at room temperature. The aorta was then carefully cut into rings (3~4 mm wide).

The aortic ring was mounted by two parallel straight stainless steel wires (0.3 mm in diameter, 5 mm in length). The lower end was anchored and the upper end was connected to a force transducer (F-60, Narco-Bio system) by glass filament. The organ bath was controlled thermostatically and filled with 50 ml of Tris-buffered Tyrode's solution containing (mM) : NaCl 158, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, Glucose 6, and Tris 5 (pH 7,4 at 37°C). The organ bath solution was maintained at 37°C and was continuously bubbled with 100% O_2. The strip was suspended under a tension of 2 g. Each preparation was allowed to recover for at least one hour. Isometric tensions were recorded on a physiograph (MK-IV, Narco-Bio system) (Chang et al, 1990).

To avoid the possible influence of the endothelium, the endothelium was removed by gently rubbing the intimal surface with a cotton ball. Successful removal of the endothelium was confirmed later by the failure of acetylcholine (10^{-6} M) to induce relaxation (Furchgott & Zawadzki, 1980).

Preparation of dispersed single smooth muscle cells

The aorta was excised, and the adventitia was removed in HEPES-buffered Tyrode's solution containing (mM): NaCl 140, KCl 4, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10, glucose 6, pH adjusted to 7.4 at 37°C. The endothelium was removed by gently rubbing the intimal surface with a cotton ball. The aorta was cut into small strips (2 mm wide and 10 mm long), and incubated in HEPES-buffered Tyrode's solution for one hour. Then small strips were incubated in a Ca-free HEPES-buffered Tyrode's solution containing 2 mg/ml collagenase (Wako), 2 mg/ml papain (Sigma), 1 mg/ml dithiotreitol (Sigma), and 5 mg/ml bovine serum albumin (Sigma) for 40 minutes. After digestion, the single cells were separated by gently stirring the muscle strips through a wide-pore pasteur pipette. The suspension of single cells was filtered through double layers of nylon mesh (pore size: 0.5 mm). The suspension was centrifuged at 1000 rpm for 5 minutes to eliminate the connective tissue debris.

Viability of the single cells was assessed by the trypan blue exclusion test (Bagby et al, 1971; Johns & Riehl, 1982). The total number of stained and viable single smooth muscle cells was then determined. All experiments were carried out within 4 hours after preparation of the cell suspension.

Measurement of ^{45}Ca uptake

^{45}Ca uptake was measured in 0.5 ml of HEPES-buffered Tyrode's solution containing ^{45}Ca (4 μCi/ml). 1~2 × 10^5 cells were incubated in the solution at 37°C for 15 minutes. Incubation was stopped by addition of 1 ml of ice-cold HEPES solution containing La^{3+} (30 mM) and followed by centrifugation at 1000 rpm for 5 minutes. The supernants were

removed and cell pellets were washed 2 times with ice-cold HEPES solution containing La^{3+} (30 mM). Cell pellets were lysed with 0.5ml of 0.5 N NaOH and added to 5.5 ml of scintillation cocktail (Luma-Gel). Radioactivity was measured with a liquid scintillation counter (Tri-Carb 350C).

The results are expressed as means ± S.E. Student's t-tests were used for statistical analysis. P values of less than 0.05 were considered to be statistically significant.

Drugs used were L-arginine, 8-Br-cGMP, sodium nitroprusside, norepinephrine, papain, dithiothreitol, bovine serum albumin (Sigma), collagenase (Wako), and ^{45}Ca (specific activity; 18.7 mCi/mg, New England Nuclear).

RESULTS

Endothelium-dependent relaxation

An endothelium-dependent relaxation was observed by applying ACh(10^{-9}~10^{-5} M) to aortic rings precontracted with 10^{-6} M norepinephrine (NE) in both WKY and SHR(Fig. 1). As shown in Fig. 1, the endothelium-dependent relaxation induced by ACh was initiated at a concentration of 10^{-8} M ACh and showed maximal relaxation at 10^{-7} M ACh in either strain. In the SHR, however, the amplitude of relaxation was less than that in WKY. As the concentration of ACh increased above 10^{-7} M, the relaxation was reduced progressively in the SHR.

Effects of L-arginine on the endothelium-dependent responses

The effects of L-arginine (the substrate for synthesis of nitric oxide) on the endothelium-dependent relaxation were observed in aortic rings pretreated with L-arginine(10^{-3} M) for 15 minutes(Fig. 2). Endothelium-dependent relaxation was measured by applying acetylcholine

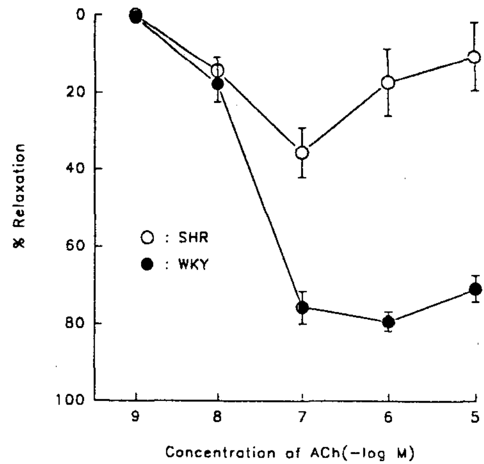

Fig. 1. Endothelium-dependent relaxation induced by acetylcholine (ACh) in aortic rings with intact endothelium of spotaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY). Ordinate represents as a percentage relaxation of norepinephrine (10^{-6} M)-induced contraction. Each point represents the mean value of 8 experiments ± S.E.M.

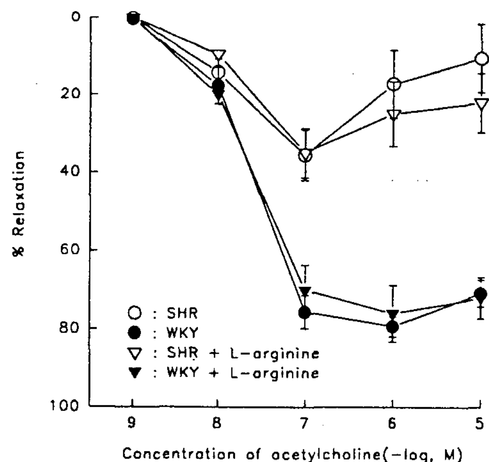

Fig. 2. Effects of L-arginine (10^{-3} M) on the endothelium-dependent relaxations induced by acetylcholine (ACh) in aortic rings with intact endothelium of spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY). Ordinate represents as a percentage relaxation of norepinephrine (10^{-6} M)-induced contraction. Each point represents the mean value of 5 experiments ± S.E.M.

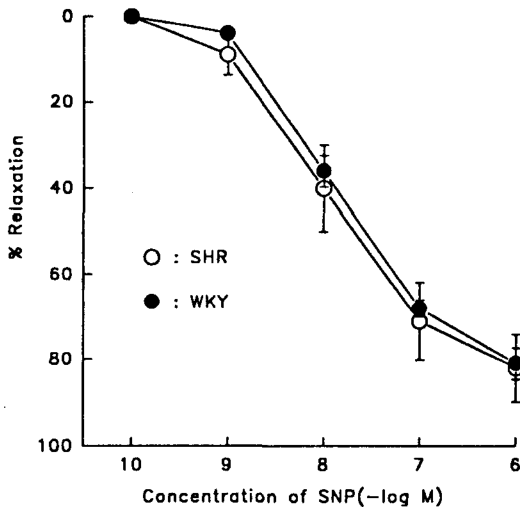

Fig. 3. Effects of sodium nitroprusside (SNP) on the 40mM K^+-induced contraction in aortic rings with rubbed endothelium of spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY). Ordinate represents as a percentage relaxation of 40 mM K^+-induced contraction. Each point represents the mean value of 5 experiments ± S.E.M.

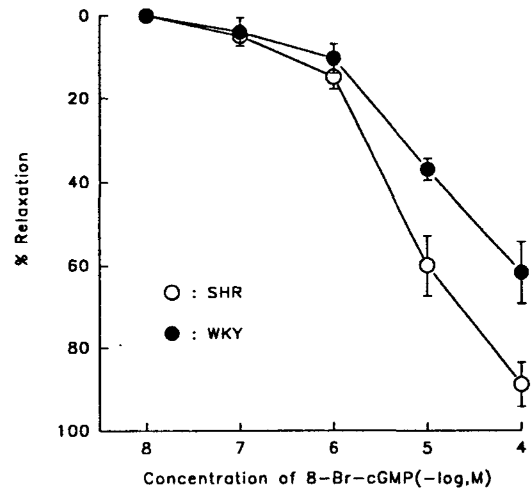

Fig. 4. Effects of 8-bromo-cGMP on the 40 mM K^+-induced contraction in aortic rings with rubbed endothelium of spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY). Ordinate represents as a percentage relaxation of 40 mM K^+-induced contraction. Each point represents the mean value of 5 experiments ± S.E.M.

(ACh) after precontraction by 10^{-6} M norepinephrine (NE). As shown in Fig. 2, Larginine(10^{-3}M) did not affect the endothelium-dependent relaxation induced by ACh in aortic rings of either WKY or SHR strains.

Relaxation response to nitrovasodilator

The relaxation of rat aortic rings by nitrovasodilator was observed by applying sodium nitroprusside (SNP) to aortic rings precontracted with 40 mM K^+-Tyrode's solution(Fig. 3). As shown in Fig. 3, SNP induced concentration-dependent relaxation (10^{-10}~10^{-6} M) of aortic rings in both SHR and WKY. The relaxation curves produced by SNP were not significantly different between SHR and WKY.

Relaxation response to 8-Br-cGMP on high K^+-induced contraction

The relaxation of rat aortic rings by 8-Br-cGMP was observed by applying 8-Br-cGMP to aortic rings precontracted with 40 mM K^+-Tyrode's solution (Fig. 4). As shown in Fig. 4, 8-Br-cGMP induced concentration-dependent relaxation (10^{-6}~10^{-4} M) of aortic rings in both SHR and WKY. The relaxation by 8-Br-cGMP in SHR was greater than that in WKY.

Relaxation response to 8-Br-cGMP on NE-induced contractions

In order to examine the effect of cGMP on the agonist-induced contractions, aortic rings were contracted by NE(10^{-6} M) in normal Tyrode's solution or Ca-free Tyrode's solution after pretreatment with 8-Br-cGMP(10^{-4} M)(Fig. 5). As shown in Fig. 5, the pretreatment of 8-Br-cGMP(10^{-4} M) suppressed norepinephrine-induced contractions in the normal Tyrode's solution as well as in the Ca-free Tyrode's solution. The suppression by 8-Br-cGMP in SHR was greater than that in WKY.

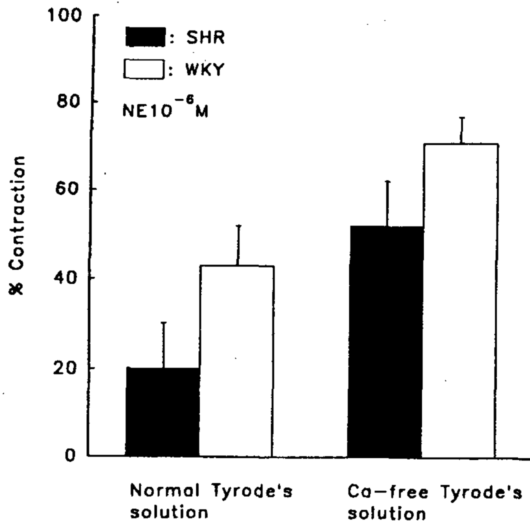

Fig. 5. Effect of 8-Br-cGMP(10^{-4} M) on the norepinephrine (10^{-6} M)-induced contraction in normal Tyrode's solution and Ca-free Tyrode's solution in aortic rings with rubbed endothelium of spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY). Ordinate represents as a percentage contraction of norepinephrine (10^{-6} M)-induced contraction in the normal Tyrode's solution and Ca-free Tyrode's solution, respectively. Each bar represents the mean value of 5 experiments ± S.E.M.

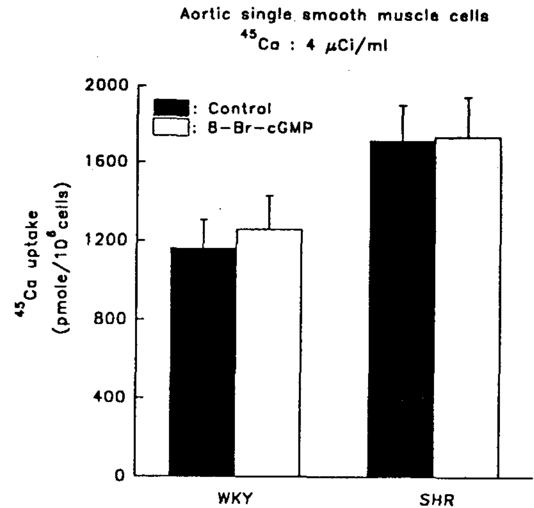

Fig. 6. Effects of 8-Br-cGMP (10^{-4} M) on the basal ^{45}Ca uptake in single aortic smooth muscle cells of spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY). Each bar represents the mean value of 5 experiments ± S.E.M.

Effects of 8-Br-cGMP on the ^{45}Ca uptake

In order to investigate the effects of 8-Br-cGMP on basal ^{45}Ca uptake, basal ^{45}Ca uptake was measured for 15 minutes in single aortic smooth muscle cells loaded with ^{45}Ca and pretreated with 8-Br-cGMP(10^{-4} M) for 30 minutes (Fig. 6). As shown in Fig 6, basal ^{45}Ca uptake in the single aortic smooth muscle cells of SHR was significantly greater than that of WKY. The pretreatment with 8-Br-cGMP did not suppress the basal ^{45}Ca uptake in the single aortic smooth muscle cells in either strain.

Also the effects of 8-Br-cGMP on high K^+-stimulated ^{45}Ca uptake was investigated in single aortic smooth muscle cell pretreated with 8-Br-cGMP (10^{-4} M) for 30 minutes, ^{45}Ca up-

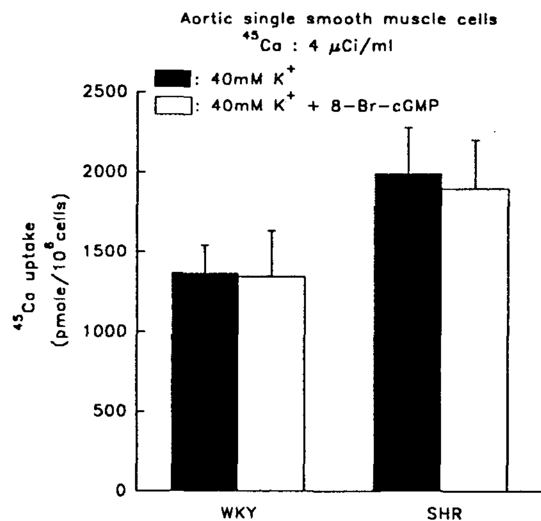

Fig. 7. Effects of 8-Br-cGMP (10^{-4} M) on the 40 mM K^+-stimulated ^{45}Ca uptake in single aortic smooth muscle cells of spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY). Each bar represents the mean value of 5 experiments ± S.E.M.

take was measured with loading of ^{45}Ca and high K^+ (40 mM K^+)(Fig 7). As shown in Fig. 7, 40mM K^+-stimulated ^{45}Ca uptake in the single aortic smooth muscle cells of SHR was significantly greater than that of WKY. The pretreatment with 8-Br-cGMP did not significantly suppress 40 mM K^+-stimulated ^{45}Ca uptake in the single aortic smooth muscle cells in either strain.

DISCUSSION

A decrease in endothelium-dependent relaxation by ACh has been previously reported in various models of hypertension. (Konishi & Su, 1983; Winquist et al, 1984; Lockette et al, 1986; Luscher & Vanhoutte, 1986). In the present study, endothelium-dependent relaxation induced by ACh was initiated at a concentration of 10^{-8} M ACh and showed maximal relaxation at 10^{-7} M ACh in both strains. However, the amplitude of relaxation in the SHR was significantly less than that in the WKY (Fig. 1). This could be related to a decreased release of EDRF, to a reduced vascular responsiveness to the EDRF, and/or to the concomitant release of an endothelium-derived contracting factor in the hypertensive strain. Furthermore, as the concentration of ACh increased above 10^{-7} M, the relaxation was reduced progressively in SHR (Fig. 1). This finding suggested the concomitant release of an endothelium-derived contracting factor (Lüscher & Vanhoutte, 1986) or rapid degradation of endothelium-derived relaxing factors at the high concentration of ACh in SHR (Gryglewski et al, 1986).

Endothelium-derived relaxing factor is synthesized from the amino acid L-arginine catalyzed by nitric oxide synthase (Palmer et al, 1988; Palmer & Moncada, 1989). The nitric oxide synthesis pathway can be stimulated by the addition of L-arginine (Rees et al, 1989; Moore et al, 1990). If the impaired endothelium-dependent relaxation in SHR is due to a deficiency of substrate for nitric oxide synthesis in the endothelial cells, the relaxation could be recovered to the level of normotensive rat by the supply of L-arginine into endothelial cells. However, L-arginine(10^{-3}M) did not increase endothelium-dependent relaxation in the aorta of SHR (Fig. 2). This finding suggests that the cause of impaired endothelium-dependent relaxation in SHR is not due to a deficiency of substrate for nitric oxide synthesis in the endothelial cells.

Endothelium-derived relaxing factor, nitric oxide, is a potent activator of soluble guanylate cyclase and increases intracellular levels of cGMP in target tissue (Ganz et al, 1986). A hypertensive mechanism in SHR that has been postulated is that a reduced vascular response to EDRF results from a reduced relaxing response of cGMP (Lockette et al, 1986; Otsuka et al, 1988). Previous reports have shown that the relaxing response to nitrovasodilators is not dependent on the presence of endothelial cells (Rubanyi and Vanhoutte, 1985) but is associated with cGMP accumulation (Kukovetz et al, 1979). In the present study, relaxing responses to SNP in the aortic rings without endothelium were not significantly different in SHR and WKY (Fig. 3). This findings suggest that the SNP-dependent cGMP pathway in SHR is not impaired.

8-Br-cGMP, a cell membrane-permeable derivative form of cGMP, directly relaxed arterial strips independent of the presence of endothelial cells (Schultz et al, 1979; Napoli et al, 1980). In the present study, the relaxant responses to 8-Br-cGMP to 40mM K^+-induced contraction and NE-induced contraction in the Ca-free Tyrode's solution and normal Tyrode's solution were significantly greater in SHR than that in WKY(Fig 4.5). This finding suggests that the cGMP-dependent pathway in the smooth muscle cells of SHR is accentuated. The fact that the relaxing response to 8-Br-cGMP in SHR was greater than that in WKY might be a

compensatory mechanism for the increased vascular tone in SHR(Noon et al, 1978; Chang, 1994). It is therefore strongly suggested that the impairment of endothelium-dependent relaxation by ACh was not due to a decreased vascular response to endothelium-derived relaxing factors in the aorta of SHR.

In the ^{45}Ca uptake study, basal and 40mM K^+-stimulated ^{45}Ca uptake in single aortic vascular smooth muscle was greater in SHR than in WKY. The fact that a high ^{45}Ca uptake in SHR may contribute to the development of high basal tone has been previously reported (Chang, 1994).

It is known that cGMP activates cGMP-dependent protein kinases in the vascular smooth muscle (Fiscus & Murad, 1988). A number of studies have demonstrated that vascular relaxation by cGMP may be brought about by a decrease of intracellular Ca concentration via an increase of sarcoplasmic reticular Ca ATPase activity (Raeymaekers et al, 1988), or by an inhibition of Ca influx via voltage-operated Ca channels (Clapp & Gurney, 1991). Also it has been shown that cGMP may lead to a reduction of Ca sensitivity by dephosphorylation of myosin light chain (Rapaport et al, 1983; Fiscus et al, 1984, Yanagisawa et al, 1989). However, the precise mechanism by which cGMP/cGMP- dependent protein kinase causes relaxation of vascular smooth muscle remains unresolved.

In the present study, NE or high K^+-induced contractions in either strain were suppressed by the pretreatment of 8-Br-cGMP (10^{-4} M). However, the basal- and 40 mM K^+-stimulated ^{45}Ca uptakes were not affected by the pretreatment with 8-Br-cGMP(10^{-4} M) in either strain. Simultaneous measurements of tension and intracellular Ca concentrations have been performed in canine coronary arteries (Yanagisawa et al, 1989). These measurements showed that vasorelaxation by cGMP occured without any reduction in intracellular Ca concentration. Therefore our findings suggested that cGMP might not affect Ca movement through the plasma membrane but might decrease Ca sensitivity in vascular smooth muscle cells of SHR and WKY.

In conclusion, cGMP may decrease Ca sensitivity in the vascular smooth muscle cells of WKY and SHR and impaired endothelium-dependent relaxation in the aorta of SHR seems unlikely to be due to a decreased vascular response to endothelium-dependent relaxing factors.

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