on day 1, and subsequently subsided on day 3 (Fig. 4a). Although inflammatory cell infiltration was not significantly different between MI/LacZ and MI/TNFR1 mice on day 1, significantly more infiltrating cells remained in MI/TNFR1 myocardium on day 3, which might reflect reduced apoptosis by TNFR1 treatment (Fig. 4b). Immunohistochemical staining revealed that most of these retained infiltrating cells on day 3 were macrophages (Fig. 4c). As summarized in Fig. 4d, TNFR1 treatment significantly increased the number of macrophages in infarct myocardium on day 3.

3.1.5. Degradation of extracellular matrix with further activation of MMP-9

Collagen was visualized in LV cross-section using Picrosirius red staining (Fig. 5a). Collagen volume fraction was not affected by MI, but was significantly reduced in MI/TNFR1 mice on day 3 (Fig. 5b).

To further elucidate the mechanisms of reduced myocardial fibrosis in MI/TNFR1 mice, the activities of MMP-2 and -9 were evaluated in infarct myocardium on day 3 using gelatin zymography (Fig. 5c). The activities of MMP-2 and -9 increased significantly after MI (Fig. 5d). Treatment with soluble TNF receptors did not affect MMP-2 but significantly further activated MMP-9. Immunohistochemical staining identified macrophages as one of the major sources of MMP-9 in infarct myocardium (Fig. 5e).

Taken together, these results suggest that pre-MI treatment with TNFR1 prevents apoptosis of infiltrating cells in infarct myocardium, resulting in the retention of macrophages and further activation of MMP-9, which may further degrade and finally rupture the infarct myocardium.

3.2. Post-MI treatment protocol

3.2.1. Exacerbation of cardiac dysfunction and remodeling after MI

Post-MI treatment protocol was conducted to evaluate effects of TNFR1 on ventricular remodeling, because pre-MI treatment significantly increased ventricular rupture and precluded late phase analysis. In this study, 102 mice underwent coronary ligation, and 28 died within 7 days. The surviving mice ($n=74$) were randomly assigned to AdLacZ or AdTNFR1 injection on day 7. Four of 36 MI/LacZ and 9 of 38 MI/TNFR1 mice died of congestive heart failure by the end of 4 weeks after ligation (Fig. 6, $p=0.16$). None of the sham-operated mice died. Plasma levels of human TNFR1 3 weeks after inoculation with AdTNFR1 (or 4 weeks after MI or sham operation) were 71.1±28.5 (SD) μg/ml, which were similar to our previous report [18]. Percent infarct area was not different between MI/LacZ and MI/TNFR1 mice on day 28 (Table 3).

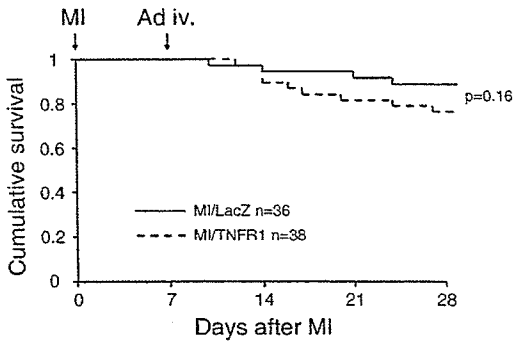

Fig. 6. Kaplan–Meier survival curves of coronary ligated mice with or without TNFR1 post-MI treatment.

Table 3
Characteristics of animal models (day 28)

	Sham/ LacZ	Sham/ TNFRI	MI/ LacZ	MI/ TNFRI
Echocardiographic data (under anesthesia)				
N	19	18	32	29
Heart rate (bpm)	465±11	461±13	471±16	467±14
LV EDD (mm)	3.9±0.3	4.0±0.3	5.6±0.3*	6.0±0.4*†
LV ESD (mm)	2.5±0.2	2.6±0.2	4.8±0.2*	5.3±0.5*†
Fractional shortening (%)	35.6±1.4	35.7±1.9	14.1±0.9*	11.7±1.9*†
Infarct wall thickness (mm)	–	–	0.29±0.03	0.29±0.03
Non-infarct wall thickness (mm)	0.78±0.04	0.76±0.05	0.95±0.06*	1.06±0.07*†
Hemodynamic data (Millar catheter, under anesthesia)				
N	6	6	12	11
Heart rate (bpm)	434±21	435±22	429±25	434±24
Mean aortic pressure (mm Hg)	81.8±4.3	80.5±4.6	78.3±4.3	79.2±4.5
LV EDP (mm Hg)	2.1±0.9	2.1±1.0	10.1±2.5*	14.5±3.3*†
LV$dP/dt_{max}$ (mm Hg/s)	7243±424	7084±422	5363±794*	4482±568*†
LV$dP/dt_{min}$ (mm Hg/s)	5047±274	4944±264	3864±602*	3219±424*†
Organ weight data				
N	19	18	32	29
Body wt (g)	42.0±2.1	41.9±2.4	40.7±2.5	40.6±3.1
Lung wt/Body wt (mg/g)	4.88±0.30	4.84±0.26	8.50±2.70*	10.13±2.78*†
Pleural effusion (%)	0	0	71.9	93.1
N	13	12	20	18
LV wt/Body wt (mg/g)	2.68±0.14	2.64±0.17	3.07±0.22*	3.33±0.29*†
N			12	11
Infarct area (%)	–	–	57.8±3.1	56.0±3.4*

LV, left ventricular; EDD, end-diastolic diameter; ESD, end-systolic diameter; EDP, end-diastolic pressure; wt, weight. Values are mean±SD. *$P<0.05$ vs. Sham/LacZ, †$P<0.05$ vs. MI/LacZ.

Echocardiography showed that cardiac dimensions of surviving mice on day 28 were significantly higher in MI/LacZ mice compared to Sham/LacZ or Sham/TNFR1 (Table 3). MI/TNFR1 mice showed significantly more cavity dilatation with exacerbation of contractile dysfunction compared with MI/LacZ. Pressure measurement with a Millar catheter showed no significant differences in heart rate and aortic blood pressure among 4 groups. However, LV end-diastolic pressure, which increased significantly in MI/LacZ mice, was further

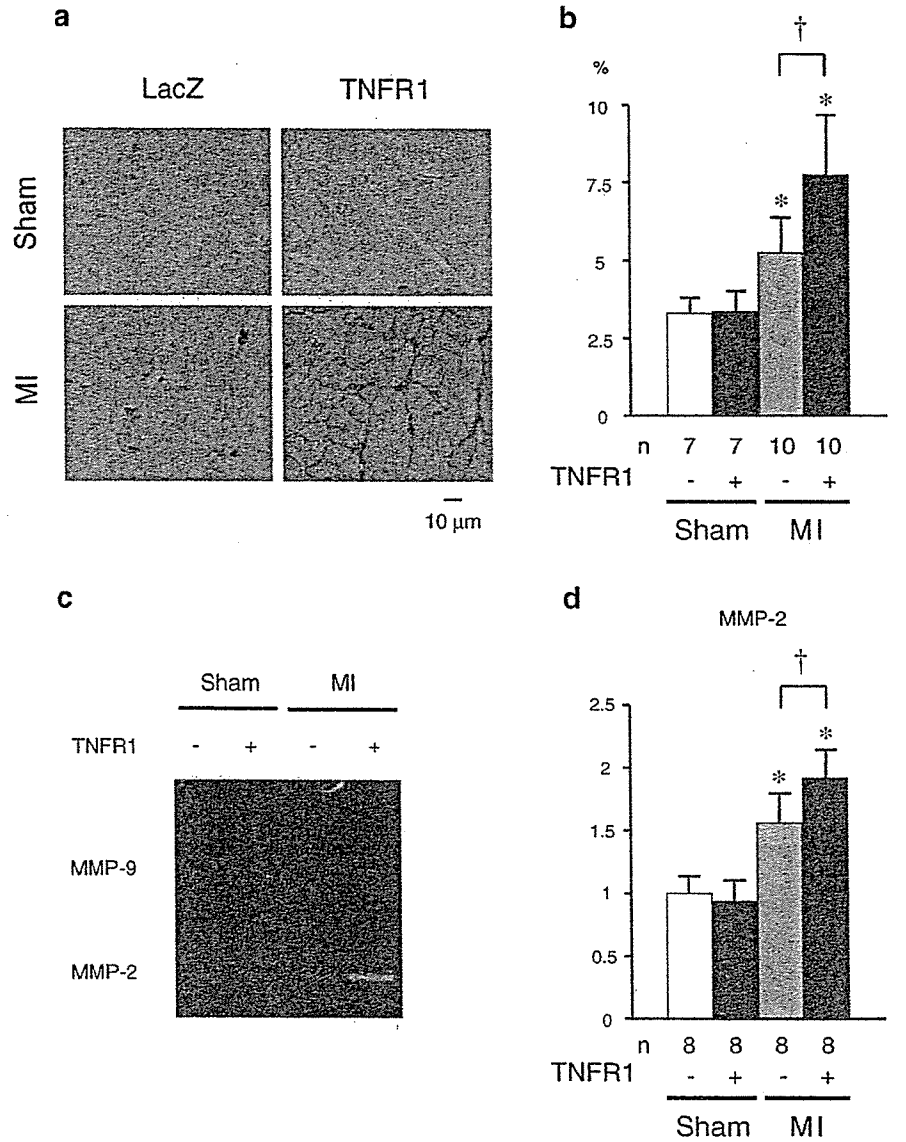

Fig. 7. Collagen volume analysis of the non-infarct myocardium on day 28 after MI: representative micrographs of Picrosirius red staining (a) and summarized data for collagen volume fraction (b). Gelatin zymography for MMP-2 and MMP-9 in non-infarct myocardium on day 28 after MI: representative gel (c) and summarized data for densitometric analysis (d). Each value is expressed as the ratio to the average of MMP-2 in Sham/LacZ mice. Values are mean±SD. TNFR1 (−) indicates post-MI treatment with AdLacZ; TNFR1 (+), post-MI treatment with AdTNFR1; Sham, sham-operated mice; MI, coronary ligated mice. *$p<0.05$ vs. Sham/LacZ mice, †$p<0.05$ vs. MI/LacZ mice.

augmented significantly in MI/TNFR1 mice. Both LV dP/dt_{max} and LV dP/dt_{min}, which decreased significantly in MI, were further lowered significantly by post-MI treatment with soluble TNF receptors. Although body weight was similar among 4 groups, LV weight/body weight ratio increased significantly in MI and further exacerbated with TNFR1 treatment. Along with increased LV end-diastolic pressure, lung weight/body weight ratio also increased significantly in MI/LacZ mice with further increment by TNFR1 treatment. These results suggest that the post-MI treatment with soluble TNF receptors exacerbates ventricular remodeling and pulmonary congestion after MI.

3.2.2. Enhanced fibrosis in non-infarct myocardium with further activation of MMP-2

Collagen was visualized in LV cross-section using Picrosirius red staining (Fig. 7a). As summarized in Fig. 7b, collagen volume fraction, which increased in non-infarct myocardium of MI/LacZ mice, was further augmented significantly by TNFR1 treatment. To further elucidate the mechanisms of increased myocardial fibrosis in MI/TNFR1 mice, MMP-2 and -9 activities were evaluated in non-infarct myocardium on day 28 using gelatin zymography (Fig. 7c). As summarized in Fig. 7d, MMP-2 activity increased significantly

after MI and was further activated by TNFR1 treatment, although MMP-9 activity was not altered.

These results indicate that post-MI treatment with soluble TNF receptors exacerbates ventricular dysfunction and remodeling, with enhanced fibrosis and further activation of MMP-2 in non-infarct myocardium.

4. Discussion

Proinflammatory cytokines including TNF-α have been implicated in the pathogenesis of cardiovascular diseases [6,7]. However, the roles of these cytokines in myocardial infarction remain controversial. In the present study, we evaluated the effects of soluble TNF receptor treatment on MI. Treatment with soluble TNFR1 neutralized the bioactivity of TNF-α that was activated after MI, and prevented apoptosis of infiltrating cells in the infarct myocardium. However, pre-MI treatment with soluble TNFR1 promoted ventricular rupture by reducing fibrosis with further activation of MMP-9. Furthermore, post-MI treatment with soluble TNFR1 exacerbated ventricular dysfunction and remodeling, and enhanced fibrosis in non-infarct myocardium with further activation of MMP-2. Because both pre- and post-MI treatments with soluble TNFR1 were deleterious in a mouse model of MI, TNF-α may play some protective roles in MI.

We used AdTNFR1 to block the effects of TNF-α after MI. We have previously confirmed the efficacy of AdTNFR1 treatment in transgenic mice with cardiac-specific overexpression of TNF-α [18]. Injection of 10^9 pfu of AdTNFR1 increased plasma levels of soluble TNFR1 substantially and ameliorated myocardial inflammation induced by TNF-α overexpression for 6 weeks [18]. In the present study, proinflammatory cytokines and chemokines including TNF-α, IL-1β, IL-6, TGF-β, MCP-1, and RANTES were up-regulated in infarct myocardium 3 days after coronary ligation. Pre-MI treatment with AdTNFR1 increased plasma levels of soluble TNFR1 as previously reported [18]. Although AdTNFR1 treatment did not affect the transcript levels of proinflammatory cytokines and chemokines, it significantly blocked the bioactivity of TNF-α in infarct myocardium. These results indicate that treatment with AdTNFR1 neutralizes the effects of TNF-α induced after MI. Furthermore, induction of proinflammatory cytokines and chemokines in infarct myocardium is not solely mediated by TNF-α.

Cardiac rupture is an acute fatal complication that occurs during the early phase after MI. Disorganized infarct healing and the resultant deficiency or disruption of extracellular matrix (ECM) at the infarction site may lead to myocardial rupture. In the present study, we have shown that pre-MI treatment with soluble TNFR1 increases ventricular rupture after MI without affecting systemic blood pressure or heart rate, but significantly reduced collagen content of infarct myocardium with further MMP-9 activation. Because targeted disruption of MMP-9 is known to prevent ventricular rupture after MI [22], further activation of MMP-9 may be the primary cause of increased ventricular rupture in this mouse MI model. Further activation of MMP-9 may be attributed to increased macrophages in soluble TNFR1-treated infarct myocardium, because macrophages produce substantial amounts of MMP-9 [21]. Increased macrophages may result from the anti-apoptotic effects of soluble TNFR1, because apoptosis of infiltrating cells was significantly attenuated by the treatment. These results suggest that TNF-α may be necessary for proper coordination of tissue repair processes after MI.

TNF-α is a potent inducer of apoptosis in a variety of cells including macrophages and myocytes [5,20]. In the present study, we have demonstrated that treatment with soluble TNFR1 decreases the number of apoptotic cells in infarct myocardium 3 days after MI, which is consistent with the blockade of TNF-α bioactivity by the treatment. Most of the apoptotic cells were interstitial infiltrating cells including macrophages, rather than myocytes. Because the number of infiltrating cells was not different between AdLacZ- and AdTNFR1-treated infarct myocardium 1 day after MI, the increase in relative number of macrophages in AdTNFR1-treated infarct myocardium after 3 days was probably due to decreased apoptosis of infiltrating cells. In contrast, Kurrelmeyer et al. [15] reported that targeted disruption of both TNFR1 and TNFR2 increased the size of infarct myocardium by enhancing apoptosis of cardiac myocytes within 24 h after MI, suggesting that TNF-α may protect cardiac myocytes from ischemic injury by preventing apoptosis. Furthermore, pre-MI treatment with TNF-α has been shown to ameliorate ischemic/reperfusion injury with induction of MnSOD [23]. These results suggest that the pathophysiological roles of TNF-α in the subacute phase of MI (more than 3 days) may be different from those in acute ischemia (within 24 h). TNF-α may protect cardiac myocytes from cell death in acute ischemia, but promote apoptosis of infiltrating cells to resolve inflammation in the subacute phase.

Myocardial infarction leads to complex structural alterations in both infarct and non-infarct myocardium, resulting in progressive dilatation and dysfunction of the ventricle (remodeling). TNF-α is induced in the failing human heart [2]. Its role in the progression of ventricular remodeling is inferred from findings that TNF-α suppresses cardiac contractility [3], provokes myocardial hypertrophy [4], and induces apoptosis in cultured cardiac myocytes [5]. However, in the present study, treatment with soluble TNFR1 further exacerbated ventricular dysfunction and remodeling even when given after the acute phase. These results suggest that TNF-α may protect infarct and non-infarct myocardium from the progression of remodeling. The dynamics of synthesis and breakdown of ECM proteins play an important role in post-MI LV remodeling. In particular, increased expression and activation of MMPs have been implicated in this process [21]. Several studies have demonstrated that MMPs are involved not only in cardiac rupture [19,22] but also in LV remodeling and failure [24,25]. Among the known MMPs, MMP-2 and MMP-9 have been shown to play an important role in post-MI remodeling [19,24]. Although MMP-9 is

mainly expressed in infiltrating inflammatory cells such as neutrophils and macrophages, MMP-2 is ubiquitously distributed in cardiac myocytes and fibroblasts and is up-regulated after MI. In the present study, we have demonstrated that post-MI treatment with soluble TNFR1 further activates MMP-2 in non-infarct myocardium, while the pre-MI treatment augments MMP-9 in infarct myocardium. Furthermore, we have previously reported that targeted disruption of MMP-2 as well as MMP-9 attenuates not only ventricular rupture but also ventricular remodeling after MI [19]. Therefore, exacerbation of ventricular remodeling and dysfunction after TNFR1 treatment may be mediated by further activation of MMP-2, although the precise mechanism by which MMP-2 is further activated remains undetermined.

The present findings contradict the results of Sun et al. [12] using TNF-α knockout mice. Targeted disruption of TNF-α by gene knockout significantly reduced acute cardiac rupture and improved chronic left ventricular dysfunction after MI, accompanied by a reduction of cardiac inflammatory cell infiltration, cytokine expression, and MMP-9 activity. Chronically, TNF-α knockout mice also showed less fibrosis and apoptosis in the myocardium remote from the infarct zone, which contributed to improved ventricular function [12]. These results are thoroughly opposite to ours. Although gene targeting completely eliminated TNF-α in myocardium, the absence of TNF-α during embryogenesis and development may have altered other signaling pathways to secure physiological growth of these mice. Therefore, the differences between the present study and the previous report may be attributed to the methods adopted to block the effects of TNF-α.

TNF-α initiates its biological effects by binding two distinct cell surface receptors with approximate molecular masses of 55 kDa (TNFR1) and 75 kDa (TNFR2) [1]. Both receptors are expressed in most cell types, including cardiac myocytes. Although most biological activities of TNF-α are signaled through TNFR1, the role of TNFR2 remains unclear. Targeted disruption of TNFR1 has been shown to reduce ventricular rupture and remodeling after MI [13]. Furthermore Higuchi et al. [26] have recently demonstrated that ablation of TNFR1 ameliorated heart failure and improved survival while ablation of the TNFR2 gene exacerbated heart failure and reduced survival of TNF-α transgenic mice, suggesting a cardioprotective role of TNFR2-mediated signaling. Therefore, exacerbation of ventricular rupture and remodeling observed in the present study may have derived from blockade of TNFR2-mediated signaling. Because the dissociation constants of TNFR1 and TNFR2 are $2–5\times10^{-10}$ and $3–7\times10^{-11}$, respectively [1], high levels of TNF-α interact with both TNFR1 and TNFR2, while low levels may only stimulate TNFR2 pathways. In other words, low levels of soluble TNF receptors may only block cytotoxic TNFR1, whereas high levels of soluble TNF receptors may also block the protective TNFR2. Because plasma levels of soluble TNF receptors in the present study (about 500 μg/ml) were more than 100 times higher than clinical plasma levels (approximately 0.3–3 μg/ml) [27], these high levels may block the protective TNFR2 resulting in deleterious results. Using lower doses of soluble TNF receptors may produce different results.

Another explanation for the negative results of the present study might be intrinsic toxicity of soluble TNF receptors [7]. Soluble TNF receptor also acts as a carrier protein that stabilizes TNF-α resulting in the accumulation of high concentrations of immunoreactive TNF-α [18]. Because binding of TNF-α–TNFR complexes is reversible [28], the increase in level of TNF-α–TNFR complex may lead to an increase in the duration of TNF bioactivity. Specifically, this might be the case for soluble TNFR2 fusion proteins, including etanercept that was used in RENEWAL [8]. Studies of the binding of TNF-α with soluble TNF receptor fusion proteins have shown that TNFR2 exchanges bound TNF-α about 50- to 100-fold faster than TNFR1 [29]. Thus, although both fusion proteins in equilibrium bind TNF-α with high affinity, the TNF-α–TNFR1 fusion protein complex is kinetically more stable than the TNFR2 fusion construct. Because the soluble TNF receptors used in the present study were TNFR1, the intrinsic toxicity might have been less than that if TNFR2 were used. To confirm this hypothesis, we measured the bioactivity of TNF-α in the myocardium, and found that the bioactivity was significantly reduced by the treatment. Therefore, this might not be the case in the present study.

Although the negative results of anti-TNF clinical trials in patients with heart failure prompted us to conduct the present study, there are several important aspects that are different between the present study and the clinical trials. First, acute myocardial infarction was used as a model of heart failure in the present study, while patients with chronic heart failure were recruited in the clinical trials. The roles of proinflammatory cytokines might be different in acute and chronic phases of myocardial infarction. Second, the dosage of soluble TNF receptors used in the present study was more than 100 times higher than that used in the clinical trials. Effects of high doses should be different from those of lower doses, which remain undetermined in this mouse MI model. Therefore, caution has to be exercised in interpreting the present results in association with the clinical trials.

In conclusion, treatment with soluble TNF receptors increases ventricular rupture and exacerbates cardiac remodeling in a murine model of MI. Because TNF-α seems to play both cytotoxic and protective roles in cardiovascular diseases, further studies are required to elucidate the optimal approach to modulate proinflammatory cytokines in clinical practice.

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Pressor response induced by central angiotensin II is mediated by activation of Rho/Rho-kinase pathway via AT_1 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 AT_1 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, AT_1 and AT_2. The cardiovascular effects of angiotensin II are widely considered to result from AT_1 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 AT_1 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 μl/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 μl/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 ± 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

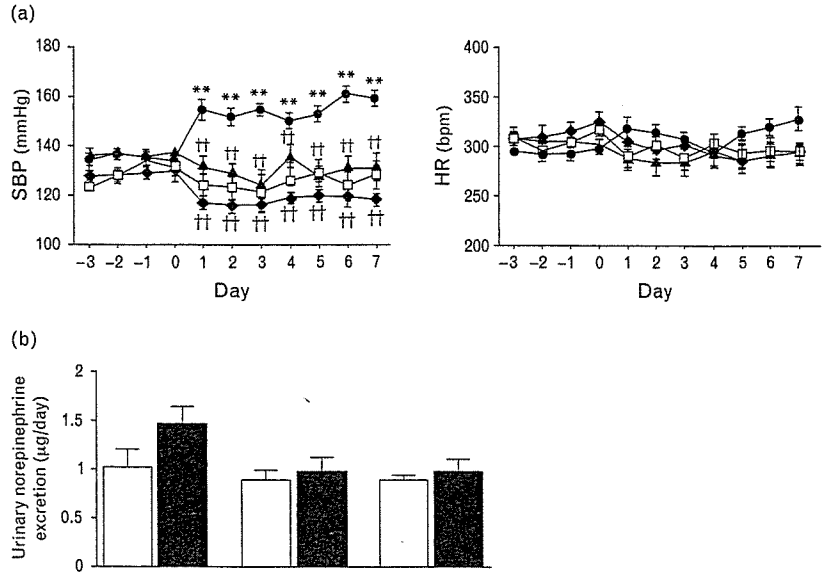
Intracisternal infusion of angiotensin II and co-infusion of Y-27632 or valsartan in Wistar–Kyoto rats

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



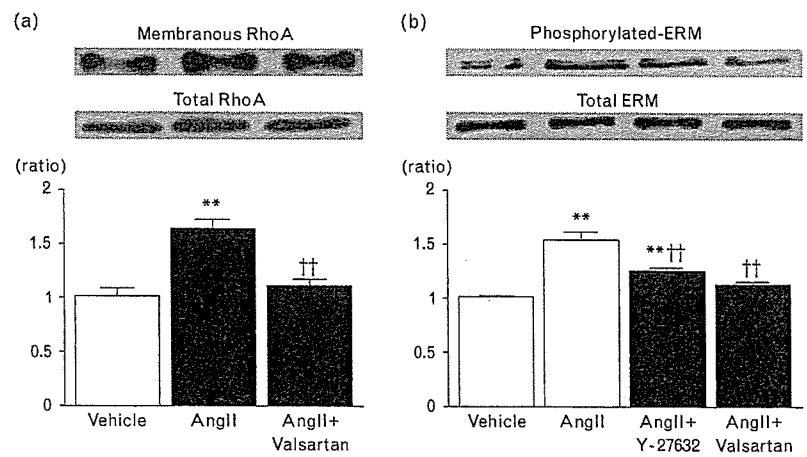
(a) Time course of systolic blood pressure (SBP) and heart rate (HR) before and during the intracisternal infusion of vehicle, angiotensin II (AngII), co-infusion of angiotensin II and Y-27632, or co-infusion of angiotensin II and valsartan ($n = 10$ for each). $^{**}P < 0.01$ compared with the vehicle group; $^{\dagger\dagger}P < 0.01$ 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; $^{\dagger\dagger}P < 0.01$ 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).

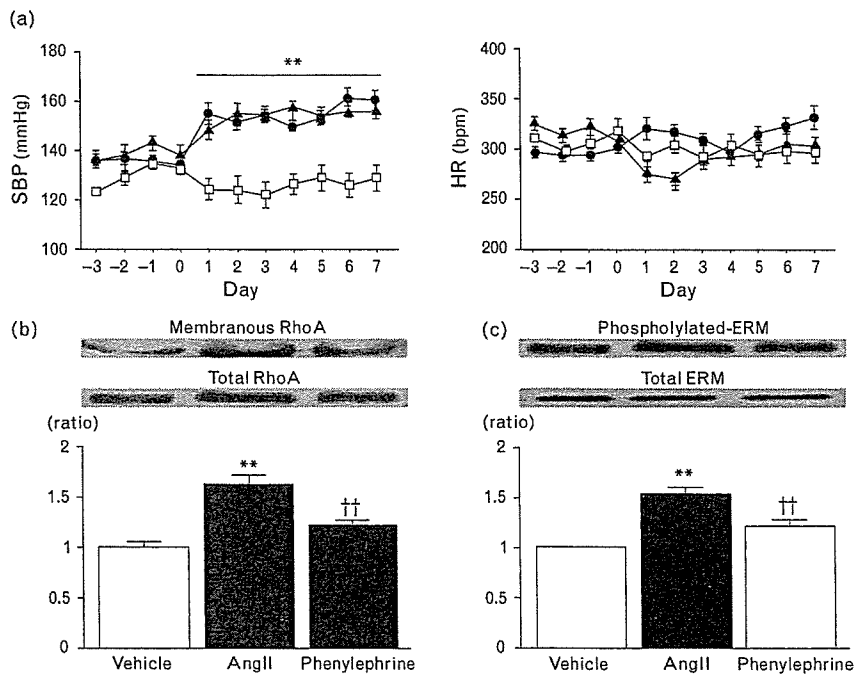
Rho/Rho-kinase activity in the brainstem of spontaneously hypertensive rats

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 AT_1 receptor blockade in Wistar–Kyoto and spontaneously hypertensive rats

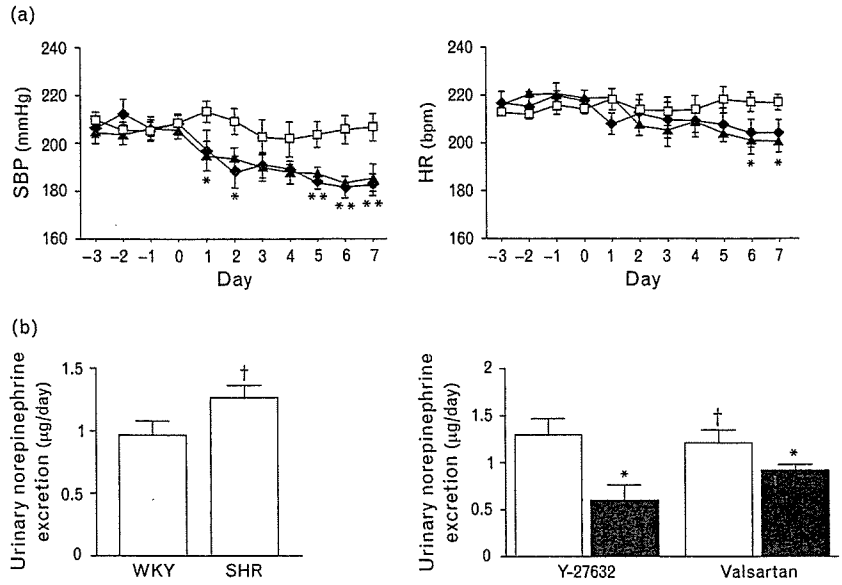
The intracisternal infusion of valsartan reduced SBP in WKY. The magnitude of the decrease, however, was significantly higher in SHR (210 ± 2 to 182 ± 5 mmHg) than in WKY (122 ± 6 to 110 ± 3 mmHg). The intracisternal infusion of valsartan significantly reduced heart rate in SHR (346 ± 10 to 316 ± 14 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; $^{\dagger\dagger}P < 0.01$ compared with the angiotensin II group.

Fig. 4

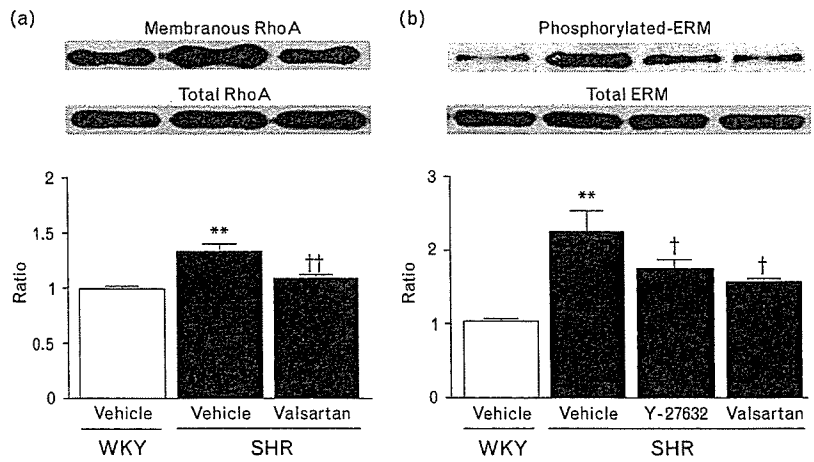


(a) Time course of systolic blood pressure (SBP) and heart rate (HR) before and during the intracisternal infusion of vehicle, Y-27632, or valsartan in SHR ($n=5$ for each). $^{*}P<0.05$; $^{**}P<0.01$ compared with the vehicle group, respectively. □ Vehicle; ▲ Y-27632; ◆ valsartan. (b) Urinary norepinephrine 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). $^{\dagger}P<0.05$ compared with the values of WKY; $^{*}P<0.05$ compared with pretreatment values. □ Before; ■ after.

Discussion

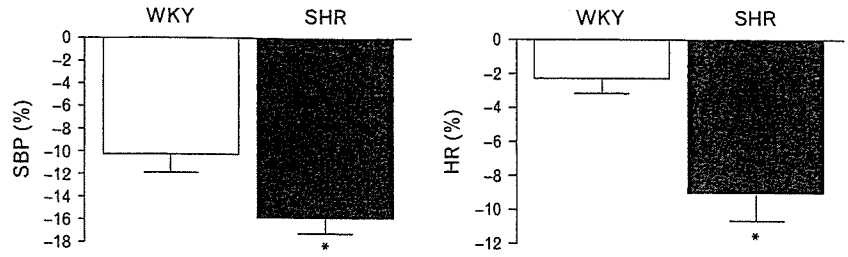
In the present study, pressor and sympathoexcitatory responses evoked by the intracisternal infusion of angiotensin II were abolished by the co-infusion of Y-27632 in WKY. The effects of angiotensin II on these responses were mediated by AT_1 receptors, because valsartan

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; $^{\dagger}P<0.05$; $^{\dagger\dagger}P<0.01$ 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 AT_1 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

Dihydropiridine 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 hidralazine, 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 ß-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 ß-tubulin or ß-actin protein.

Statistical Analysis

All values are expressed as mean ± 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,

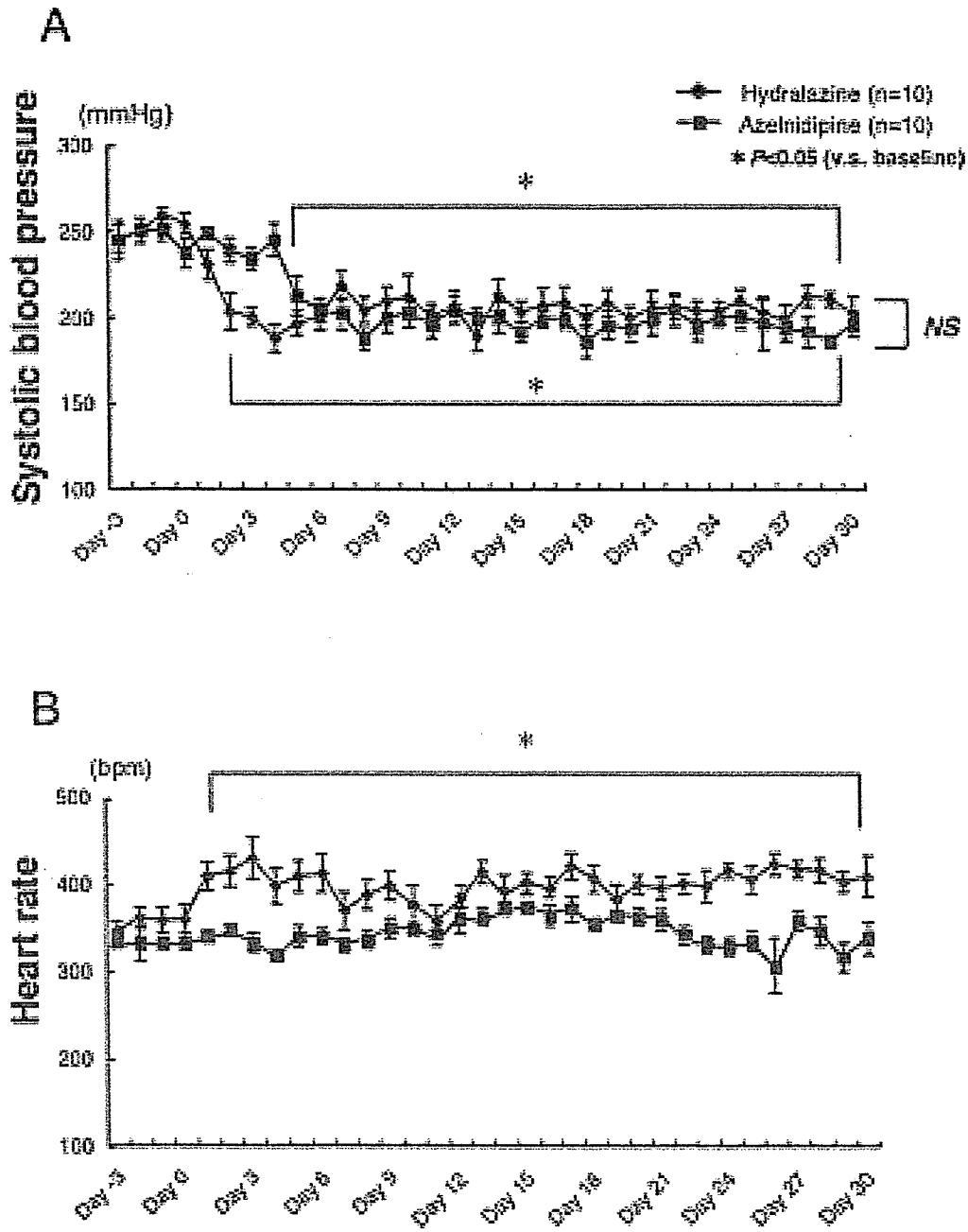

Figure 1. Time courses in systolic blood pressure (a) or heart rate (b) induced by treatment with hydralazine or azelnidipine.

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

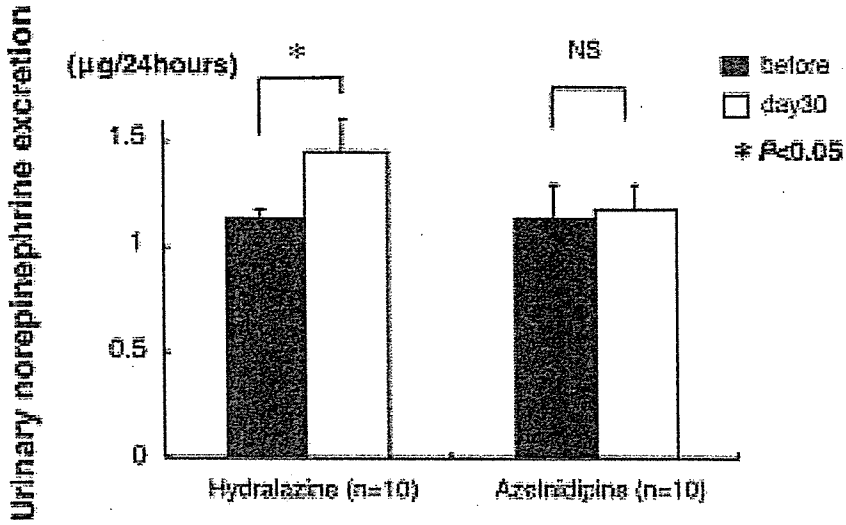

Figure 2. Twenty-four-hour urinary norepinephrine excretion before and after treatment with hydralazine or azelnidipine.

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 Ser^{1177} and the dephosphorylation of Thr^{495} 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

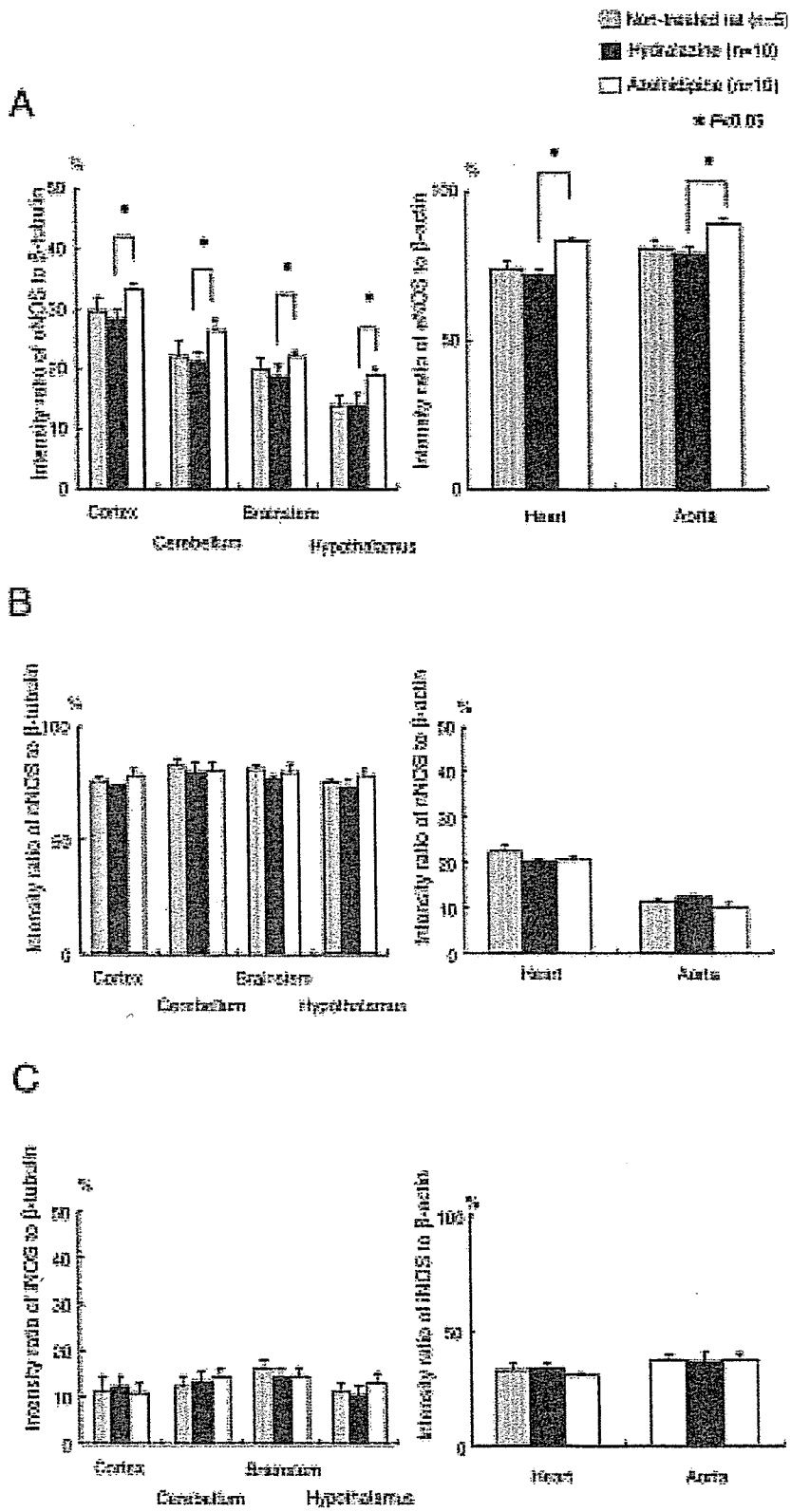

Figure 3. NOS expression levels evaluated by Western blot analysis in the brain (cortex, cerebellum, brain stem, and hypothalamus), heart, and aorta in non-treated rats and rats treated with hydralazine or azelnidipine for 30 days; a: eNOS expression levels, b: nNOS expression levels, c: iNOS expression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

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