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European Journal of Pharmacology 374 (1999) 49–58

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Differential effects of tyrosine kinase inhibitors on contraction and relaxation of the aortas of normotensive and hypertensive rats

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Received 14 April 1999; accepted 22 April 1999

Abstract

The contribution of tyrosine kinase activity to vasoreactivity in normotensive Wistar–Kyoto (WKY) and spontaneously hypertensive (SHR) rats was investigated on isolated aortic preparations by the use of two tyrosine kinase inhibitors: methyl-2,5-dihydroxycinnamate (30 μM) and genistein (30 μM). The pretreatment of endothelium denuded aorta with methyl-2,5-dihydroxycinnamate reduced the sensitivity of the rings to noradrenaline to a larger extent in SHR than in WKY. The relaxing effects evoked by methyl-2,5-dihydroxycinnamate and genistein on the sustained contraction induced by endothelin-1 were also more pronounced in SHR denuded rings. Furthermore, in presence of methyl-2,5-dihydroxycinnamate, the endothelium-independent contractile responses to equipotent doses of cyclopiazonic acid were more depressed in SHR than in WKY. In WKY and SHR endothelium-intact aortas contracted with either phenylephrine or endothelin-1, carbachol and cyclopiazonic acid evoked endothelium derived relaxing factor (EDRF)/nitric oxide (NO)-dependent relaxations which were reduced by pretreatment of the rings with methyl-2,5-dihydroxycinnamate or genistein. These inhibitory effects were larger in WKY rings and more important on the cyclopiazonic acid response. In addition, sodium orthovanadate (30 μM) potentiated the noradrenaline-mediated contractions of endothelium-denuded SHR rings and reduced the cyclopiazonic acid-induced relaxation of endothelium-intact WKY rings. The present study suggests a regulatory role for tyrosine kinase in the smooth muscle contraction and the endothelium-dependent relaxation in WKY and SHR aortas and demonstrates the existence of a different relationship in the effect of tyrosine kinase inhibitors on vasoreactivity between SHR and WKY. We propose that an increase in the tyrosine kinase activity in SHR could lead to an enhanced reactivity of Ca^{2+}-linked contractile mechanisms. In addition, our results suggest a link between the loss of tyrosine kinase activity and the altered endothelium-dependent relaxation associated with hypertension.

Keywords: Tyrosine kinase; Endothelium; Noradrenaline; Endothelin-1; Cyclopiazonic acid; Spontaneously hypertensive rat (SHR)

1. Introduction

One of the critical components of cellular signalling is the activation of protein tyrosine kinase, which phosphorylates various intracellular protein substrates leading to a number of cellular events (Malloy et al., 1993; Hollenberg, 1994). Tyrosine phosphorylation was originally associated with cell growth, differentiation, or proliferation. Studies using selective pharmacological tyrosine kinase inhibitors have shown that these enzymes were involved in the constrictor effects elicited by a range of agonists such as noradrenaline, angiotensin II, endothelin-1, serotonin and vasopressin on a variety of isolated vascular preparations (Saifeddine et al., 1992; Abebe and Agrawal, 1995; Semenchuk and Di Salvo, 1995; Jinsi et al., 1996; Sauro et al., 1996; Savineau et al., 1996; Watts et al., 1996). It has been also proposed that tyrosine kinase mediates the pressor response to angiotensin II (Sauro et al., 1996). These data and other studies performed on non-vascular smooth muscle such as gastric smooth muscle (Yang et al., 1993) and bronchial airways (Chopra et al., 1997) support the notion that activation of tyrosine kinase signalling pathways plays a primordial role for contractile process (Savineau et al., 1996; Watts et al., 1996).

Hypertension is associated with some changes in vascular reactivity. These changes involved both the smooth

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00304-0

muscle cells and the endothelial cells. An increased reactivity to vasoconstrictor agonists and a decreased influence of endothelium-derived relaxing factors (EDRF) have been observed in resistance vessels of spontaneously hypertensive rats (SHR). However, conflicting results have been reported concerning the conducting vessels such as aorta (Spector et al., 1969; Shibata et al., 1973; Sim and Singh, 1987; Auguet et al., 1989). Nevertheless, a qualitative modification of the intracellular Ca^{2+} regulation may, at least in part, contribute to the arterial smooth muscle reactivity in experimental models of hypertension (Bohr and Webb, 1988; Auguet et al., 1989).

Endothelium regulates local vascular tone mainly by its ability to synthesise and release vasoactive agents like nitric oxide (NO). In this regard, there is a close relationship between endothelium-dependent relaxation and NO (Furchgott and Zawadzki, 1980). On the other hand, a selective impairment of endothelium-dependent vasodilatation is intimately involved in the pathogenesis of hypertension in experimental animal models (Konishi and Su, 1983; Sim and Singh, 1987) and humans (Panza et al., 1994). The differences in vasorelaxation between hypertensive and normotensive animals may be due to disturbances in the receptor-effector coupling and the effector activity of the L-arginine-EDRF/NO pathway. Although much is known about the involvement of Ca^{2+} in the signalling pathway that transduces the agonist stimulation in the production of EDRF/NO, the mechanisms underlying the impaired endothelium- and NO-dependent relaxations in pathological conditions are poorly understood. In this regard, the finding that tyrosine kinase related mechanisms contribute to the endothelium- and NO-dependent relaxations (Hisayama et al., 1995) make tyrosine kinase a likely candidate for vasorelaxant impairment in hypertensive vessels.

The working hypothesis is that multiple cellular sites of impairment beyond the smooth muscle and endothelial receptors linked to the homeostasis of intracellular calcium may be involved in the alterations of the vasoreactivity in hypertensive vessels. Thus, the aim of the present study was to gain insight into the relative importance of the tyrosine kinase-mediated pathway in vascular smooth muscle contraction and endothelium-dependent relaxation in a model of experimental hypertension, the SHR and in its normotensive Wistar–Kyoto (WKY) control rat.

2. Materials and methods

2.1. Tissue preparation

Male SHR and normotensive control WKY rats (Charles River, France) of 14–17 weeks of age were used. Rats were stunned and exsanguinated. The thoracic aorta freed of fat and of adhering connective tissues was cut into rings 3 mm wide. In some rings the endothelium was removed by gently rubbing the intimal surface with the tip of small forceps.

The isolated rings were suspended (2 g passive tension) in organ bath containing 20 ml of Krebs–Henseleit solution at 37°C and gassed with 95% O_2/5% CO_2. Contractile responses were measured by using isometric force transducers connected to a data-collection system (IOS, EMKA, Paris, France). After mounting, the vessel segments were allowed to equilibrate for 60 min in bath medium. After equilibration, each vascular preparation was precontracted by a submaximal concentration of phenylephrine (1 μM) before the experimental protocols were started. When the contraction was stable, carbachol (10 μM) was tested in order to verify the integrity of endothelium (Furchgott and Zawadzki, 1980). The failure of carbachol to relax rubbed aortic rings was considered as a proof of endothelium disruption. The end of this contraction–relaxation cycle was considered as onset time of the incubation period preceding experiments.

2.2. Experimental procedures

To examine the role of protein tyrosine kinase in modulating smooth muscle contraction, protein tyrosine kinase inhibitors were incubated with aortic rings before the addition of noradrenaline or phenylephrine. In other experiments, protein tyrosine kinase inhibitors were added 3 min after the beginning of the sustained phase of endothelin-1-induced contraction. The effects of the protein tyrosine kinase inhibitors were compared to control rings in the absence of the inhibitor.

2.2.1. Experiments in endothelium-denuded rings

In a first series of experiments, endothelium-free rings from WKY or SHR aortas were exposed after a 30 min incubation period to cumulative concentrations of noradrenaline (1 nM to 3 μM). The concentration–response curves to noradrenaline were constructed in the presence or absence of methyl-2,5-dihydroxycinnamate (30 μM) added in the bath 15 min before the addition of the first dose of noradrenaline. Experiments were also performed in rings exposed to a single concentration of endothelin-1 (10 nM), a concentration sufficient to elicit a stable contraction. Then methyl-2,5-dihydroxycinnamate (30 μM; WKY $n = 6$, SHR $n = 6$) or genistein (30 μM; WKY $n = 6$, SHR $n = 6$) were added at the plateau evoked by the peptide. In parallel studies we have examined the responses of endothelin-1-contracted aortic rings to daidzein, a structural analogue of genistein (30 μM).

In another series of experiments, cumulative concentration–response curves were recorded by increasing the concentrations of cyclopiazonic acid from 0.3 μM to 100 μM in WKY ($n = 6$) and SHR ($n = 6$) rings without endothelium. On the other hand, the contractile effects evoked by a single concentration of cyclopiazonic acid (WKY: 30 μM; SHR: 10 μM) were studied after exposition of the

rings to methyl-2,5-dihydroxycinnamate (30 μM) for 15 min. The concentrations of cyclopiazonic acid were different in WKY and SHR and chosen to provide equipotent contractions according to the previous concentration–response curves to the constrictor agent obtained in the different species.

In some experiments, the contractile responses to KCl (10–100 mM) and phorbol 12,13 dibutyrate (10 nM–3 μM) were investigated in the presence and the absence of methyl-2,5-dihydroxycinnamate (30 μM) and genistein (30 μM) in WKY and SHR. The inhibitors were added 15 min before the addition of the first dose of the compound.

2.2.2. Experiments in endothelium-intact aortic rings

In the second part of our study, the vasorelaxant effects produced by carbachol or cyclopiazonic acid were studied in endothelium-intact aortic rings from WKY and SHR. In a first series of experiments, in both types of preparations, carbachol (0.1 nM–0.3 μM) or cyclopiazonic acid (0.1 nM–0.3 μM) were added cumulatively after the contraction to phenylephrine (1 μM) had reached a plateau. In some experiments, the dose–response curves to these vasorelaxant agents were obtained in preparations pretreated with methyl-2,5-dihydroxycinnamate (15 min, 30 μM). Subsequently, endothelium-independent relaxation responses to sodium nitroprusside (a nitric oxide donor, 0.1 nM–1 μM) were established in the presence or absence of methyl-2,5-dihydroxycinnamate (15 min, 30 μM). In the second series of experiments the rings with endothelium from WKY ($n = 4$) and SHR ($n = 4$) were exposed to a single concentration of endothelin-1 (10 nM). Then, cyclopiazonic acid was added at the plateau evoked by the peptide after incubation of the preparations with genistein (30 μM). In complementary experiments, we challenged endothelium-intact precontracted rings with a single concentration of cyclopiazonic acid (0.1 μM) after incubation of the preparations for 30 min with N^G-nitro-L-arginine (NLA: 100 μM; WKY $n = 2$; SHR $n = 3$).

2.2.3. Experiments in aortic rings treated with a tyrosine phosphatase inhibitor

We have examined the effects of an inhibitor of tyrosine phosphatase, sodium orthovanadate (30 μM) on vasoreactivity. SHR aortic rings without endothelium were incubated for 15 min with sodium orthovanadate before addition of noradrenaline (30 nM). In parallel experiments phenylephrine (3 μM)-precontracted WKY rings with endothelium were used to study relaxation to cyclopiazonic acid (100 μM) in the absence and presence of sodium orthovanadate (30 μM). The inhibitor was introduced into the bath medium 15 min before phenylephrine.

2.3. Data and statistical analysis

All values are presented as means ± S.E.M. in tension (g). Comparisons of differences between 2 groups of data were performed using a two-way analysis of variance. The EC_{50} mean values ± S.E.M. for agonists were calculated from the maximal contractile response to each agonist by computer analysis of mean tension values (In-house statistical computer program). P values < 0.05 were considered significant.

2.4. Drugs

The following drugs were used. Noradrenaline bitartrate, phenylephrine hydrochloride, carbachol chloride, genistein, methyl-2,5-dihydroxycinnamate, cyclopiazonic acid, daidzein, sodium orthovanadate and sodium nitroprusside were purchased from Sigma-Aldrich Chimie (St. Quentin-Fallavier, France). Phorbol 12,13-dibutyrate was obtained from Research Biochemicals International (Illkirch, France) and endothelin-1 from Peptide Institute (Japan). Noradrenaline, phenylephrine and carbachol were prepared daily in deionized water and kept in ice until use. The pH of the solution of sodium orthovanadate was adjusted to 9.7 and boiled until the solution turned clear. Cyclopiazonic acid, genistein and phorbol 12,13 dibutyrate were dissolved in 10% dimethylsulfoxide as stock solution. Control vascular preparations were treated with the corresponding concentration of the solvent studied simultaneously. In all experiments the final concentration of solvent in the organ bath did not exceed 0.1%.

3. Results

3.1. Effects of tyrosine kinase inhibitors on vasoconstriction in endothelium-denuded rings

3.1.1. Effects on the responses to noradrenaline

In aortic endothelium-denuded rings from WKY and SHR, noradrenaline (1 nM–3 μM) produced concentration-dependent contractions (WKY: $EC_{50} = 5.80 \pm 0.83$ nM; SHR: $EC_{50} = 6.20 \pm 0.97$ nM; $n = 6$). The maximal contractile responses to noradrenaline of aortas from hypertensive rats were not different from responses of normotensive ($E_{max} = 1.12 \pm 0.10$ g in SHR rings and 1.13 ± 0.06 g in WKY rings; $n = 6$; Fig. 1).

Methyl-2,5-dihydroxycinnamate (30 μM) did not change the baseline tone of the WKY ($n = 6$) and SHR ($n = 6$) vascular preparations but shifted to the right the noradrenaline dose–response curves (Fig. 1). When compared to their corresponding controls, the shift was twice larger in SHR ($EC_{50} = 28.90 \pm 1.00$ nM; $P < 0.001$) than in WKY ($EC_{50} = 9.30 \pm 0.97$ nM; $P < 0.01$) with significant modifications in the maximal constrictor responses (WKY: $E_{max} = 0.95 \pm 0.02$ g, $P < 0.05$; SHR: $E_{max} = 0.73 \pm 0.05$ g, $P < 0.001$).

3.1.2. Effects upon contractions to endothelin-1

Endothelin-1 (10 nM) produced stable contractions in endothelium-denuded aortic rings from WKY ($E_{max} = 1.36$

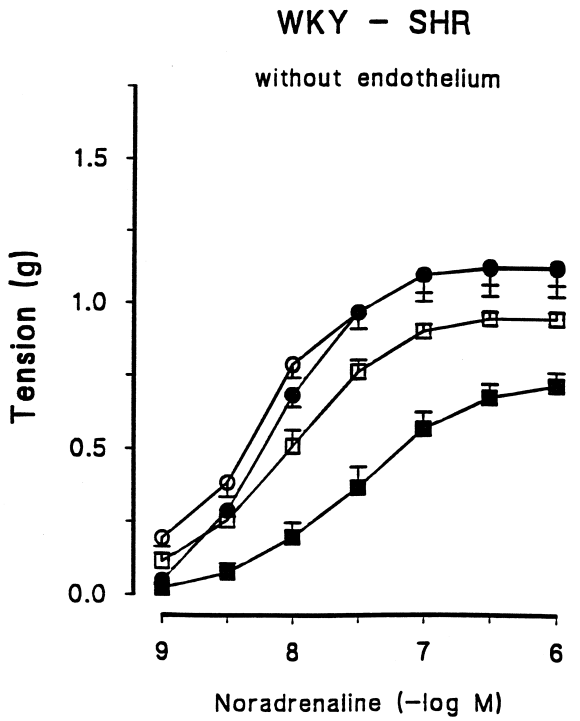

Fig. 1. Effect of methyl-2,5-dihydroxycinnamate on the contractile responses to noradrenaline in rings without endothelium from Wistar Kyoto rats (WKY, open symbols, $n = 6$) and spontaneously hypertensive rats (SHR, solid symbols, $n = 6$). Noradrenaline was added cumulatively alone (circles) or in the presence of the tyrosine kinase inhibitor (square). Symbols represent the mean ± S.E.M.

± 0.07 g, $n = 11$) and SHR ($E_{max} = 1.31 \pm 0.08$ g, $n = 11$). At this dose, tension developed slowly reaching a sustained plateau after about 5 min.

The addition of tyrosine kinase inhibitors upon the sustained contraction phase evoked by endothelin-1 accelerated the decrease in smooth muscle tension in comparison to control rings. After 5 min, the decreases in tone produced by methyl-2,5-dihydroxycinnamate (30 μM) were greater in SHR than in WKY (44.0 ± 5.5% vs. 18.6 ± 4.16%; $n = 6$; Fig. 2A). Similar results were observed with genistein (30 μM, SHR: 41.5 ± 3.7%, $n = 5$; WKY: 21.8 ± 2.7%, $n = 5$; Fig. 2B).

Daidzein that differs from genistein in one hydroxyl group and does not inhibit the activity of tyrosine kinase, only slightly attenuated (less than 10%) the contractile responses evoked by endothelin-1 (10 nM; data not shown).

3.1.3. Effects on the contractions to cyclopiazonic acid

Cyclopiazonic acid (0.3 μM–100 μM) produced contractions in aortic endothelium denuded rings from WKY and SHR. Although the maximal contractile responses to cyclopiazonic acid were similar in both species (WKY: 0.61 ± 0.08 g, SHR: 0.63 ± 0.06 g; $n = 6$), the tensions developed to lower concentrations of cyclopiazonic acid (3 and 10 μM) were significantly enhanced in SHR (3 μM: +82.7 ± 26.8%, $P < 0.001$; 10 μM: +74.7 ± 23.2%, $P < 0.001$; $n = 6$) compared to normotensive controls (Fig. 3).

The contractile responses elicited by equipotent doses of cyclopiazonic acid in WKY (30 μM: 0.61 ± 0.06 g,

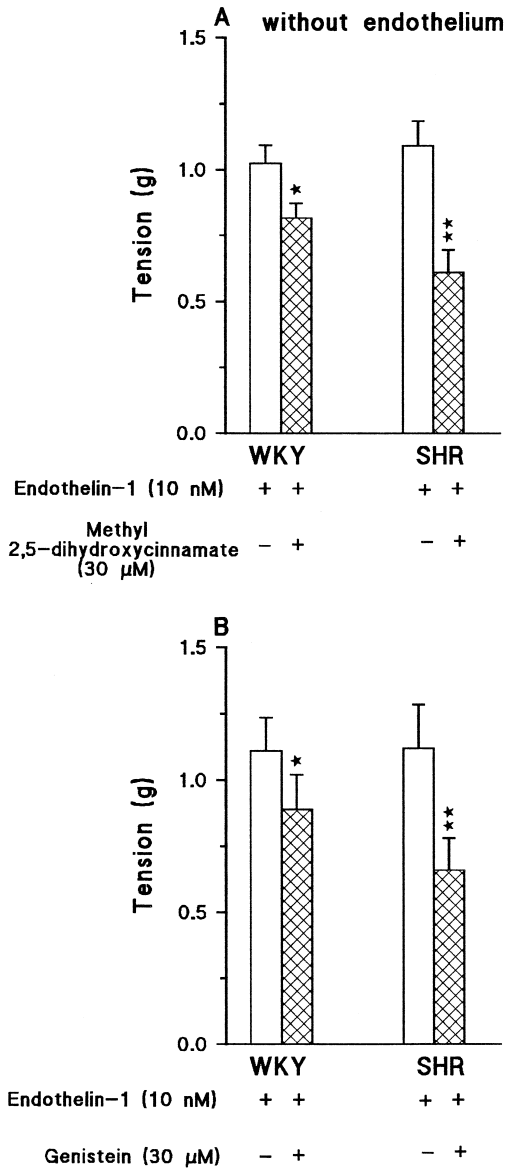

Fig. 2. Bar graphs show comparison of tension to a single concentration of endothelin-1 (10 nM) in endothelium-denuded rings of aortas from WKY and SHR. The experiments were performed under (1) control conditions (open bar, $n = 6$), (2) in the presence of methyl-2,5-dihydroxycinnamate (30 μM, cross-hatched bar, $n = 6$, (A), (3) in the presence of genistein (30 μM, cross-hatched bars, $n = 5$, (B). The inhibitors of tyrosine kinase or the solvent were added at the beginning of the sustained phase elicited by endothelin-1. Five minutes after, the decreases in smooth muscle tension were compared between solvent-treated (control) rings and tyrosine kinase inhibitor-treated rings. Results are expressed in tension (g) and are presented as means ± S.E.M. $^{*}P < 0.05$ and $^{**}P < 0.01$ for significant differences between tyrosine kinase inhibitor treated preparations and their respective controls.

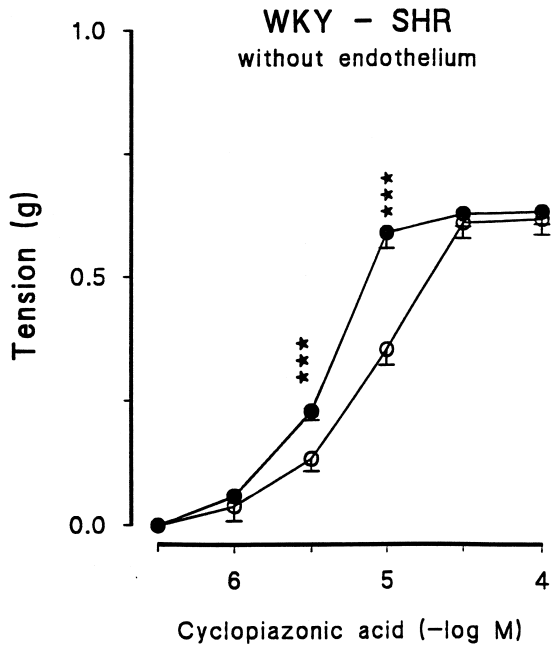

Fig. 3. Concentration–response curves for the contractile effect of cyclopiazonic acid (1 μM–100 μM) in WKY (open circles, $n = 6$) and SHR (solid circles, $n = 6$) endothelium-denuded rings. $^{***}P < 0.001$ at 3 and 10 μM.

$n = 4$) and SHR (10 μM: 0.64 ± 0.12 g, $n = 4$) rings were attenuated by pre-exposure of the rings to methyl-2,5-dihydroxycinnamate (30 μM). This inhibitory effect was greater in SHR (32.01 ± 5.50%, $n = 4$) than in WKY (15.30 ± 2.30%, $n = 4$).

3.1.4. Effects on the contractions to KCl and phorbol 12,13-dibutyrate

Methyl-2,5-dihydroxycinnamate (30 μM) and genistein (30 μM) did not affect the contractile responses evoked by KCl (10–100 mM) in aortic endothelium denuded rings from WKY ($n = 2$) and SHR ($n = 3$).

The tensions elicited by phorbol 12,13-dibutyrate (10 nM–3 μM) in endothelium denuded rings from WKY ($n = 4$) and SHR ($n = 4$) were not significantly modified by methyl-2,5-dihydroxycinnamate (30 μM) or genistein (30 μM versus 1 μM of phorbol 12,13-dibutyrate).

3.2. Effects of tyrosine kinase inhibitors on vasorelaxation in endothelium-intact rings

3.2.1. Effects on the endothelium-dependent relaxations to carbachol

In WKY and SHR endothelium-intact aortic rings precontracted with phenylephrine (3 μM), carbachol (0.1 nM–0.3 μM) produced concentration-dependent relaxations (WKY: $EC_{50} = 4.80 \pm 0.95$ nM; SHR: $EC_{50} = 8.12 \pm 0.91$ nM; $n = 8$; Fig. 4). The maximal relaxations to carbachol were greater ($P < 0.01$) in WKY (96.00 ± 2.15%; $n = 8$) in comparison to SHR (75.00 ± 1.82%; $n = 8$).

In the presence of methyl-2,5-dihydroxycinnamate (30 μM), the concentration–relaxation curves of carbachol were shifted to the right. This effect was larger in WKY than in SHR. When compared to control rings (Fig. 4), in WKY, $EC_{50} = 9.5 \pm 0.9$ nM ($n = 8$, $P < 0.01$) and in SHR, $EC_{50} = 10.5 \pm 0.8$ nM, ($n = 8$, not significant).

The endothelial specificity of the effects of methyl-2,5-dihydroxycinnamate is supported by the lack of effect of this inhibitor on the vasodilator responses to sodium nitroprusside on endothelium-denuded rings (data not shown).

3.2.2. Effects on the relaxation responses to cyclopiazonic acid

In endothelium-intact aortic rings precontracted with phenylephrine (3 μM), cyclopiazonic acid (0.1 nM to 0.3 μM) produced concentration-dependent relaxations (WKY: $EC_{50} = 2.20 \pm 0.36$ nM, $E_{max} = 0.17 \pm 0.03$ g; SHR: $EC_{50} = 1.44 \pm 0.09$ nM; $E_{max} = 0.62 \pm 0.06$ g; $n = 6$; Fig. 5). The relaxing responses to the cumulative addition of cyclopiazonic acid expressed as percentage in inhibitions of

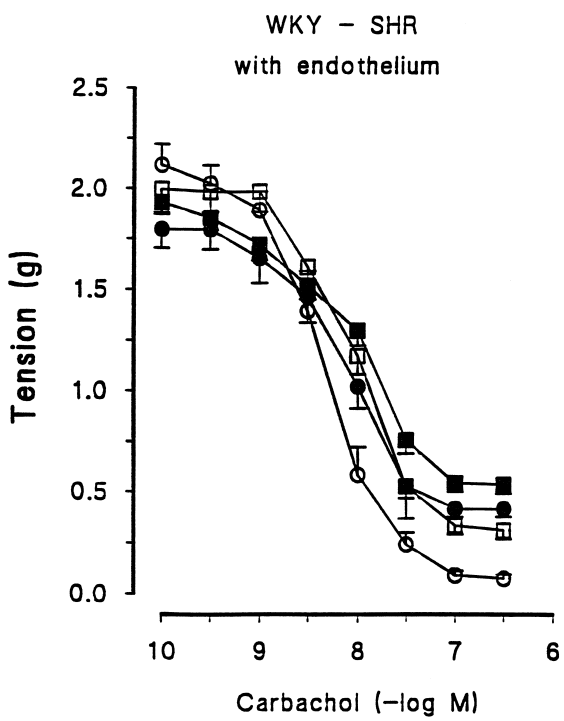

Fig. 4. Concentration–response curves for the vasorelaxation effect of carbachol (0.1 nM–0.3 μM) on the phenylephrine-evoked contraction in aortic rings with endothelium from Wistar–Kyoto rats (WKY, open symbols, $n = 8$) and spontaneously hypertensive rats (SHR, solid symbols, $n = 8$). The experiments were performed under (1) control conditions (circles), (2) in the presence of methyl-2,5-dihydroxycinnamate (30 μM, squares) introduced in the bath 15 min before the addition of phenylephrine (3 μM). Symbols represent the mean ± S.E.M..

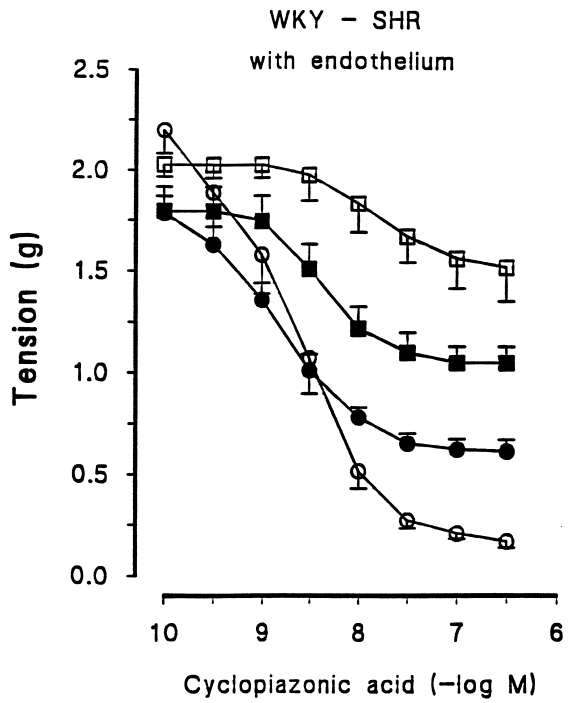

Fig. 5. Concentration–response curves for the vasorelaxation effect of cyclopiazonic acid (0.1 nM–0.3 μM) on the phenylephrine-evoked contraction in aortic rings with endothelium from Wistar Kyoto rats (WKY, open symbols, $n = 8$) and spontaneously hypertensive rats (SHR, solid symbols, $n = 8$). The experiments were performed under (1) control conditions (circles), (2) in the presence of methyl-2,5-dihydroxycinnamate (30 μM) (squares). Symbols represent the mean ± S.E.M..

the phenylephrine-induced contraction were significantly greater ($P < 0.001$) in WKY (94.30 ± 3.42%) than in SHR (65.50 ± 2.70%). The relaxation evoked by cyclopiazonic acid (0.1 μM) was abolished by pretreatment of the vascular preparations for 30 min with NLA (100 μM).

Methyl-2,5-dihydroxycinnamate (30 μM) shifted to the right the cyclopiazonic acid-induced dose–response curves. The shift was larger in WKY ($EC_{50} = 15.10 \pm 0.73$ nM, $n = 6$, $P < 0.001$ in comparison to controls) than in SHR ($EC_{50} = 4.16 \pm 0.21$ nM, $n = 6$, $P < 0.01$). Although the level of tension of both precontracted preparations relaxed by cyclopiazonic acid (3 nM; Fig. 5) was similar in WKY (1.07 ± 0.18 g, $n = 6$) and SHR (1.01 ± 0.08 g, $n = 6$) endothelium-intact rings, the inhibitory effects elicited by methyl-2,5-dihydroxycinnamate (30 μM), expressed as percentages of the phenylephrine-induced contractions, were significantly higher ($P < 0.01$) in WKY (50%, $n = 6$) than in SHR (27%, $n = 6$) treated rings.

In aortic rings with endothelium precontracted with endothelin-1 (0.1 μM; WKY: 1.76 ± 0.11 g; SHR: 1.68 ± 0.11 g, $n = 4$), the relaxing effects elicited by cyclopiazonic acid (10 nM) in WKY (0.63 ± 0.09 g) and SHR (0.96 ± 0.10 g) were reduced in vascular preparations treated by genistein (30 μM) (WKY: 1.08 ± 0.11 g, $P < 0.001$; SHR: 1.21 ± 0.11 g, $P < 0.05$; $n = 4$).

3.3. Effects of a tyrosine phosphatase inhibitor on vasoreactivity in WKY and SHR aortas

The addition of sodium orthovanadate (30 μM) in the organ bath did not modify the resting tension of SHR rings without endothelium and of WKY rings with endothelium.

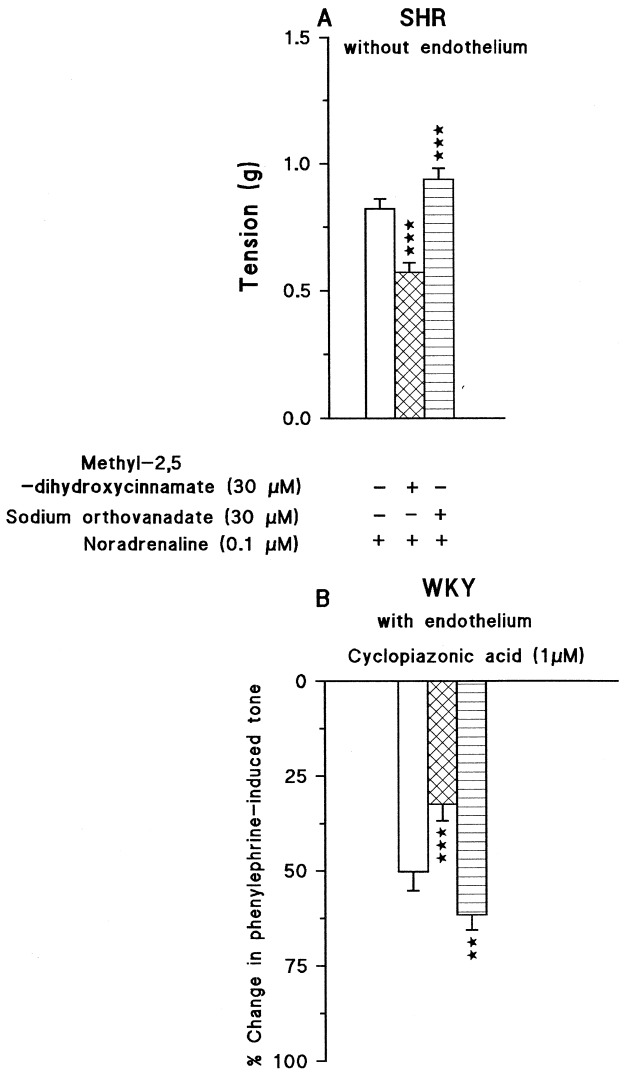

Fig. 6. (A) Contractile responses to noradrenaline in SHR aortic rings without endothelium. Experiments were conducted in rings untreated (open bar) or pretreated with methyl-2,5-dihydroxycinnamate (cross-hatched bar) or sodium orthovanadate (hatched bar). Data are expressed as mean ± S.E.M.. (B) Depressor effect of cyclopiazonic acid (1 μM) on tension development in WKY aortic rings with endothelium contracted with phenylephrine (3 μM). Experiments were conducted in rings untreated (open bar: phenylephrine = 2.1 ± 0.1 g, post-cyclopiazonic acid = 1.03 ± 0.11 g) or pretreated with methyl-2,5-dihydroxycinnamate (cross-hatched bar: phenylephrine = 2.05 ± 0.12 g, post-cyclopiazonic acid = 0.81 ± 0.07 g) or sodium orthovanadate (hatched bar: phenylephrine = 2.10 ± 0.08 g, post-cyclopiazonic acid = 1.37 ± 0.08 g). Data are expressed as percentage change in phenylephrine-induced tone and are presented as mean ± S.E.M. of six separate experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control rings.

In SHR rings without endothelium, the constrictor responses to noradrenaline (0.1 μM, 0.82 ± 0.04 g, $n = 8$) were potentiated in presence of sodium orthovanadate (30 μM, 0.94 ± 0.04 g, $n = 8$, $P < 0.001$, Fig. 6A).

Phenylephrine (3 μM) produced comparable maximal tension in WKY aortic rings with endothelium either bathed in control solution or in media containing sodium orthovanadate (30 μM). In control precontracted rings, cyclopiazonic acid (1 μM) produced a relaxation of 50.27 ± 5% ($n = 6$). When vessels were pretreated with sodium orthovanadate (30 μM), the relaxant effect evoked by cyclopiazonic acid was increased to 61.6 ± 4% ($n = 6$, $P < 0.05$, Fig. 6B).

4. Discussion

The present study shows a different sensitivity to tyrosine kinase inhibitors in SHR and WKY. However, opposite alteration in the modulatory role of tyrosine kinase inhibitors on contraction and relaxation was observed in the two strains. Contractions to noradrenaline, endothelin-1 and cyclopiazonic acid were more sensitive to tyrosine kinase inhibitors in aortic rings from SHR in comparison to WKY whereas endothelium-dependent relaxations were less sensitive to tyrosine kinase inhibitors in SHR than in WKY. These results may indicate that the contribution of the protein tyrosine kinase in the regulation of vasoreactivity depends on the hypertensive state of the animal and on the vascular cell studied.

Although no drug is completely specific, our experimental approach was to use tyrosine kinase inhibitors in functional experiments. In this regard, the use of two drugs of different chemical classes acting at two different sites of inhibition may support the conclusion that tyrosine kinase is the cellular target of their action. Methyl-2,5-dihydroxycinnamate, a stable erbstatin analogue, binds to the recognition site of the enzyme. Moreover, methyl-2,5-dihydroxycinnamate exhibits specific inhibitory effect against epidermal growth factor receptor (EGFR) kinase. There is growing evidence that EGFR kinase is present in rat aorta. Indeed, in cultured rat aortic smooth muscle cells the tyrosine phosphorylation of several proteins and the mediated synthesis of DNA caused by EGF are affected by tyrosine kinase inhibitors (Watts et al., 1996; Panek et al., 1997). On the other hand, Florian and Watts (1999) have shown that EGF induced potent contractions in arteries from two forms of experimental hypertension, the DOCA-salt and one kidney, one clip (1K, 1C) rats. Genistein is an ATP analogue which blocks the ATP binding site of a variety of tyrosine kinases. Since a physiological balance between the protein phosphorylation process provided by tyrosine kinases and the protein dephosphorylation facilitated by tyrosine phosphatases may influence the signalling pathways, sodium orthovanadate, a blocker of tyrosine phosphatase that inhibits the activity of tyrosine kinases (Swarup et al., 1982), was used as an additional pharmacological tool to analyse the specific role of tyrosine phosphorylation in the vasoreactivity of WKY and SHR aorta.

Our results show that methyl-2,5-dihydroxycinnamate inhibited the contractile responses elicited by noradrenaline in WKY and SHR endothelium-denuded aortic rings. This effect was 2-fold larger in SHR than in WKY. In addition, the sustained plateau elicited by a single dose of endothelin-1 was more depressed by methyl-2,5-dihydroxycinnamate and genistein in SHR than in WKY. Daidzein, the inactive analogue of genistein did not reverse endothelin-1-induced contraction. Furthermore, sodium orthovanadate enhanced the contractions evoked by noradrenaline in SHR aortic rings without endothelium. However, non-specific inhibitory mechanisms may confound the interpretation of the results obtained with the tyrosine kinase inhibitors. Indeed, it has been reported that high concentrations of genistein could inhibit protein kinase C (Akiyama et al., 1987). This seems unlikely in our experiments since in SHR endothelium-denuded rings, genistein and methyl-2,5-dihydroxycinnamate did not affect the contractile responses evoked by phorbol 12,13-dibutyrate, a protein kinase C activator. On the other hand, tyrosine kinase inhibitors did not change KCl-induced contraction.

Taken together, our findings point to a role of tyrosine kinases in the contractile responses to different agonists such as noradrenaline and endothelin-1. Moreover, this role appeared to be higher in SHR aorta compared to WKY. The present data are consistent with the previously demonstrated ability of tyrosine kinase to modulate the contractile responses of agonists in vascular tissue of Sprague–Dawley rats (Abebe and Agrawal, 1995; Watts et al., 1996; Ohanian et al., 1997). Since tyrosine kinase activity contributes to the vascular effects of EGF, our findings are in line with a recent study demonstrating that EGF-induced contractions were greater in aorta from hypertensive than normotensive rats (Florian and Watts, 1999). However, our results are at variance with those showing that contractions of small arteries from WKY were more sensitive to tyrosine kinase inhibitors in comparison to SHR (Malloy and Sauro, 1996). This discrepancy may be explained by differences in the distribution and functional importance of tyrosine kinase depending on vascular beds and size of vessels. These conflicting results may also be explained by the diversity of the applied stimulus since it has been reported that angiotensin II- but not phenylephrine-induced contractions were altered by tyrphostin-25, an inhibitor of tyrosine kinase (Malloy and Sauro, 1996).

Hypertension is generally characterised by an increased sensitivity of vascular smooth muscle to vasoconstrictor stimuli (Folkow, 1982). However, conflicting results have been reported on the vascular reactivity of different vessels from hypertensive rats. The resistance vessels from SHR are more responsive to constrictor agonists whereas in conducting vessels such as aorta, the contractile responses

are similar or lower in SHR than in WKY (Spector et al., 1969; Shibata et al., 1973; Sim and Singh, 1987; Auguet et al., 1989; Kam et al., 1996).

Receptor-coupled vascular contraction is associated with the elevation of cytosolic concentration of free Ca^{2+} through mechanisms which mobilise both intracellular Ca^{2+} stores and the influx of extracellular Ca^{2+} (Minneman, 1988; Auguet et al., 1989). In the present study, there is no discernible quantitative hypertension-related effect on vasoconstriction mediated by noradrenaline in aortic preparations. However, the contribution of various key cellular mechanisms to the overall contraction produced by noradrenaline may be modified in SHR aorta (Auguet et al., 1989). This possibility is substantiated in our experiments by the findings that (i) the tension developed by cyclopiazonic acid, a selective inhibitor of sarcoplasmic reticulum Ca^{2+} ATPase, was greater in the preparations from SHR compared to WKY and (ii) that the inhibition of tyrosine kinase by methyl-2,5-dihydroxycinnamate attenuated to a greater magnitude the elevation of the tone caused by cyclopiazonic acid in SHR than in WKY. These results suggest that the mechanism of Ca^{2+}-influx linked to store depletion and its associated tyrosine-kinase sensitivity were increased in SHR aortic rings stimulated by constrictor agonists. However, our observations do not rule out the possibility of an elevated Ca^{2+} storage in SHR sarcoplasmic reticulum with the consequent enhanced release of Ca^{2+} from this store, or an increased sensitivity of the Ca^{2+}-pump to cyclopiazonic acid in SHR compared to WKY.

In the second part of our study, cyclopiazonic acid was shown to cause endothelium- and NO-dependent relaxations in constricted rings from WKY and SHR aortas. These results are consistent with previous reported findings obtained in the aorta of Wistar rats (Moritoki et al., 1994; Zheng et al., 1994). In addition, it is well established that depletion of internal Ca^{2+} stores by Ca^{2+} ATPase inhibitors is the signal of Ca^{2+} entry (Dolor et al., 1992; Schilling et al., 1992; Zhang et al., 1994) and the subsequent production of NO in endothelial cells (Hutcheson and Griffith, 1997). In the present study, methyl-2,5-dihydroxycinnamate and genistein inhibited the endothelium-dependent vasorelaxant responses evoked by carbachol and cyclopiazonic acid on rings stimulated by phenylephrine or endothelin-1. In addition, sodium orthovanadate enhanced the extent of the relaxation evoked by noradrenaline in WKY rings with endothelium. These results are in accordance with some postulating that the cellular events leading to the vascular release of EDRF/NO involve the activation of the tyrosine kinase pathway (Hisayama et al., 1995). However, the possibility that the signalling mechanism for the augmented cyclopiazonic acid induced relaxation after pretreatment of WKY rings with sodium orthovanadate may involve the inhibitory effect of vanadate on sarcoplasmic reticulum Ca^{2+}-ATPase could not be ruled out (Raeymaekers et al., 1983).

The endothelium-dependent effects of the different tyrosine kinase inhibitors investigated were larger in WKY than in SHR. Of note is that the inhibitory effects of methyl-2,5-dihydroxycinnamate were more important on the endothelium-dependent relaxations evoked by cyclopiazonic acid compared to carbachol in WKY aorta. Thus, it seems that the tyrosine kinase triggered process accounts only for a mild part of the carbachol-induced endothelium-dependent vasorelaxation.

Selective impairment of endothelium-dependent vasodilatation is intimately involved in the pathogenesis of hypertension (Konishi and Su, 1983; Panza et al., 1994). Different mechanisms have been proposed to elucidate endothelial dysfunction in SHR as the decrease in the formation of NO by NO synthase (Konishi and Su, 1983) or an increased production of endothelium-derived contracting factor by cyclooxygenase-1 or cyclooxygenase-2 (Ge et al., 1995; Zerrouk et al., 1998). However, cellular defects unrelated to the intrinsic NO synthase activity may be involved in the alteration of the endothelium-dependent vasoreactivity. In this regard, some findings clearly link changes in calcium handling to vasoactive responses characteristic of the impairment of the endothelial cell function and the reduced synthesis of EDRF/NO (Miwa et al., 1997). Of interest is the recent data suggesting that this impairment could be associated with the defect in the signalling pathways beyond the endothelial receptors (Cardillo et al., 1998). A decreased level of resting cytosolic Ca^{2+} concentration and an altered intracellular Ca^{2+} release pathway have been reported in SHR endothelial cells (Liu et al., 1995; Wang et al., 1995). With respect to inhibition by tyrosine kinase inhibitors of carbachol and cyclopiazonic acid endothelium-dependent relaxation, our data rise the question of a possible impairment of tyrosine phosphorylation in SHR. In support to this hypothesis are data reported in a restenosis model characterised by an altered endothelium-dependent relaxation. In this model, the contribution of tyrosine kinase in response to acetylcholine was lesser in regenerated endothelial cells from carotid arteries (Belmadani et al., 1997). This finding suggests some shared similarities in the mechanisms of endothelial dysfunction between vessels of different pathological models.

In conclusion, the present study suggests a regulatory role for tyrosine kinase in the smooth muscle contraction and the endothelium-dependent relaxation in WKY and SHR aortas. Moreover, our results demonstrate the existence of different effects of tyrosine kinase inhibitors on vasoreactivity between SHR and WKY. We propose that an increase in the tyrosine kinase activity in SHR could lead to a modified reactivity of Ca^{2+}-linked contractile mechanisms. In addition, our results allow to evidence a link between the loss of tyrosine kinase activity and the altered endothelium-dependent relaxation in SHR. Finally, the possibility that altered vasorelaxation in hypertension might involve defects as the impairment of tyrosine-phos-

phorylation regulatory mechanisms should be worth considered.

Acknowledgements

This study was supported by a grant from Institut Henri Beaufour (IPSEN group, France).

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