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Pressor response induced by central angiotensin II is mediated by activation of Rho/Rho-kinase pathway via AT₁ receptors

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Objectives The brain renin-angiotensin system plays an important role in cardiovascular regulation and the pathogenesis of hypertension. Angiotensin II activates the Rho/Rho-kinase pathway in vascular smooth muscle cells and cardiomyocytes *in vitro*. The aim of the present study was to determine whether angiotensin II in the brainstem activates the Rho/Rho-kinase pathway, and, if so, whether this mechanism is involved in the central pressor action of angiotensin II.

Methods and results Angiotensin II infused intracisternally for 7 days in Wistar-Kyoto rats (WKY) increased systolic blood pressure (SBP) and urinary norepinephrine excretion. These responses were abolished by the co-infusion of Y-27632, a specific Rho-kinase inhibitor, or valsartan. The intracisternal infusion of Y-27632 or valsartan also reduced SBP and norepinephrine excretion in spontaneously hypertensive rats (SHR). Western blot analysis was performed to examine the expression levels of membranous RhoA and phosphorylated ezrin, radixin, and moesin (p-ERM), which reflects Rho/Rho-kinase activity. The expression levels of membranous RhoA and p-ERM in the brainstem were significantly greater in both angiotensin II-treated WKY and SHR than in vehicle-treated WKY. Valsartan reduced the expression levels of membranous RhoA and p-ERM in angiotensin II-treated WKY and SHR. Y-27632 reduced the

expression levels of p-ERM in angiotensin II-treated WKY and SHR.

Conclusions These results suggest that the pressor response induced by intracisternally infused angiotensin II is substantially mediated by the activation of the Rho/Rho-kinase pathway via AT₁ receptors of the brainstem in WKY, and that this pathway might be involved in the hypertensive mechanisms of SHR. *J Hypertens* 25:399-406 © 2007 Lippincott Williams & Wilkins.

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Introduction

Angiotensin II is critical for the regulation of vascular tone, blood pressure, and volume homeostasis. The physiological actions of angiotensin II occur through its binding to two main cell surface receptors, AT₁ and AT₂. The cardiovascular effects of angiotensin II are widely considered to result from AT₁ receptor activation. The brain renin-angiotensin system is also considered pivotal in cardiovascular regulation and is important in the pathogenesis of hypertension [1,2]. The specific mechanisms by which increased brain renin-angiotensin system activity results in hypertension are not well understood, but include increased sympathetic vasomotor tone and impaired arterial baroreflex function.

The involvement of the small guanosine triphosphatase Rho and its downstream effector Rho-kinase [3] is implicated in various cellular functions, including actin

cytoskeleton organization [4], cell adhesion and motility [5,6], myosin light chain phosphorylation, and smooth muscle contraction [7]. Y-27632, a specific Rho-kinase inhibitor [8,9], dramatically reduces hypertension in rat hypertension models [9]. In addition, Rho-kinase activity is increased in hypertensive blood vessels [10], and the inhibition of Rho-kinase induces preferential forearm vasodilation in hypertensive patients [11]. Activation of the Rho/Rho-kinase pathway thus contributes to the pathophysiology of hypertension [9-11]. Recent results indicate that the Rho/Rho-kinase pathway is involved in AT₁ receptor signaling. The Rho/Rho-kinase pathway is activated in angiotensin II-induced hypertrophy of vascular smooth muscle cells and cardiomyocytes [12,13]. RhoA and Rho-kinase are also found in the central nervous system [14,15]. Rho/Rho-kinase is present in the nucleus of the solitary tract (NTS) in the brainstem, and activation of the Rho/Rho-kinase pathway in the

NTS contributes to the neural mechanisms of hypertension in spontaneously hypertensive rats (SHR) and in a hypertensive rat model of chronic nitric oxide synthase inhibition [16,17]. Furthermore, inhibition of this pathway in the NTS augments baroreflex control of the heart rate and enhances glutamate sensitivity in SHR [18,19]. It remains to be determined, however, whether activation of the Rho/Rho-kinase pathway also contributes to neurogenic hypertensive mechanisms caused by angiotensin II. The aim of the present study was to elucidate the role of the Rho/Rho-kinase pathway in the brainstem on the central effects of angiotensin II. For this purpose, we infused angiotensin II intracisternally for 7 days with a mini-osmotic pump in Wistar-Kyoto rats (WKY). Then, Y-27632 or valsartan was infused intracisternally in angiotensin II-treated WKY or SHR. Systolic blood pressure (SBP) and heart rate were measured using the tail-cuff method. Urinary norepinephrine excretion was measured as a marker of sympathetic nerve activity. Finally, we evaluated RhoA and Rho-kinase activity in the brainstem by western blot analysis.

Methods

This study was reviewed and approved by the Committee on Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, and was conducted according to the Guidelines for Animal Experiments of Kyushu University. Male WKY or SHR (280–340 g, 16–20 weeks old) were used in the present study. Rats were obtained from an established colony at the Animal Research Institute of Kyushu University Faculty of Medicine (Fukuoka, Japan) [16,17].

Continuous infusion experiments with angiotensin II, Y-27632, valsartan, and phenylephrine

Angiotensin II (0.1 mg/kg per day) or vehicle (artificial cerebrospinal fluid) were infused intracisternally (0.25 μ l/h) for 7 days with a mini-osmotic pump (Alzet model 1002; Durect Corporation, Cupertino, California, USA) in WKY. Y-27632 (5 or 10 mmol/l) or valsartan (0.5 mg/kg per day) were infused intracisternally (0.25 μ l/h) in angiotensin II-treated WKY or SHR. The rats were anaesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). The mini-osmotic pump, filled with angiotensin II, vehicle, Y-27632, or valsartan was implanted subcutaneously in the back and connected to a polyethylene tube (PE10). A small hole was then made in the atlanto-occipital membrane, which covers the dorsal surface of the medulla, and the tip of the tube was placed intracisternally and fixed in place with tissue adhesive. At the end of the infusion, we confirmed intracisternal delivery of drug in each rat by checking the connection of the tube and the placement of its tip. Phenylephrine (1 mg/kg per day) was infused subcutaneously for 7 days using a mini-osmotic pump (Alzet model 2001; Durect Corporation) in WKY. After full recovery from anaesthesia, the rats were free to move about in their cages.

Measurement of blood pressure, heart rate, and urinary norepinephrine excretion

SBP and heart rate were measured using the tail-cuff method before and during treatment. We measured SBP and heart rate blinded to the group membership of the rats. We collected urine for 24 h using a metabolic cage. We measured the urinary norepinephrine concentration by high-performance liquid chromatography before and at day 7, and calculated the urinary norepinephrine excretion for 24 h, as described previously [20].

Western blot analysis

We performed western blot analysis for phosphorylated ezrin, radixin, and moesin (p-ERM) or total ezrin, radixin, and moesin family members (ezrin, radixin, and moesin), which are target proteins of Rho-kinase [21] and membranous or total RhoA (1:1000; Santa Cruz Biotechnology, Santa Cruz, California, USA), in the brainstem (a coronal section; 3 mm caudal to 2 mm rostral to the obex), as described previously [16].

Statistical analysis

All values are expressed as means \pm SEM. Two-way analysis of variance was used to compare SBP and heart rate between any two groups. Comparisons between any two mean values were performed using Bonferroni's correction for multiple comparisons. A paired *t*-test was used to compare 24-h urinary norepinephrine excretion before and at day 7 during treatment. Differences were considered to be statistically significant when $P < 0.05$.

Results

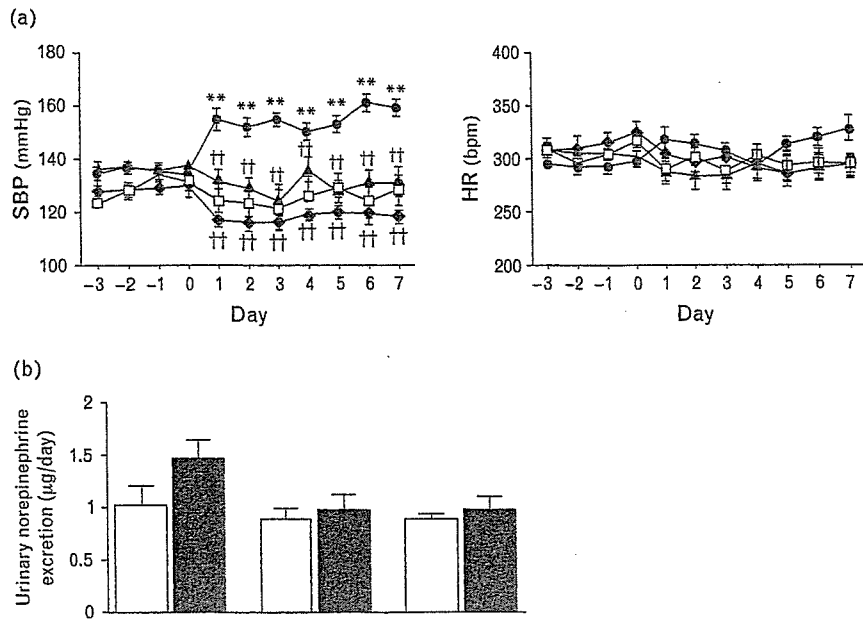
Intracisternal infusion of angiotensin II and co-infusion of Y-27632 or valsartan in Wistar-Kyoto rats

Figure 1a shows the time course of SBP and heart rate after the intracisternal infusion of angiotensin II or vehicle in WKY. SBP was significantly increased in angiotensin II-treated rats compared with vehicle-treated rats. The co-infusion of Y-27632 (5 mmol/l) or valsartan significantly attenuated the increase in SBP induced by angiotensin II. There were no significant differences in heart rate between any two groups. The intracisternal infusion of angiotensin II significantly increased urinary norepinephrine excretion. This response was abolished by the co-infusion of Y-27632 or valsartan (Fig. 1b).

Effect of angiotensin II on Rho/Rho-kinase activity in the brainstem

To confirm the effects of the intracisternal infusion of angiotensin II on Rho/Rho-kinase activity in the brainstem, we examined the expression levels of membranous RhoA, which represent RhoA activity, and the expression levels of p-ERM, which represent Rho-kinase activity. The expression levels of membranous RhoA in the

Fig. 1

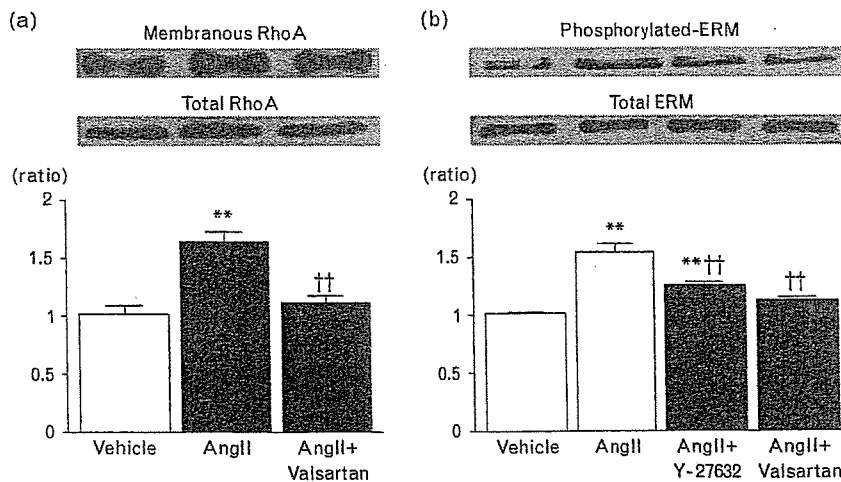


(a) Time course of systolic blood pressure (SBP) and heart rate (HR) before and during the intracisternal infusion of vehicle, angiotensin II (AngII), co-infusion of angiotensin II and Y-27632, or co-infusion of angiotensin II and valsartan ($n = 10$ for each). $**P < 0.01$ compared with the vehicle group; $††P < 0.01$ compared with the angiotensin II group. □ Vehicle; ● AngII; ▲ AngII and Y-27632; ◆ AngII and valsartan. (b) Urinary norepinephrine excretion before and at day 7 during the intracisternal infusion of angiotensin II, co-infusion of angiotensin II and Y-27632, or co-infusion of angiotensin II and valsartan ($n = 6$ per group). $*P < 0.05$ compared with pretreatment values. □ Before; ■ after.

brainstem were greater in angiotensin II-treated rats than in vehicle-treated rats (Fig. 2a). Furthermore, the expression levels of p-ERM in the brainstem were greater in angiotensin II-treated rats than in vehicle-treated rats (Fig. 2b).

Inhibitory effects of Y-27632 or valsartan on Rho/Rho-kinase activation by angiotensin II
 Valsartan significantly reduced the expression levels of membranous RhoA in angiotensin II-treated rats (Fig. 2a). In addition, Y-27632 or valsartan significantly reduced the

Fig. 2



(a) Expression levels of membranous RhoA and total RhoA evaluated by Western blot analysis during the intracisternal infusion of vehicle, angiotensin II (AngII), or co-infusion of angiotensin II and valsartan ($n = 5$ for each). The graph shows the ratio of membranous RhoA to total RhoA. (b) Expression levels of phosphorylated ezrin, radixin, and moesin (ERM) and total ERM evaluated by western blot analysis during the intracisternal infusion of vehicle, angiotensin II ($n = 10$ for each), co-infusion of angiotensin II and Y-27632, or co-infusion of angiotensin II and valsartan ($n = 8$ for each). The graph shows the ratio of p-ERM to total ERM. Data are expressed as the relative ratio to vehicle, which was assigned a value of 1. $**P < 0.01$ compared with the vehicle group; $††P < 0.01$ compared with the angiotensin II group.

expression levels of p-ERM in angiotensin II-treated rats (Fig. 2b).

Effect of subcutaneous infusion of phenylephrine on Rho/Rho-kinase activity

To examine whether activation of the Rho/Rho-kinase pathway in the brainstem was caused by the pressure response, phenylephrine was infused subcutaneously in WKY. The subcutaneous infusion of phenylephrine increased SBP to the same level as the intracisternal infusion of angiotensin II (Fig. 3a). The subcutaneous infusion of phenylephrine, however, did not alter the expression levels of membranous RhoA and p-ERM in the brainstem (Fig. 3b).

Intracisternal infusion of Y-27632 or valsartan in spontaneously hypertensive rats

The intracisternal infusion of Y-27632 (10 mmol/l) or valsartan significantly reduced SBP and heart rate in SHR (Fig. 4a). Urinary norepinephrine excretion was significantly higher in SHR than in WKY. Furthermore, the intracisternal infusion of Y-27632 or valsartan significantly reduced urinary norepinephrine excretion in SHR (Fig. 4b).

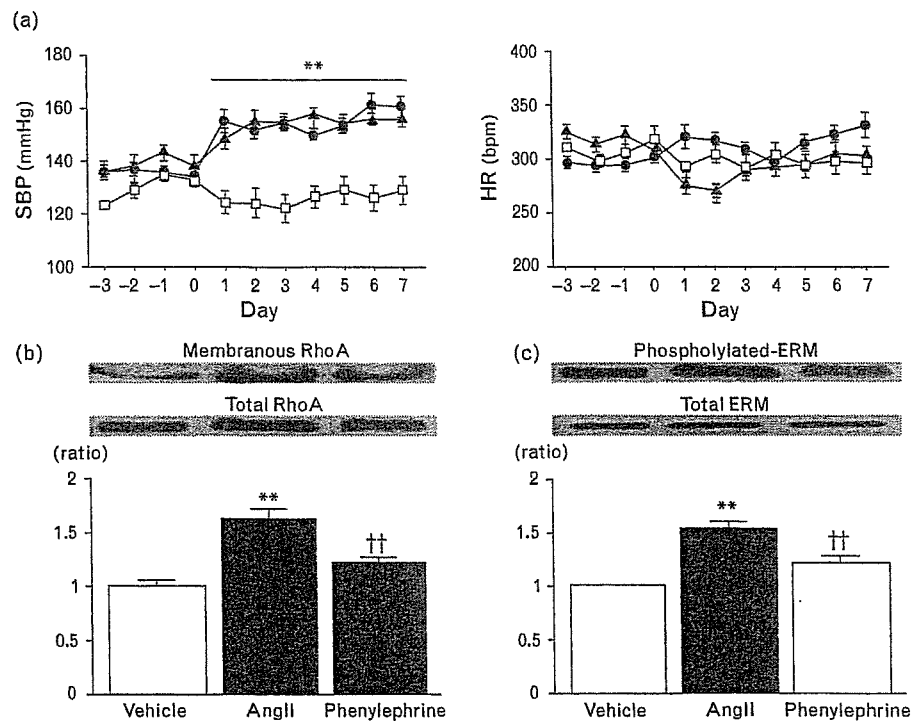
Rho/Rho-kinase activity in the brainstem of spontaneously hypertensive rats

The expression levels of membranous RhoA in the brainstem were greater in vehicle-treated SHR than in vehicle-treated WKY. The intracisternal infusion of valsartan reduced the expression levels of membranous RhoA in SHR (Fig. 5a). Furthermore, the expression levels of p-ERM in the brainstem were greater in vehicle-treated SHR than in vehicle-treated WKY. The intracisternal infusion of Y-27632 or valsartan reduced the expression levels of p-ERM in SHR (Fig. 5b).

Effect of central AT₁ receptor blockade in Wistar-Kyoto and spontaneously hypertensive rats

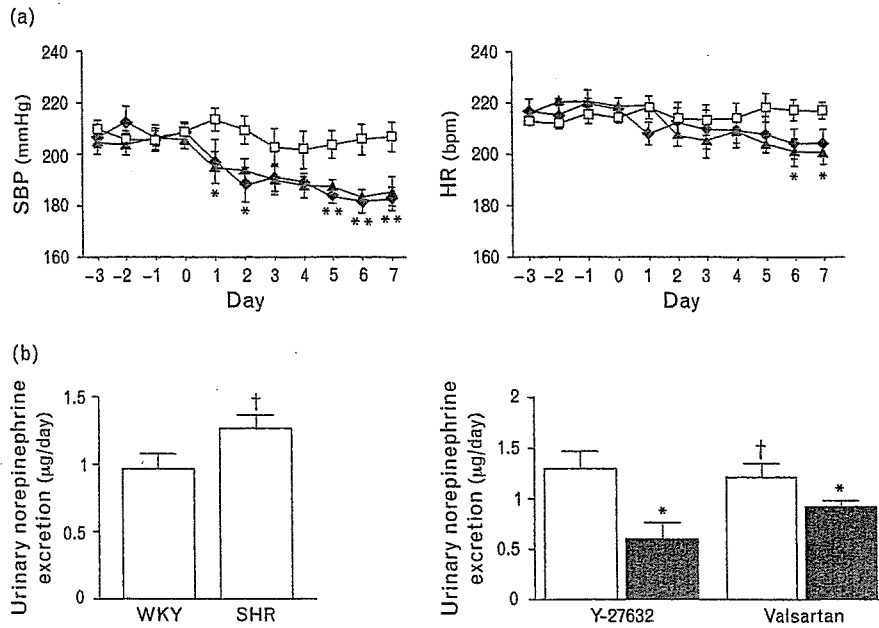
The intracisternal infusion of valsartan reduced SBP in WKY. The magnitude of the decrease, however, was significantly higher in SHR (210 ± 2 to 182 ± 5 mmHg) than in WKY (122 ± 6 to 110 ± 3 mmHg). The intracisternal infusion of valsartan significantly reduced heart rate in SHR (346 ± 10 to 316 ± 14 bpm), but not in WKY (317 ± 16 to 310 ± 10 bpm; Fig. 6).

Fig. 3



(a) Time course of systolic blood pressure (SBP) and heart rate (HR) before and during the intracisternal infusion of vehicle, angiotensin II, or subcutaneous infusion of phenylephrine ($n = 10$ for each). □ Vehicle; ● angiotensin II; ▲ phenylephrine. (b) Expression levels of membranous RhoA and total RhoA evaluated by Western blot analysis during the intracisternal infusion of vehicle, angiotensin II, or subcutaneous infusion of phenylephrine ($n = 5$ for each). The graph shows the ratio of membranous RhoA to total RhoA. Data are expressed as the relative ratio to vehicle, which was assigned a value of 1. (c) Expression levels of phosphorylated ezrin, radixin, and moesin (ERM) and total ERM evaluated by western blot analysis during the intracisternal infusion of vehicle, angiotensin II, or subcutaneous infusion of phenylephrine ($n = 10$ for each). The graph shows the ratio of p-ERM to total ERM. Data are expressed as the relative ratio to vehicle, which was assigned a value of 1. The results of vehicle and angiotensin II infusion are obtained from Fig. 1 and Fig. 2 for comparison. ** $P < 0.01$ compared with the vehicle group; †† $P < 0.01$ compared with the angiotensin II group.

Fig. 4



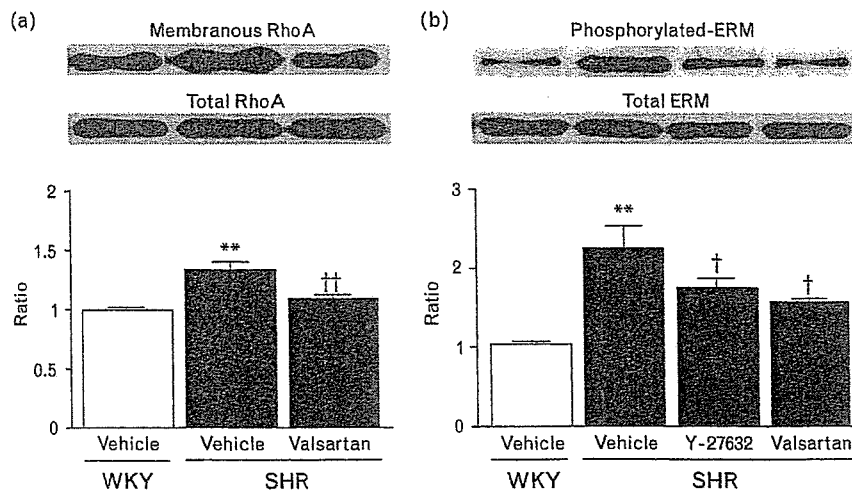
(a) Time course of systolic blood pressure (SBP) and heart rate (HR) before and during the intracisternal infusion of vehicle, Y-27632, or valsartan in SHR ($n=5$ for each). * $P < 0.05$; ** $P < 0.01$ compared with the vehicle group, respectively. □ Vehicle; ▲ Y-27632; ◆ valsartan. (b) Urinary norepinephrine excretion in Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) ($n=5$ for each; left), and before and at day 7 during the intracisternal infusion of Y-27632 or valsartan in SHR ($n=5$ per group; right). † $P < 0.05$ compared with the values of WKY; * $P < 0.05$ compared with pretreatment values. □ Before; ■ after.

Discussion

In the present study, pressor and sympathoexcitatory responses evoked by the intracisternal infusion of angio-

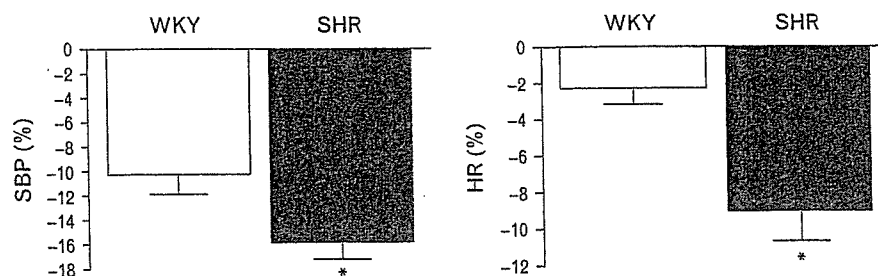
tensin II were abolished by the co-infusion of Y-27632 in WKY. The effects of angiotensin II on these responses were mediated by AT_1 receptors, because valsartan

Fig. 5



(a) Expression levels of membranous RhoA and total RhoA evaluated by western blot analysis in Wistar-Kyoto rats (WKY; vehicle) and spontaneously hypertensive rats (SHR; during the intracisternal infusion of vehicle or valsartan; $n=5$ for each). The graph shows the ratio of membranous RhoA to total RhoA. (b) Expression levels of p-ERM and total ERM evaluated by western blot analysis in WKY (vehicle) and SHR (during the intracisternal infusion of vehicle, Y-27632, or valsartan; $n=5$ for each). The graph shows the ratio of p-ERM to total ERM. Data are expressed as the relative ratio to WKY, which was assigned a value of 1. ** $P < 0.01$ compared with the WKY group; † $P < 0.05$; †† $P < 0.01$ compared with the vehicle-treated SHR group, respectively.

Fig. 6



Changes in systolic blood pressure (SBP) and heart rate (HR) at day 7 during the intracisternal infusion of valsartan in Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR; $n=5$ for each). Data are expressed as the relative ratio to day 0, which was assigned a value of 100%. * $P < 0.05$ compared with the WKY group.

blocked the responses. The expression level of membranous RhoA represents RhoA activity [22]. The expression was significantly increased by the intracisternal infusion of angiotensin II and blocked by the co-infusion of valsartan in WKY. The expression level of p-ERM represents Rho-kinase activity and was also significantly increased by the intracisternal infusion of angiotensin II in WKY. This response was blocked by the co-infusion of either valsartan or Y-27632. Furthermore, the intracisternal infusion of Y-27632 reduced SBP and urinary norepinephrine excretion in SHR. Y-27632 reduced the expression levels of p-ERM in the brainstem of SHR. Valsartan reduced the expression levels of both membranous RhoA and p-ERM. Taken together, these results suggest that exogenous angiotensin II applied to the brainstem activates the brain Rho/Rho-kinase pathway, as in the aorta and heart [12,13], and thereby increases SBP through the sympathetic nervous system via AT_1 receptors in WKY. Moreover, the inhibition of endogenous AT_1 receptors in the brainstem reduces increased RhoA and Rho-kinase activity in SHR, thereby at least partly inhibiting SBP in SHR.

The brain renin-angiotensin system is closely associated with blood pressure regulation via the sympathetic nervous system, although it is also related to other physiological responses, such as drinking behaviour via the vasopressin system [23]. Although vasopressin might be involved in the pressor response to the intracisternal infusion of angiotensin II [24], resetting of the arterial baroreflex towards higher pressures occurs with the long-term infusion of angiotensin II, and this resetting permits high sympathetic nerve activity [25]. Our results are consistent with these previously reported findings, indicating that centrally administered angiotensin II elicits pressor and sympathoexcitatory responses via activation of the Rho/Rho-kinase pathway in WKY. Furthermore, activation of the brain renin-angiotensin system is one of the hypertensive mechanisms in animal models of hypertension [26,27]. Angiotensin immunoreactivity [28] and AT_1 receptor expression levels [29] are greater in

the brainstem of SHR than WKY. Whereas there is some evidence that the brain renin-angiotensin system is not an important factor in causing hypertension [30,31], we demonstrated that the intracisternal infusion of valsartan reduced SBP and heart rate to a greater extent in SHR than in WKY. This result suggests that the endogenous brain renin-angiotensin system is activated in SHR.

The sites of action of the intracisternal infusion of angiotensin II are not clear from the results of the present study. AT_1 receptors appear to dominate in cardiovascular control regions [32], however, and many nuclei in these regions are activated by the central infusion of angiotensin II, as evaluated by *c-fos* expression, which is a marker of neuronal excitation [33]. The NTS and rostral ventrolateral medulla are possible candidates. The microinjection of angiotensin II into the NTS [34] or rostral ventrolateral medulla [35] elicits pressor and sympathoexcitatory responses. This is particularly apparent in animal models of hypertension [36,37]. Although some studies have indicated that the microinjection of angiotensin II into the NTS produces a depressor effect [38], the response to the microinjection of angiotensin II into the NTS exhibits a complicated dose-response relationship [39]. We previously reported that the microinjection of Y-27632 into the NTS decreases blood pressure in SHR and nitro-L-arginine methyl ester-treated rats [16,17], in which the endogenous renin-angiotensin system is activated. Although we did not specifically examine the NTS in the present study, activation of the Rho/Rho-kinase pathway in the NTS by angiotensin II might contribute, at least partly, to the increase in blood pressure.

Activation of the Rho/Rho-kinase pathway in the brainstem is not caused by the pressor response *per se*, because the subcutaneous infusion of phenylephrine does not activate this pathway. It is possible that the pressor response was centrally elicited by other substances, such as *N*-methyl-D-aspartate or an α_2 antagonist, which are not considered to be related to the Rho/Rho-kinase

pathway, but might also activate this pathway. Further studies are needed to clarify this issue.

Activation of AT₁ receptors also alters signaling pathways other than the Rho/Rho-kinase pathway, such as the mitogen-activated protein kinase and nicotinamide adenine dinucleotide phosphate, reduced form oxidase pathways [40,41]. Nicotinamide adenine dinucleotide phosphate, reduced form oxidase pathways stimulates another Rho family member, rac1 [42], which enhances superoxide anion generation [43]. Interaction between Rho and other Rho family members such as rac1 or cdc42 have been reported *in vitro* [44]. The type of physiological response involved in their interaction *in vivo*, however, remains unknown. There are some studies that do not support an antihypertensive effect of Y-27632. Y-27632 (3 mg/kg per day) did not affect SBP in Dahl salt-sensitive hypertensive rats [45]. In nitro-L-arginine methyl ester-treated rats, however, Y-27632 (30 mg/kg per day) reduces blood pressure, whereas lower doses of Y-27632 (10 mg/kg per day) do not affect blood pressure [46]. Therefore, Y-27632 appears to have a dose-dependent antihypertensive effect, although we cannot exclude the possibility that activation of the Rho/Rho-kinase pathway differs among the hypertensive models.

The precise mechanism(s) by which angiotensin II activates RhoA and Rho-kinase is unknown, particularly in the brain. Rho-kinase plays an important role in angiotensin II-induced messenger RNA expression of monocyte chemoattractant protein 1, transforming growth factor 1, and plasminogen activator inhibitor 1 [46–48]. In cultured human coronary vascular smooth muscle cells, the expression of Rho-kinase is enhanced by inflammatory stimuli, such as angiotensin II and IL-1 [49]. Complex signal transduction pathways might thus be involved in producing the final physiological responses.

In conclusion, the results of the present study suggest that centrally administered angiotensin II activates the Rho/Rho-kinase pathway in the brainstem nuclei, thereby increasing central sympathetic outflow and blood pressure. In addition, it is possible that inhibition of endogenous Rho-kinase in the brainstem decreases central sympathetic outflow, thereby decreasing blood pressure in SHR.

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Long-Acting Calcium Channel Blocker, Azelnidipine, Increases Endothelial Nitric Oxide Synthase in the Brain and Inhibits Sympathetic Nerve Activity

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Nitric oxide (NO) in the central nervous system inhibits sympathetic nerve activity, thereby decreasing blood pressure. It is unknown, however, whether orally administered antihypertensive treatment alters NO synthase (NOS) expression, particularly in the brain, and how changes in NOS expression affects sympathetic nerve activity. Azelnidipine, a recently developed long-acting dihydropyridine calcium channel blocker, does not cause baroreflex-induced tachycardia. The aim of the present study was to determine whether antihypertensive treatment with azelnidipine alters endothelial NOS (eNOS), neuronal NOS (nNOS), or inducible NOS (iNOS) expression in the brain, and how changes in NOS affect sympathetic nerve activity. Azelnidipine (20 mg/kg/day) or hydralazine (20 mg/kg/day) was orally administered for 30 days in stroke-prone spontaneously hypertensive rats (SHRSP). Blood pressure and heart rate were measured by the tail cuff method. Urinary norepinephrine excretion was measured as a marker of sympathetic nerve activity. Western blot analysis was performed to examine eNOS, nNOS, or iNOS expression levels in the brain (cortex, cerebellum, hypothalamus, and the brain stem), heart, and aorta. The extent of blood pressure reduction was similar between the two groups. Heart rate increased in the hydralazine-treated group but did not change in the azelnidipine-treated group. Urinary norepinephrine excretion was significantly increased only in the hydralazine-treated group. Treatment with azelnidipine significantly increased eNOS expression levels in the brain, heart, and aorta, but did not alter nNOS or iNOS expression levels. Treatment with hydralazine did not change any of the NOS expression levels. These results suggest that antihypertensive treatment with azelnidipine attenuates reflex-induced sympathetic activation and enhances eNOS expression levels in the brain as well as in the heart and aorta.

Keywords blood pressure, heart rate, brain, nitric oxide, sympathetic nervous system

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Introduction

Dihydropyridine calcium channel blockers are widely used for the treatment of hypertension or angina pectoris and are generally accepted as useful for blood pressure reduction (1–3). There is some concern, however, regarding reflex tachycardia and sympathoexcitation because of the rapid reduction of blood pressure, which might be related to cardiovascular events when dihydropyridines are used in patients with cardiovascular diseases (4).

In animal models of hypertension and patients with hypertension, nitric oxide (NO) production is abnormal, leading to hypertensive vascular lesion formation (5). NO in the brain, such as brain stem and hypothalamus, inhibits sympathetic nerve activity, thereby reducing blood pressure (6,7). It is not known, however, whether antihypertensive treatment alters NO synthase (NOS) expression or how changes in NOS expression affect sympathetic nerve activity. Azelnidipine is a recently developed long-acting calcium channel blocker (8–10) that does not elicit reflex tachycardia (11,12). The aim of the present study, therefore, was to determine whether antihypertensive treatment with azelnidipine alters each type of NOS (i.e., endothelial, neuronal, and inducible) expression in the brain, heart, and aorta, and to examine the relationship between changes in NOS expression levels and changes in sympathetic nerve activity.

Materials and Methods

This study was approved by the Committee of Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, and conducted according to the Guidelines for Animal Experiments of Kyushu University.

General Preparation

Male SHRSP/Izm (14 weeks old, SLC Japan, Hamamatsu, Japan) were placed on standard feed supplemented with azelnidipine and had free access to drinking water. The animals received azelnidipine at a dose of 20 mg/kg of body weight per day, which was calculated according to the daily food intake. As a control group, other SHRSP/Izm were fed standard feed and received hydralazine (20 mg/kg/day) in the drinking water. Treatment was started when the rats were 14 weeks of age and continued for 30 days.

Measurement of Blood Pressure, Heart Rate, and Urinary Norepinephrine Excretion

Systolic blood pressure and heart rate evaluated with the tail-cuff method was measured before and after treatment with azelnidipine in SHRSP. Urine was collected for 24 hours while housing the animals in a metabolic cage. Urinary norepinephrine concentration was measured before and after treatment with azelnidipine or hydralazine by high-performance liquid chromatography, and calculated urinary norepinephrine excretion was used as a marker of sympathetic nerve activity.

Western Blot Analysis for eNOS, nNOS, and iNOS

At day 30 after beginning treatment with azelnidipine or hydralazine, the animals were deeply anesthetized with sodium pentobarbital (100 mg/kg IP) and perfused transcardially with phosphate-buffered saline (PBS). The descending aorta and heart were removed, and the brain was divided into four sections: cortex, cerebellum, hypothalamus, and brain stem. All

tissues were homogenized and then sonicated in lysing buffer (pH 7.4) containing 40 mmol/L HEPES, 1% Triton X-100, 10% glycerol, and 1mmol/L phenylmethylsulfonyl fluoride. The tissue lysates were centrifuged at 6000 rpm for five minutes at 4°C with a microcentrifuge. The supernatant was collected, and the protein concentration was determined with a BCA protein assay kit (Pierce Chemical, Rockford, Illinois, USA). An aliquot of 30 µg of protein from each sample was separated on 12% sodium dodecyl sulfate-polyacrylamide gels. Proteins were subsequently transferred onto polyvinylidene difluoride membranes (Immobilon-P membrane, Millipore, Billerica, Massachusetts, USA). Membranes were incubated for two hours with mouse IgG monoclonal antibody to endothelial NOS (eNOS; 1:2500, Santa Cruz Biotechnology, Santa Cruz, California, USA), neuronal NOS (nNOS; 1:2500, Santa Cruz Biotechnology), or inducible NOS (iNOS; 1:500, Santa Cruz Biotechnology). As an internal control, rabbit IgG polyclonal antibody to β -tubulin (1:5000, Santa Cruz Biotechnology) for the brain tissues or rabbit IgG polyclonal antibody to β -actin (1:10000, Santa Cruz Biotechnology) for the aorta and heart was used. Membranes were then washed and incubated with a horseradish peroxidase-conjugated horse anti-mouse IgG antibody (1:10000) for ten minutes. Immunoreactivity was detected by enhanced chemiluminescence autoradiography (ECL plus Western blotting detection kit; Amersham, Aylesbury, UK). The densitometric average was normalized to the values obtained from the analysis of β -tubulin or β -actin protein.

Statistical Analysis

All values are expressed as mean \pm SEM. A two-way analysis of variance (ANOVA) was used to compare the systolic blood pressure and heart rate between the azelnidipine- and hydralazine-treated groups. Comparisons between any two mean values were performed using Bonferroni's correction for multiple comparisons. ANOVA was used to compare the eNOS, nNOS, and iNOS expression levels in conjunction with a post hoc test using Scheffe's correction. A paired *t*-test was performed to compare the urinary norepinephrine excretion before and after treatment. Differences were considered to be statistically significant when *p* was less than 0.05.

Results

Effect of Azelnidipine on Blood Pressure, Heart Rate, and Urinary Norepinephrine Excretion

Systolic blood pressure was decreased to similar levels in the azelnidipine-treated and hydralazine-treated groups (see Figure 1a; -45 ± 15 vs. -43 ± 8 mmHg, *n*=10 for each). By contrast, heart rate was not significantly altered after azelnidipine treatment but increased after hydralazine treatment (see Figure 1b). Hydralazine treatment significantly increased urinary norepinephrine excretion in SHRSP, but azelnidipine treatment did not (hydralazine: 1.17 ± 0.02 vs. 1.45 ± 0.08 µg/24hours; azelnidipine: 1.15 ± 0.19 vs. 1.18 ± 0.09 µg/24hours, *n*=10 each, *p* < 0.05; see Figure 2).

Effect of Azelnidipine on the eNOS, nNOS, and iNOS Expression in the Brain, Heart, and Aorta

Azelnidipine treatment significantly increased eNOS expression in the cortex, cerebellum, hypothalamus, and brain stem, though hydralazine treatment did not (see Figure 3a). In the heart and aorta, azelnidipine treatment also increased eNOS expression. Treatment with azelnidipine or hydralazine did not alter nNOS expression in the cortex, cerebellum,

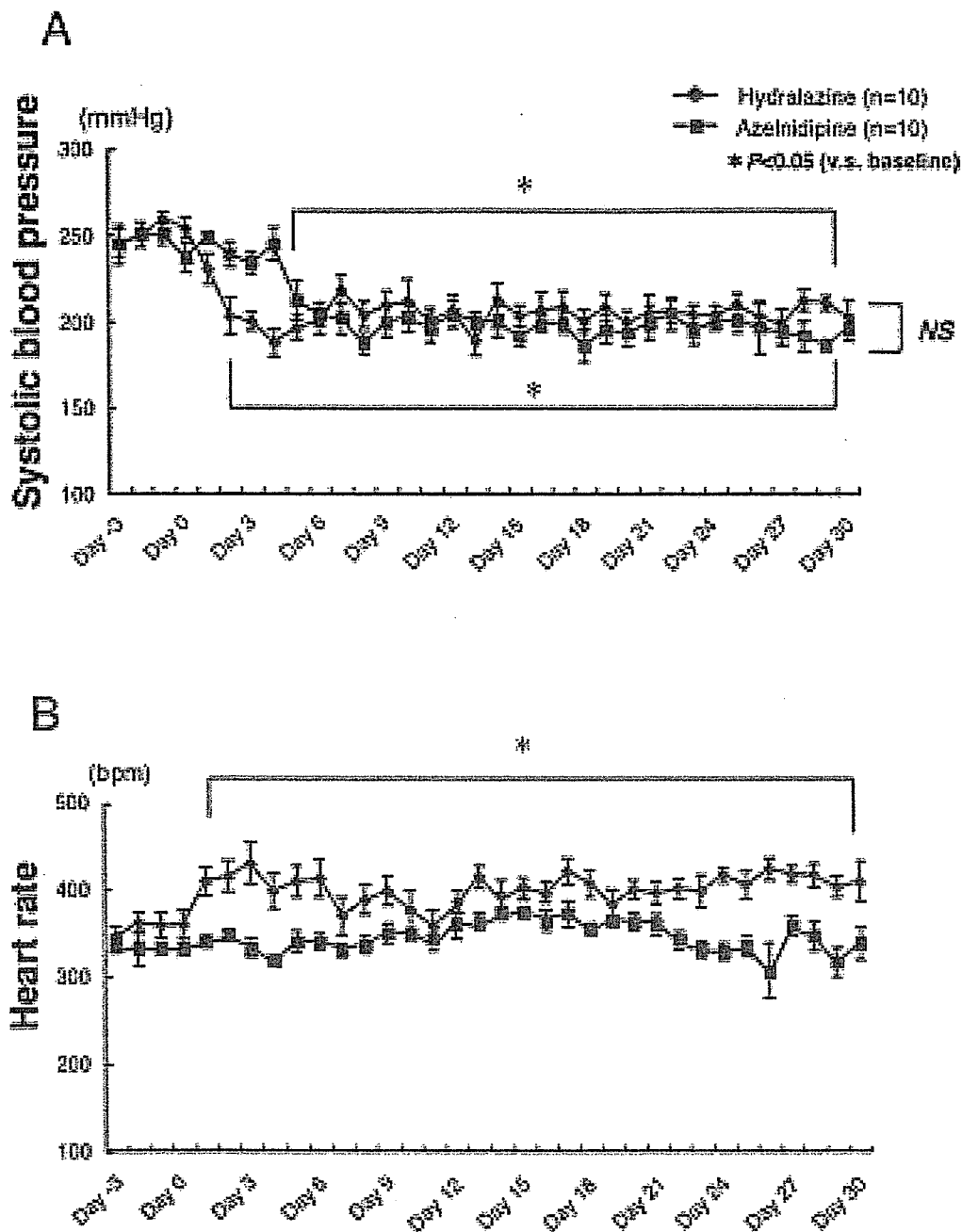


Figure 1. Time courses in systolic blood pressure (a) or heart rate (b) induced by treatment with hydralazine or azelnidipine.

hypothalamus, brain stem, aorta, or heart (see Figure 3b). iNOS expression was low compared with eNOS and nNOS. After treatment with either drug, iNOS expression was not changed in the brain, heart, or aorta (see Figure 3c).

Discussion

The major finding of the present study was that antihypertensive treatment with azelnidipine did not elicit reflex-induced tachycardia, but antihypertensive treatment with

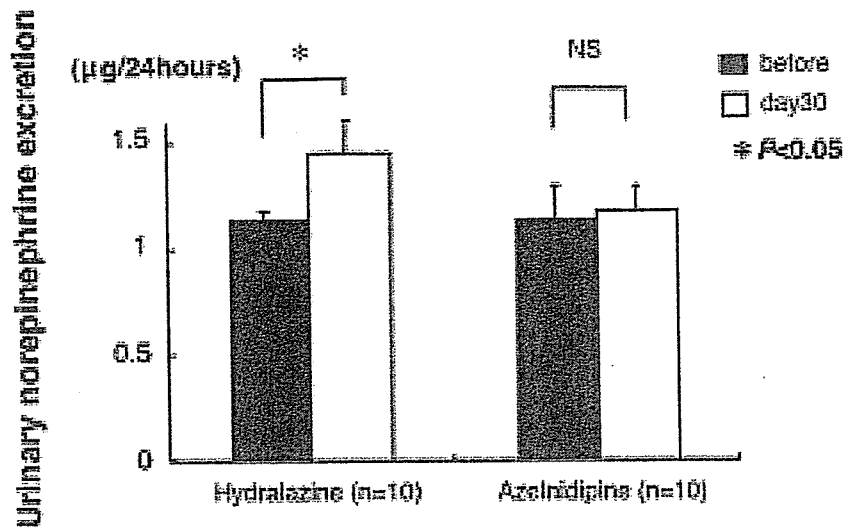


Figure 2. Twenty-four-hour urinary norepinephrine excretion before and after treatment with hydralazine or azelnidipine.

hydralazine induced tachycardia and a similar reduction in blood pressure. The increase in urinary norepinephrine excretion supports the idea that hydralazine treatment produces the reflex-induced activation of the sympathetic nervous system. In contrast, azelnidipine did not increase urinary norepinephrine excretion. Another important finding of the present study was that azelnidipine treatment significantly increased the expression levels of eNOS but not nNOS or iNOS in the brain, heart, and aorta. Hydralazine did not have this effect. Because NO in the brain inhibits sympathetic nerve activity and NO produced by eNOS can diffuse and affect neurons, NO in the brain, including the hypothalamus and the brain stem, is involved at least in part in the mechanisms by which azelnidipine does not elicit the reflex-induced activation of the sympathetic nervous system.

An increase in eNOS expression does not necessarily indicate a similar increase in eNOS activity. This study demonstrated, however, that the overexpression of eNOS in the brain stem nuclei, such as the nucleus tractus solitarius and rostral ventrolateral medulla, has hypotensive and sympathoinhibitory effects (6,7). In those studies, an increase in NO production was confirmed. In support of these findings, Kobayashi et al. reported that benidipine stimulates eNOS and improves coronary circulation in 2K-1C hypertensive rats (13). In their study, benidipine increased both eNOS mRNA expression and eNOS activity (13). Furthermore, another long-acting calcium channel blocker, amlodipine, increased both eNOS expression and activity (14,15). Amlodipine enhances eNOS generation by inducing changes in the phosphorylation of Ser¹¹⁷⁷ and the dephosphorylation of Thr⁴⁹⁵ in cultured cells (16). Further studies are necessary to clarify whether a similar mechanism(s) is involved in the azelnidipine-induced upregulation of eNOS. It is also possible that NO produced by the upregulation of eNOS in the peripheral vasculature might affect the peripheral nervous system beyond the endothelium (17).

The present study does not elucidate mechanism(s) by which azelnidipine upregulates eNOS expression. Azelnidipine is a highly lipophilic dihydropyridine (10,11), which penetrates vascular walls and might alter signal transduction stimulated by the tissue renin-angiotensin system. The dihydropyridine calcium channel blockers have sympathoinhibitory

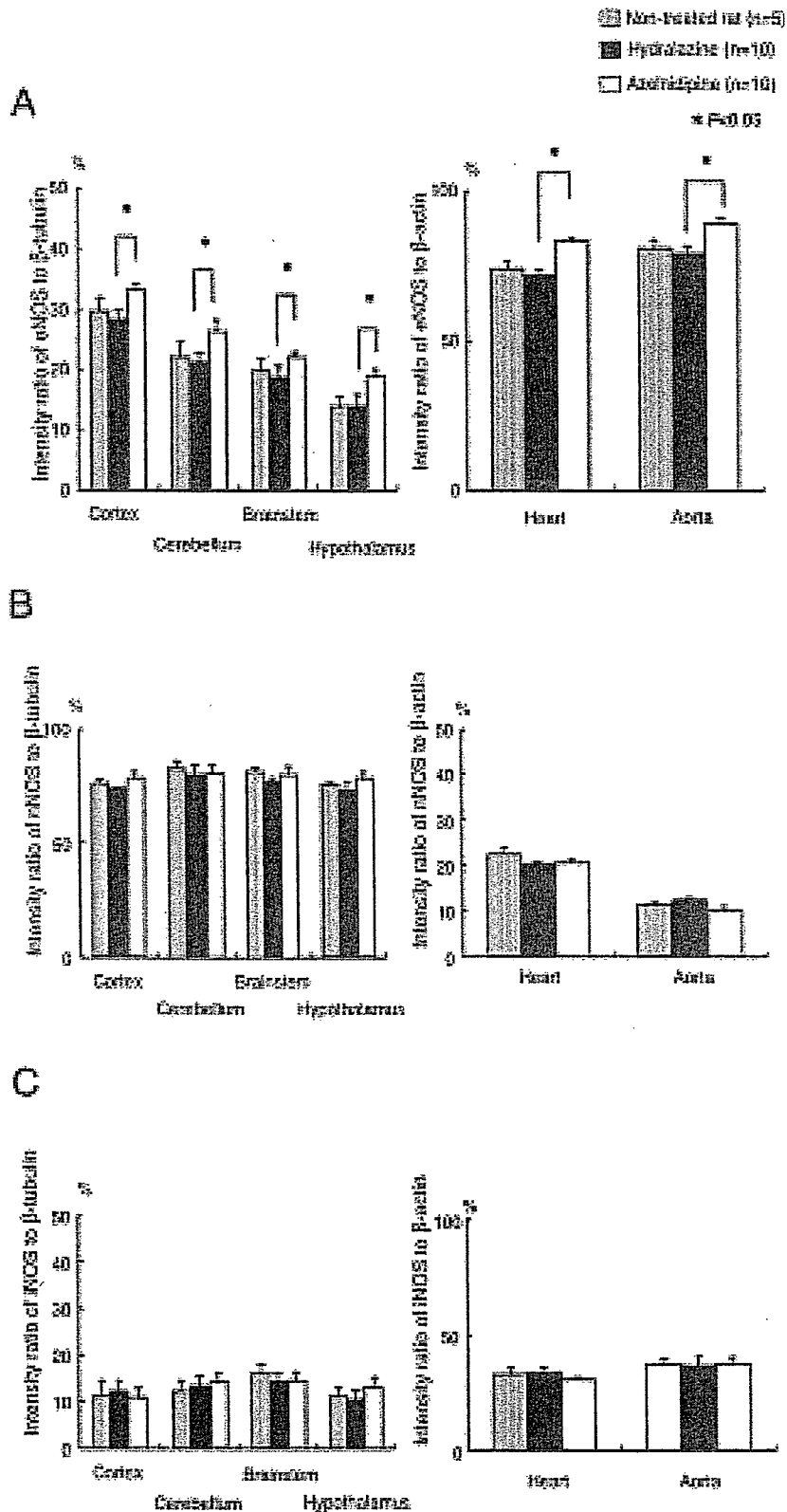


Figure 3. NOS expression levels evaluated by Western blot analysis in the brain (cortex, cerebellum, brain stem, and hypothalamus), heart, and aorta in non-treated rats and rats treated with hydralazine or azenidipine for 30 days; a: eNOS expression levels, b: nNOS expression levels, c: iNOS expression levels.

actions (18–21). These actions are suggested to be mediated by the central nervous system. For example, peripherally administered nifedipine penetrates into the central nervous system to inhibit the sympathetic nervous system (20). Intracerebroventricularly administered nifedipine or amlodipine decreases blood pressure, heart rate, and renal sympathetic nerve activity dose-dependently (19,21). There are L-type voltage-gated calcium channels in the central nervous system, and dihydropyridines act on those receptors (22,23). Lipophilic dihydropyridines such as nifedipine, nimodipine, and amlodipine penetrate the blood brain barrier after peripheral administration. Thus, azelnidipine also crosses the blood brain barrier and acts on L-type voltage gated calcium channels in the central nervous system, thereby inhibiting sympathetic nerve activity. The direct microinjection of calcium channel blockers into the brain stem reduces blood pressure (24). The present results support this concept. The upregulation of eNOS elicited by azelnidipine might also be involved in the mechanisms of the sympathoinhibition. Orally administered atorvastatin also induces depressor and sympathoinhibitory effects and upregulates eNOS in SHRSP (25).

A lack of sympathoinhibition caused by antihypertensive drugs, such as hydralazine or fast-acting dihydropyridines, elicits rapid decreases in blood pressure by vasodilation and thereby evokes arterial baroreflex-induced sympathoexcitation. Lipophilicity as well as pharmacokinetics might determine the extent of the central action of the dihydropyridines. Thus, the short half-life of some dihydropyridines is not sufficient to reach the brain tissue to induce sympathoinhibition. In fact, azelnidipine induced a gradual decrease in blood pressure compared with hydralazine in the present study. In the clinical setting, azelnidipine reduces blood pressure without the augmentation of the sympathetic nervous system in patients with essential hypertension (11). There might be some differences in the effects of eNOS expression or sympathetic nerve activity among calcium channel blockers because the effects of azelnidipine on sympathoinhibition or eNOS expression were not compared with those of other calcium channel blockers. Chronic treatment with other long-acting calcium channel blockers, however, does not change sympathetic nerve activity in hypertensive patients (26), suggesting that treatment with azelnidipine will have favorable effects, similar to amlodipine.

In conclusion, antihypertensive treatment with azelnidipine, a new type of calcium channel blocker, decreases blood pressure without reflex-induced sympathoexcitation and upregulates eNOS expression in the brain, heart, and aorta. An increase in NO production in the central nervous system might be involved, at least in part, in the sympathoinhibitory action of azelnidipine.

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Stent-Based Local Delivery of Nuclear Factor- κ B Decoy Attenuates In-Stent Restenosis in Hypercholesterolemic Rabbits

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Background—Nuclear factor- κ B (NF- κ B) plays a critical role in the vascular response to injury. However, the role of NF- κ B in the mechanism of in-stent restenosis remains unclear. We therefore tested the hypothesis that blockade of NF- κ B by stent-based delivery of a cis-element “decoy” of NF- κ B reduces in-stent neointimal formation.

Methods and Results—Stents were coated with a polymer containing or not containing NF- κ B decoy, which represented a fast-release formulation (<7 days). Bare, polymer-coated, and NF- κ B decoy-eluting stents were implanted in iliac arteries of hypercholesterolemic rabbits. Increased NF- κ B activity was noted at early stages after stenting, which was suppressed by stent-based delivery of NF- κ B decoy. NF- κ B decoy-eluting stents also reduced monocyte infiltration and monocyte chemoattractant protein-1 expression and suppressed CD14 activation on circulating leukocytes. Importantly, NF- κ B decoy-eluting stents attenuated neointimal formation on day 28. There was no evidence of an incomplete healing process (persistent inflammation, hemorrhage, fibrin deposition, impaired endothelial regeneration) at the site of NF- κ B decoy-eluting stents. Transfection of NF- κ B decoy suppressed proliferation of human coronary artery smooth muscle cells in vitro. No systemic adverse effects of NF- κ B decoy were detected.

Conclusions—Stent-based local delivery of NF- κ B decoy reduced in-stent neointimal formation with no evidence of incomplete healing. These data suggest that this strategy may be a practical and promising means for prevention of in-stent restenosis in humans. (*Circulation*. 2006;114:2773-2779.)

Key Words: inflammation ■ monocytes ■ restenosis ■ myocytes, smooth muscle

Each year, >1.5 million patients worldwide undergo percutaneous coronary intervention for atherothrombotic lesions. Local drug delivery by drug-eluting stents is now becoming a useful strategy for prevention of restenosis because of promising results in animal studies and clinical trials.^{1,2} Currently marketed first-generation drug-eluting stents use antiproliferative drugs including rapamycin, its analogues, or paclitaxel. The current antiproliferative strategies are no longer a panacea, however, because this strategy involves potential problems such as impaired endothelial regeneration and an incomplete healing process (excessive inflammation and fibrin deposition) associated with increased risk of stent thrombosis.³⁻⁵ Lack of long-term effects of sirolimus-eluting stents due to delayed inflammation and proliferation has been reported in a porcine coronary model.⁶

Clinical Perspective p 2779

Recent experimental and clinical studies suggest that inhibition of stent-associated inflammation (monocyte recruitment and activation) can be a promising next-generation approach.⁷⁻⁹ Nuclear factor- κ B (NF- κ B) is a redox-sensitive transcription factor that regulates inflammation and thus plays a critical role in the vascular response to injury.¹⁰ Activated NF- κ B is detected in human atherosclerotic and restenotic lesions of smooth muscle cells, monocytes, and endothelial cells.¹¹ In contrast, activated NF- κ B is rarely detected in normal uninjured arteries. After vascular injury, rapid activation of NF- κ B in smooth muscle cells correlates with proliferation of smooth muscle cells and induced expression of NF- κ B-dependent genes.¹² Recently, blockade of NF- κ B by transfection of adenoviral inhibitor- κ B or NF- κ B “decoy” oligodeoxynucleotides attenuated restenotic changes

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(neointimal formation) after balloon injury in animal models associated with reduced NF- κ B-dependent genes like monocyte chemoattractant protein-1 (MCP-1).^{13,14} As a clinically feasible technique, however, these gene transfer approaches are usually hampered by prolonged arterial occlusions. It has not yet been directly determined whether blockade of NF- κ B inhibits neointima formation after stenting. This is important because the mechanisms underlying neointima formation differ between balloon injury and stent-induced injury, and stenting is the most frequently performed vascular interventional technique.

We therefore created the NF- κ B decoy-eluting stent using polymer technology that facilitates local delivery of the NF- κ B decoy oligodeoxynucleotide during stent expansion by balloon dilatation. This decoy-eluting stent strategy is a clinically feasible approach. Although local gene delivery from a polymeric plasmid DNA-coated stent has been reported, local vascular transfer of decoy oligodeoxynucleotide by polymeric-coated stent has not been reported thus far. We herein report inhibition of in-stent neointimal formation by stent-based local transfer of NF- κ B decoy in vivo.

The aims of this study were (1) to create a NF- κ B decoy-eluting metallic stent by the use of water-soluble polymer; (2) to evaluate the in vivo blockade of NF- κ B activation by NF- κ B decoy-eluting stent implantations; and (3) to determine whether the NF- κ B decoy-eluting stent attenuates stent-associated inflammation and neointimal formation in vivo.

Methods

Stent Preparation

The NF- κ B decoy sequences are 5'-CCTTGAAGGGA-TTCCCTCC-3' and 3'-GGAAGTCCCTAAAGGGAGG-5'. GG-GATTCC is the consensus sequence for the NF- κ B binding site. The decoy is directed against the NF- κ B binding site in the promoter region that corresponds to NF- κ B-responsive genes.^{15,16} The decoy works to inhibit binding of this transcription factor to the promoter region.^{15,16} The NF- κ B decoy oligodeoxynucleotides have been shown to bind to free NF- κ B, preventing NF- κ B transactivation of the cytokine genes. Because NF- κ B is activated immediately after stenting, we designed the NF- κ B decoy-eluting stent as an early-release formulation. The 15-mm-long stainless steel balloon-expandable stent (Kawasumi Co, Osaka, Japan) was dip-coated with multiple thin layers of polyurethane containing or not containing NF- κ B decoy (500 to 600 μ g per stent) under sterile conditions. Another layer of decoy-free polyurethane was applied on top of the decoy-polyurethane matrix. We selected polyurethane as a polymer matrix material for stent coating because (1) it is water soluble and therefore stably absorbs NF- κ B decoy oligodeoxynucleotides and (2) metallic stents coated with polyurethane containing DNA are reported to be useful for transgene delivery to the iliac arterial wall of rabbits.¹⁷ In addition, of a number of polymer matrix materials evaluated for stent coating, polyurethane has been shown to prevent the thrombosis and inflammation that can occur with uncoated stents and some polymers used for stent coating.¹⁸

The coated stent was then mounted over a 3-mm balloon catheter. Uncoated bare stent mounted over the same balloon catheter was used as a control. Before implantation, all stents were sterilized with the use of ethylene oxide.

In Vitro Kinetics

In vitro kinetics studies were performed by placing a NF- κ B decoy-coated stent in Tris-EDTA buffer at 37°C. The stent was periodically removed from the buffer, and the decoy eluted into the buffer was measured by a high-performance liquid chromatography

system. The incremental quantities of the decoy released from the stent were plotted against time (n=8).

Stent Implantation in Animal Models

The experiments were reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine, and were performed according to the US National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Male Japanese white rabbits (KBT Oriental, Tokyo, Japan) weighing 3.0 to 3.5 kg were fed a high-cholesterol diet containing 1% cholesterol and 3% peanut oil for 2 weeks before stent implantation. Animals were anesthetized and were divided randomly into 3 groups, which underwent deployment of either an uncoated bare metal stent (n=22); decoy-free polyurethane-coated stent (n=22); or NF- κ B decoy-coated stent (n=22) in the right femoral artery as described previously.⁹ All animals received aspirin at 20 mg/d until euthanasia from 3 days before the stent implantation procedure. After venous blood samples were taken, animals were killed with a lethal dose of anesthesia at days 3 (n=6 each), 10 (n=8 each), and 28 (n=8 each). Stented arterial sites and contralateral unstented sites were excised for biochemical, immunohistochemical, and morphometric analyses.

Histopathological and Immunohistochemical Analysis

The stented artery segments were processed as described previously.⁹ The segment was divided into 2 parts at the center of the stent. The proximal part was embedded in methyl methacrylate mixed with *n*-butyl methacrylate to allow for sectioning through metal stent struts. Serial sections were stained with elastica van Gieson and hematoxylin-eosin. Neointimal area, the area within the internal elastic lamina and external elastic lamina, and the lumen area were measured by computerized morphometry. A single observer who was blinded to the experiment protocol performed morphometry. All images were captured by an Olympus microscope equipped with a digital camera (HC-2500) and were analyzed with the use of Adobe Photoshop 6.0 and Scion Image 1.62 Software.

The injury and inflammatory scores were determined at each strut site, and mean values were calculated for each stented segment as previously described^{19,20} (see Table I in the online-only Data Supplement for details).

The distal part was used for immunohistochemical analysis. After stent struts were removed gently with microforceps, the tissue was dehydrated, embedded in paraffin, and cut into 5- μ m-thick slices. They were subjected to immunostaining with antibodies against rabbit monocytes/macrophages (RAM-11; Dako, Glostrup, Denmark), endothelial cells (CD31; Dako), an epitope (α -p65) on the p65 subunit of NF- κ B (α -p65; Boehringer Mannheim, Roche Diagnostics, Basel, Switzerland), MCP-1 (a gift from Dr Matsukawa, Kumamoto University), or nonimmune mouse IgG (Dako). The α -p65 monoclonal antibody recognizes an epitope on the p65 subunit that is masked by bound I- κ B.¹¹ Therefore, this antibody exclusively detects activated NF- κ B. For quantification of immunohistochemical images, care was taken to select stented sites with minor injury in the neointima induced by detachment of stent strut. Because this process of selecting sections with the least injury may introduce bias, at least 5 representative images were selected, and the percentage of immunopositive cells per total cells in each image was calculated. The average of the 5 images was reported for each animal.

Electrophoretic Mobility Shift Assay

The electrophoretic mobility gel shift assay was performed on nuclear extracts prepared immediately from rabbit femoral arteries after stent implantation with the method described previously.²¹ For competition studies, a 50-fold molar excess of unlabeled probe for NF- κ B was added. For supershift assays, 1 μ g anti-p50 or anti-p65 (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) antibodies was added and incubated for 20 minutes. Nuclear extracts of HeLa cells were used as positive control.