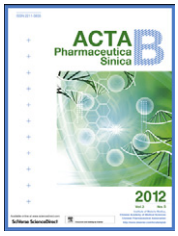
Acta Pharmaceutica Sinica B 2012;2(5):450–458

Institute of Materia Medica, Chinese Academy of Medical Sciences
Chinese Pharmaceutical Association

Acta Pharmaceutica Sinica B

www.elsevier.com/locate/apsb
www.sciencedirect.com



ORIGINAL ARTICLE

Differential effects of Rho-kinase inhibitor and angiotensin II type-1 receptor antagonist on the vascular function in hypertensive rats induced by chronic L-NAME treatment

Bainian Chen, Lili Shi, Xiaoyan Yu, Jialin Sun, Hengai Zhang, Shoubao Wang, Lianhua Fang, Guanhua Du*

National Center for Pharmaceutical Screening, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China

Received 5 February 2012; revised 21 March 2012; accepted 29 March 2012

KEY WORDS

Angiotensin II receptor antagonist;
L-NAME-induced hypertension;
Rho-kinase inhibitor;
Vascular function;
Cardiac hypertrophy

Abstract Little attention has been paid to the effect of Rho-kinase inhibitor on the vascular dysfunction of nitric oxide-deficient hypertension. We aimed to investigate whether the Rho-kinase inhibitor fasudil showed beneficial effect on the vascular dysfunction of the N^G-nitro-L-arginine methyl ester (L-NAME) treated rat, as well as to compare the differential effects of fasudil and angiotensin II receptor antagonist valsartan on vascular function. In the present study, both valsartan and fasudil exerted antihypertensive action on the L-NAME-treated rats, while only valsartan attenuated the cardiac hypertrophy. Treatment with valsartan showed improvement on vascular reactivity to norepinephrine, KCl and $CaCl_2$, whereas fasudil therapy showed little effect on vasoconstriction. Endothelium-dependent vasodilation to acetylcholine was reduced in the NO-deficient group but was normalized by the fasudil therapy. The increased expression of RhoA and Rho-kinase (ROCK) in the vasculature was corrected well to normal level by either valsartan or fasudil administration, which seemed to be at least partially responsible for the beneficial effect of the drug infusion. These findings suggest that the angiotensin II receptor antagonist interferes more

*Corresponding author. Tel./fax: +86 10 63165184.
E-mail address: dugh@imm.ac.cn (Guanhua Du).

Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

http://dx.doi.org/10.1016/j.apsb.2012.04.002

with the contractile response than Rho-kinase inhibitor, whereas inhibition of Rho-kinase activity exhibits a better improvement on vasorelaxation than blockade of angiotensin II receptor.

1. Introduction

Reduced production or activity of nitric oxide (NO) contributes to several human diseases, especially hypertension[1,2]. Therefore, NO-deficient hypertension evoked by chronic treatment with NO synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) is a preferred animal model for hypertension research[3]. This systemic hypertension may be attributed to a deficiency in NO production by the vasculature[4]. Previous reports have revealed the prevention of high blood pressure and the improvement of vascular function in NOS-blockaded animals through Ang II receptor antagonist intervention[5,6]. Furthermore, valsartan has been reported to decrease blood pressure and total peripheral resistance with improved cardiac output in L-NAME-treated mice[7]. However, little is known about the effect of Rho-kinase inhibitors on this hypertension model.

Rho-kinase inhibitors target a wide range of human ailments, including hypertension, heart failure, atherosclerosis, diabetes, cerebral ischemia, glomerulosclerosis, Alzheimer's disease, bronchial asthma and cancers[8,9]. Fasudil, a selective Rho-kinase inhibitor, is clinically used in China and Japan for treating cerebral vasospasm after subarachnoid hemorrhage. A dose-dependent vasorelaxative effect of fasudil has been validated in humans based on the result of an increased blood flow and decreased resting vascular resistance in the forearm after drug infusion[10]. However, there is no report about the effect of fasudil on the vascular function of L-NAME-induced hypertension. Thus, we aimed to investigate the beneficial potential of Rho-kinase inhibitor on the NO-deficient hypertensive rat and to compare the effects of Rho-kinase inhibitor and AT_1 blocker on the vascular function. The differential role of fasudil and valsartan in the cardiac hypertrophy was also addressed in the present study.

2. Materials and methods

2.1. Animals and treatment

Male Wistar rats weighing 120–140 g were purchased from Beijing Vital River Company, China, kept in polypropylene cages (5 per cage) and housed in the institutional animal facility with normal rodent chow and distilled water freely available. All the procedures were in accordance with the guidelines of the National Research Council and were approved by the ethical committee of Chinese Academy of Medical Sciences and Peking Union Medical College.

All the animals were suited in the environment mentioned above for two weeks before the experiment. Then they were randomly divided into two groups: control group (C, $n=15$) and L-NAME treated group ($n=54$). Rats in the L-NAME group received an intake of L-NAME (40 mg/kg, i.g.) every morning and those in the control group received distilled water intragastrically at the same time. Three weeks later, systolic blood pressure (SBP) was measured by the tail-cuff method (BP-98A, Softron, Japan) in two successive days, and those with averaged SBP < 140 mmHg in the L-NAME treated group were excluded from the following study.

The rest animals in the L-NAME treated group were randomly divided into three groups: model group (M, $n=15$), valsartan-treated group (V, $n=17$) and fasudil-treated group (F, $n=12$). Rats in the groups C and M were orally administrated with distilled water and L-NAME (40 mg/kg/day), respectively. Rats in the group V were intragastrically given L-NAME (40 mg/kg/day) plus valsartan, and those in the group F were intragastrically given L-NAME (40 mg/kg/day) plus fasudil. The doses of valsartan and fasudil were both 30 mg/kg/day. The treatments were carried out for 6 weeks and stopped two or three days before the *in vitro* experiment in order to avoid the effect of acute receptor occupancy[11].

From the start of treatments, the SBP and heart rate of all rats were determined weekly at 1 h after drug administration by the tail-cuff instrument and were calculated as average values of 3 measurements. The last SBP determination was made before therapy was discontinued.

2.2. Drugs and reagents

L-NAME, norepinephrine (NE), acetylcholine (Ach), Ang II and phorbol dibutyrate (PDBu) were all purchased from Sigma (Sigma Chemical Co., USA). Valsartan (Diovan®, Novartis) was commercially available and fasudil hydrochloride was kindly provided by Tianjin Chase Sun Pharmaceutical Company (Tianjin, China). All other chemicals were of highest grade available. Solutions used in the present study were all prepared freshly and protected from light.

2.3. Vessel preparation

Rats were weighed, anesthetized with urethane (1.0 g/kg, i.p.) and killed by decapitation. The thoracic aorta was carefully removed and placed in the cold Krebs–Henseleit (K–H) buffer with the following composition (mM): NaCl 120, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.1, $CaCl_2$ 2.5, $MgSO_4$ 1.2. The aortic rings (3 mm in length), cleaned of excess connective tissue and fat, were mounted between two stainless steel hooks in organ chambers containing 10 mL K–H solution (37 °C) with a gas mixture of 95% O_2 and 5% CO_2. One of the two steel hooks was fixed to the bottom of chambers, and the other was connected to a force transducer in order to record isometric tension (MP 100, BIOPAC, USA).

The rings were equilibrated at a resting tension of 1.2 g for 1 h. During the equilibration period, the K–H buffer was replaced every 20 min, and the resting tension was readjusted.

After the equilibration, two successive stimulations with high potassium K–H solution (80 mM), which was prepared by replacing NaCl with KCl on an equimolar basis, were applied to the aortas to record the maximum contractile response to KCl.

2.4. Vascular experimental protocols

The aortic rings were rinsed with K–H solution to return to baseline tension after the response to high potassium K–H solution. Then the cumulative dose-response curves of NE (10^{-9}–10^{-5} M) were registered on one of the two segments. After a 20-min equilibration period, the aortic rings were precontracted with 10^{-6} M NE followed by a concentration-response to acetylcholine (10^{-9}–10^{-4} M). The rings were rinsed with K–H buffer twice and equilibrated in Ca^{2+}-free K–H solution (containing 1 M EGTA) for another 20 min with fresh Ca^{2+}-free solution replaced at 5-minute intervals. A single dose of NE (10^{-6} M) was added into the organ bath to obtain the transient contractile response to intracellular Ca^{2+} released from the sarcoplasmic reticulum. Once the basal tension value was restored, the rings were challenged with 80 M KCl in fresh Ca^{2+}-free K–H buffer followed by cumulative addition of $CaCl_2$ (0.1–2.5 mM).

KCl was added in a cumulative manner (10–80 mM) to another segment of the aorta from each rat and the concentration–response curve to KCl was registered. The rings were contracted with a single concentration of Ang II (10^{-7} M) after a 20-min washout period. Then the rings were rinsed with K–H solution and allowed a recovery period for 20 min. To test the direct relaxation of vascular smooth muscle, the vessels were precontracted with 10^{-6} M NE followed by the cumulative addition of sodium nitroprusside (10^{-10}–10^{-5} M) in the chambers. Thereafter, responses to PDBu (10^{-7} M) were measured, and those rings were allowed another 20-min equilibration period between the two responses with the former vasodilator being washed away.

2.5. Histological evaluation of heart

Excised hearts were fixed with 10% neutral buffered formalin before weighing at room temperature, and embedded in paraffin. Coronal sections, 5 μm thick, were cut and mounted onto poly-L-lysine coated slides. HE (hematoxylin and eosin) and Masson's trichrome staining was performed for different groups according to the standard histochemical procedure. All histological sections of each heart were examined using a color digital camera (Canon, model EOS450D, Japan) mounted on a standard light microscope (magnification × 400). The transdiameters of 20 randomly selected cardiac myocytes from each sample stained by HE were measured using the Image J software (National Institute of Health, USA). To assess the area of interstitial fibrosis, a total of 15 fields per section stained by Masson's trichrome were evaluated using the Image-Pro Plus software (Media Cybernetics, MD, USA), and the ratio of the total area of fibrosis to the total area of the image was calculated and used for analysis. The histopathology was carried out by a researcher who was blinded to the experimental groups.

2.6. Western blot analysis of aorta

Aortas were removed from rats, immediately frozen in liquid nitrogen and homogenized to obtain tissue protein samples. Equal amounts of protein were loaded onto sodium dodecyl sulfate polyacrylamide gels, and an electrophoresis was run at 100 V for 1.5 to 2 h (Bio-Rad, Hercules, CA, USA). Proteins were then transferred to PVDF membranes (Millipore, USA). The membranes were blocked with 5% bovine serum albumin (BSA) in 0.1% Tween 20-phosphate-buffered saline for 4 h at room temperature. Blots were probed with antibodies to RhoA (Cell Signaling Technology, USA) or ROCK-I (Santa Cruz Biotechnology, USA) at 4 °C overnight. Those blots were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 2 h at 37 °C. Between steps, membranes were washed three times in tris/tween buffer. Bound antibodies were detected by the ECL method (Molecular Imager ChemiDoc XRS$^+$ System, Bio-Rad, USA), and the films were analyzed by an imaging system (Quantity One System, Bio-Rad, USA). Data were expressed as a fold of the controls after normalization to GADPH expression.

2.7. Statistical analysis

Contractions of the rat aorta were expressed as percentage of the maximal contractile response to KCl (80 mM). Relaxations were expressed as percentage dilation of NE-induced precontraction. In all series of experiments, n was the number of rats from which the tissues were obtained. Results are given as mean ± SEM. All data were analyzed by one-way ANOVA followed with the least significant difference (LSD) test for multiple comparisons. A two-tailed value of $P<0.05$ was regarded as statistically significant.

3. Results

3.1. Blood pressure, heart rate and heart index

At the end of 3-week administration of L-NAME, the rats in NO-deficiency developed sustained higher blood pressure compared to the control rats, indicating an established hypertensive state in those animals. As shown in Fig. 1, the SBP and heart rate of rats in the control group remained at the basal level during the 6-week treatment period; whereas the blood pressure of rat in the model group displayed an increase, reaching its maximum within 3 weeks. However, the heart rate of the hypertensive rats was markedly lower than the control rats. Both valsartan and fasudil treatment effectively prevented the further rising of SBP (Fig. 1A). Interestingly, valsartan treatment did not alter the heart rate, but fasudil treatment significantly increased the heart rate compared to both the control and model groups (Fig. 1B).

After 6-week treatment, the body weights were similar in all groups. The heart weights and heart-body weight ratios of the model rats were much heavier and higher than those of the control rats, while valsartan therapy reduced both of these parameters significantly. In contrast, fasudil treatment markedly increased the heart weights and heart index (heart weight/body weight) compared to the control group (Table 1).

3.2. *Vasoconstrictor-induced contractions*

In aortic rings, the cumulative concentration–response to NE (10^{-9}–10^{-5} M) was markedly decreased in the L-NAME induced hypertensive rats compared to the control rats ($P<0.05$). Administration of valsartan significantly improved the aortic contraction evoked by NE, while fasudil treatment corrected the defective constriction to some extent (Fig. 2A). For the KCl- and $CaCl_2$-induced contractile response, the aorta of the model group showed slight reduction compared to that of control group. However, there was no significant difference in all groups (Fig. 2B and C).

A single dose of Ang II and the protein kinase C activator PDBu was added to the organ chamber resulting in vasoconstriction. A long-term treatment with L-NAME decreased vascular reactivity to Ang II but significantly increased vascular response to PDBu (Fig. 3). Both valsartan and fasudil treatment showed some improvement for the vascular reactivity to Ang II and PDBu, but a significant difference between drug-treated and model groups was only observed in PDBu-induced constraction ($P<0.05$). As shown in Fig. 3, the decreased level of response to PDBu provided by fasudil therapy was greater than valsartan treatment.

NE (10^{-6} M)-induced contractile response in Ca^{2+}-free K–H solution (0.27 ± 0.03) represented $18\pm2\%$ of KCl-induced contraction, and this response was lowered by L-NAME treatment without a significant difference (Fig. 3). Valsartan treatment did not affect this response, whereas fasudil treatment markedly enhanced the contraction of aortic segments compared to the L-NAME-evoked hypertensive group.

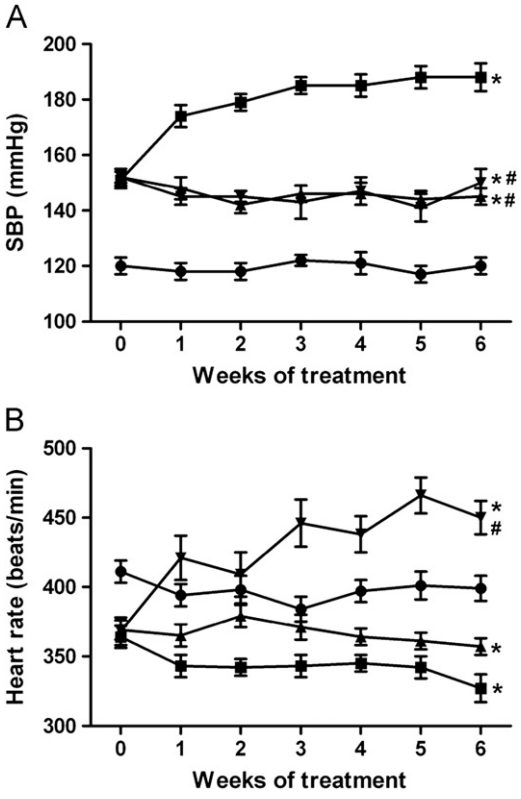


Figure 1 Time course of SBP (A) and heart rate (B) during 6 weeks of treatment in control (●), model (■), valsartan-treated (▲) and fasudil-treated groups (▼). Values are mean±SEM, $n=12$–17. $^{*}P<0.05$ compared with the control group, $^{\#}P<0.05$ compared with the model group.

3.3. *Endothelium-dependent and -independent vasorelaxation*

Endothelium-dependent relaxation of aortic rings precontracted with NE was shown in Fig. 4A. Exposure to cumulative concentrations of acetylcholine (Ach) led to a marked vasorelaxation of NE-constricted rings in the control group. In contrast, the response of aortic rings from the model group to Ach was almost abolished, indicating an impaired endothelium-dependent relaxation of the aorta in the NO-deficient rats induced by L-NAME treatment. Little improvement of this effect was observed in the valsartan-treated group, but a significantly improved vasorelaxation was provided by fasudil therapy in comparison to the model group ($P<0.05$).

The vasodilatory function of vascular smooth muscle was tested by cumulative addition of sodium nitroprusside (SNP) to the organ bath, and the results showed that there was no significant difference of endothelium-independent dilation in all groups (Fig. 4B).

3.4. *Histopathological change of heart*

Standard light microscopy was used to evaluate the development of cardiac hypertrophy. The NO-deficient rats treated by L-NAME showed dramatically increased size of cardiomyocytes compared to the rats in control group (15.69 ± 0.43 μm *vs.* 11.14 ± 0.27 μm, respectively, $P<0.01$). Moreover, disarray of hypertrophied myocytes with increscent nuclei was observed at the ventricular walls of the rats in the model group. This phenomenon was countered by 6-week treatment with valsartan, but not by fasudil therapy (Fig. 5). The cardiomyocyte diameter of

Table 1 Body weight (BW), heart weight (HW) and heart weight–body weight ratio (HW/BW) at the end of the experiment.

	BW (g)	HW (mg)	HW/BW (mg/g)
Control	479±10	1170±27	2.44±0.04
Model	495±11	1298±26**	2.62±0.05
Valsartan	490±10	1158±29##	2.36±0.04##
Fasudil	461±15	1338±38**	2.90±0.09**,#

Values are mean±SEM, $n=12$–17 in each group.
**$P<0.01$ compared with the control group.
#$P<0.05$.
##$P<0.01$ compared with the model group.

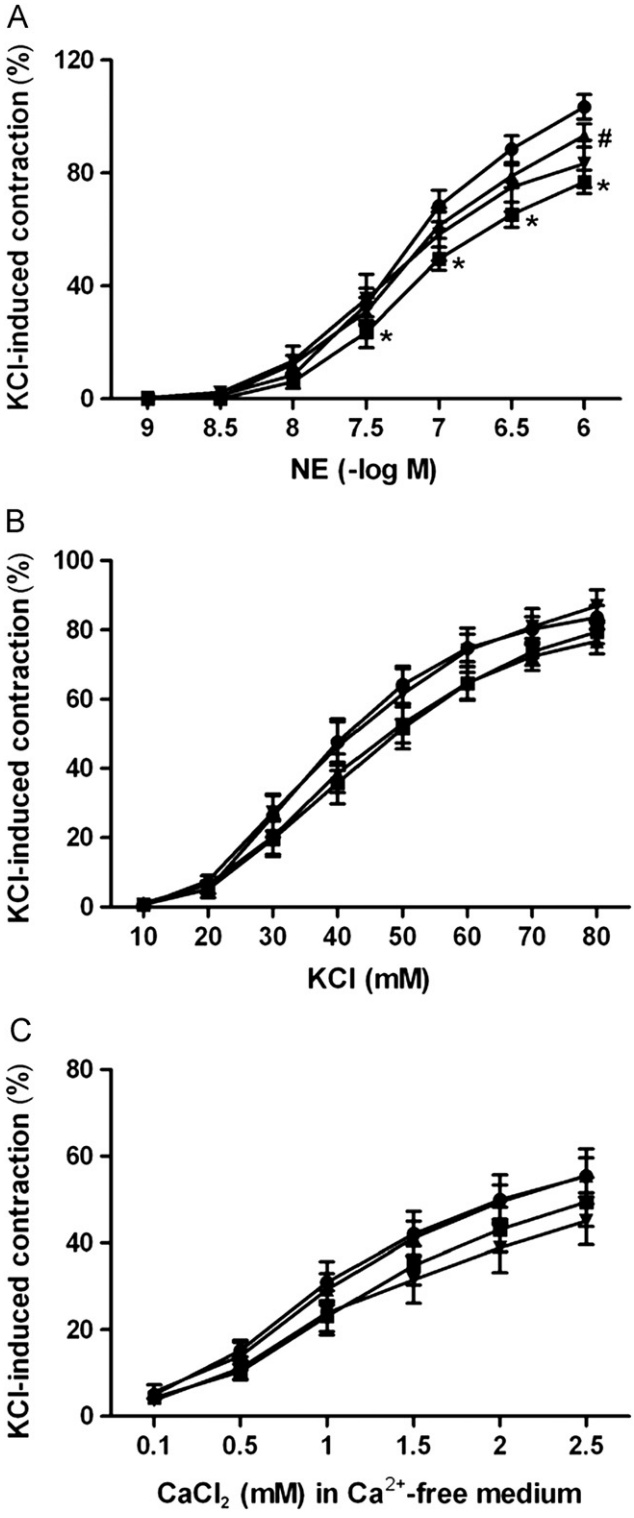


Figure 2 Cumulative concentration-response curves to NE (A), KCl (B) and $CaCl_2$ (C) in aortic rings from control (●), model (■), valsartan-treated (▲) and fasudil-treated groups (▼). Results are presented as percentage of the maximal contractile response to 80 mM KCl. Values are mean ± SEM, $n = 10–14$. $^{*}P < 0.05$ compared with the control group, $^{\#}P < 0.05$ compared with the model group.

the rats in valsartan- and fasudil-treated groups was 12.64 ± 0.26 μm and 15.18 ± 0.36 μm, respectively, the former of which was significant shorter than that of the model group ($P < 0.01$).

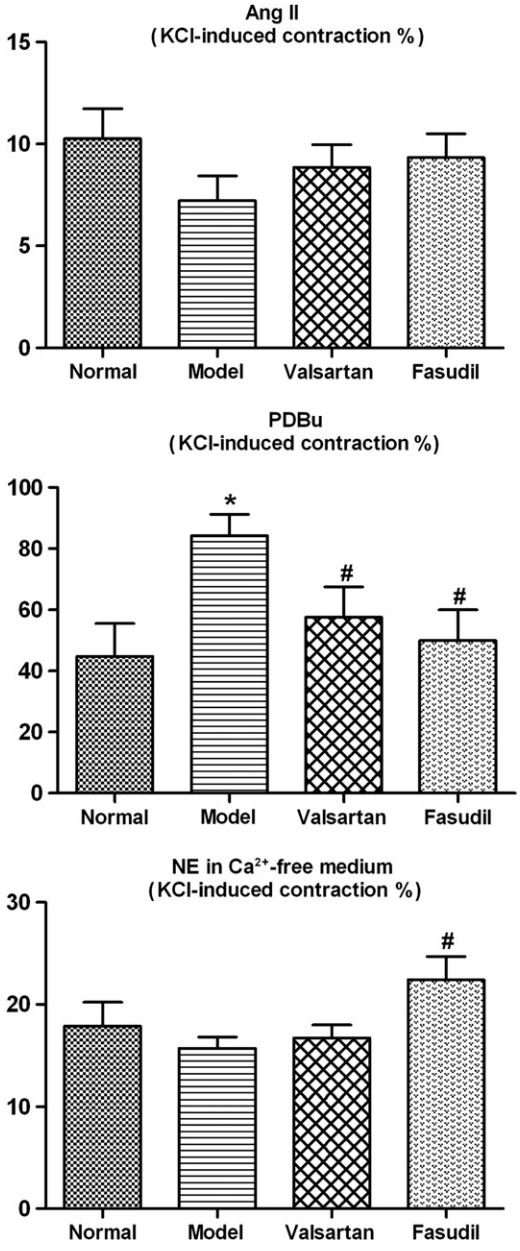


Figure 3 Contractile responses of aortic rings to Ang II (10^{-7} M), PDBu (10^{-7} M) and NE (10^{-6} M) in Ca^{2+}-free K–H solution. Results are presented as percentage of the maximal contractile response to 80 mM KCl. Values are mean ± SEM, $n = 10–14$. $^{*}P < 0.05$ compared with the control group, $^{\#}P < 0.05$ compared with the model group.

Microscopic pictures taken of myocardial fibrosis with Masson's trichrome stain are shown in Fig.6. The area of interstitial fibrosis in the model group was significantly greater than that in the control group ($P < 0.01$). Compared to the model group, myocardial fibrosis was significantly less in the valsartan- and fasudil-treated groups ($P < 0.01$).

3.5. *Expression of RhoA and ROCK-I in aorta*

Analysis with the antibody specific for RhoA revealed that the expression of RhoA in aorta was significantly increased in the

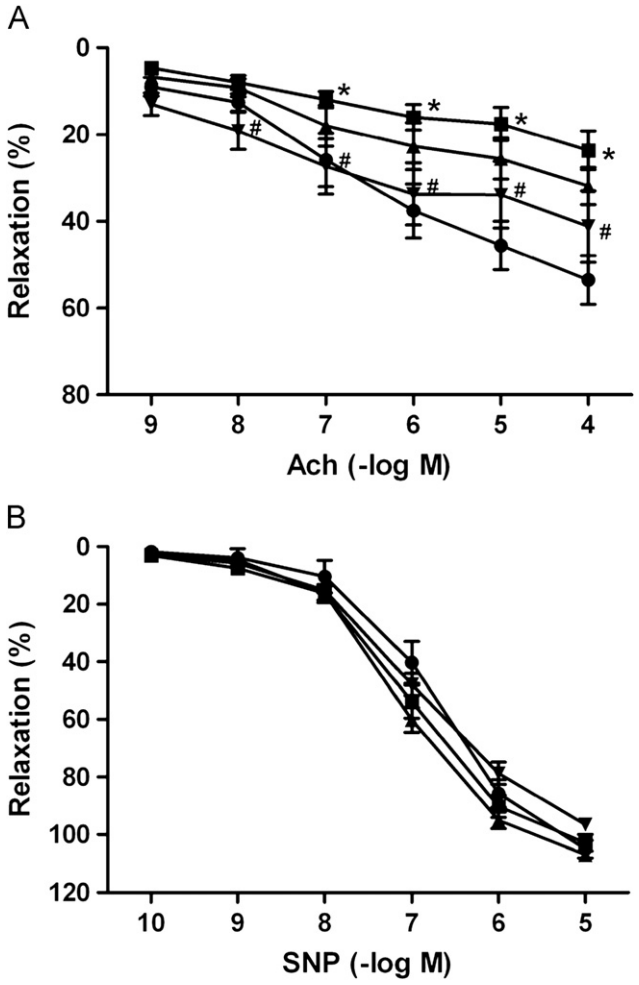


Figure 4 Cumulative concentration–response curves to Ach (A) and SNP (B) in aortic rings from control (●), model (■), valsartan-treated (▲) and fasudil-treated groups (▼). Values are mean ± SEM, $n=6$–14. $^{*}P<0.05$ compared with the control group, $^{\#}P<0.05$ compared with the model group.

L-NAME-treated rats, compared to the control rats ($P<0.05$). There was a significant reduction in vascular RhoA expression in both the valsartan and fasudil treated animals ($P<0.05$). Similarly, in the aorta of the model group, a marked augmentation of ROCK-I expression was observed, which was well corrected to the basal level by both valsartan and fasudil treatment (Fig. 7).

4. Discussion

The present study has demonstrated the changed arterial reactivity to various vasoconstrictors and vasodilators due to chronic blockade of NOS, as well as the beneficial effects of Rho-kinase inhibitor and Ang II receptor antagonist on the vascular hyporesponsiveness. To the best of our knowledge, this is the first report of the effects of Rho-kinase inhibitor on vascular function in the L-NAME-induced hypertensive rat.

Long-term administration of L-NAME caused a sustained elevation in SBP and a marked decrease in the heart rate of normotensive rats in our study, in accordance with other reports[3,5,6]. Previous data showed that Ang II receptor antagonist prevented the development of hypertension induced by L-NAME treatment[5]. Badejo et al.[12] reported that fasudil caused a dose-dependent decrease in pulmonary and systemic arterial pressure in normotensive rats. In the present study, we found that a daily intake of either valsartan or fasudil (30 mg/kg) restored the increased SBP to close the normal level in the chronic NO-deficient rats.

Vascular dysfunction is one of the complicated features in hypertension. A large body of research has shown the abnormality in vascular reactivity of L-NAME-induced hypertensive rats to the alpha-adrenergic receptor agonist, Ang II and drugs acting beyond receptor activation[13]. Henrion et al.[4] observed that chronic NOS blockade induced a hyporeactivity of the blood vessel to various stimulus. Further research confirmed that the hyporesponsiveness of aorta might develop as a result of high-dose, chronic L-NAME administration[13]. The present study found that the cumulative concentration–response to NE was

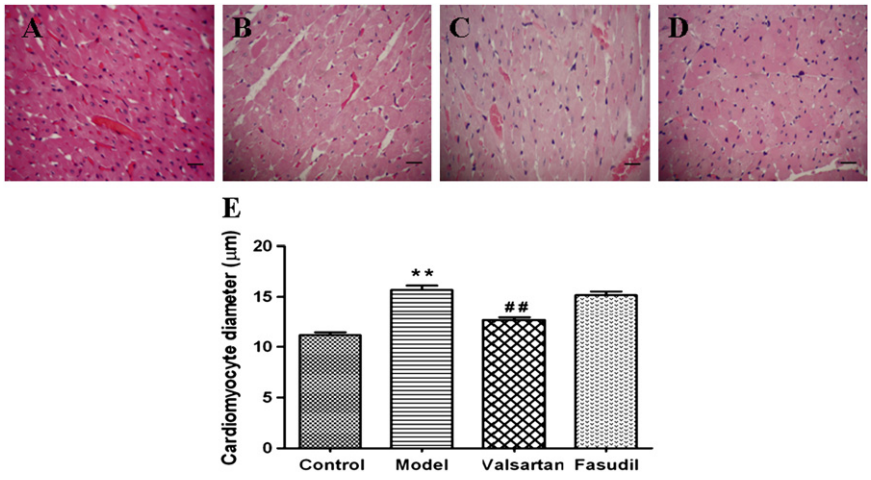


Figure 5 Histopathological changes of cardiomyocytes in different groups (a bar indicates 25 μm). (A)–(D), representative photomicrographs of myocytes by HE staining from control (A), model (B), valsartan-treated (C) and fasudil-treated (D) groups, respectively. (E) Cardiomyocyte diameter for different groups. Values are mean ± SEM, $n=3$. $^{**}P<0.01$ compared with the control group, $^{\#\#}P<0.01$ compared with the model group.

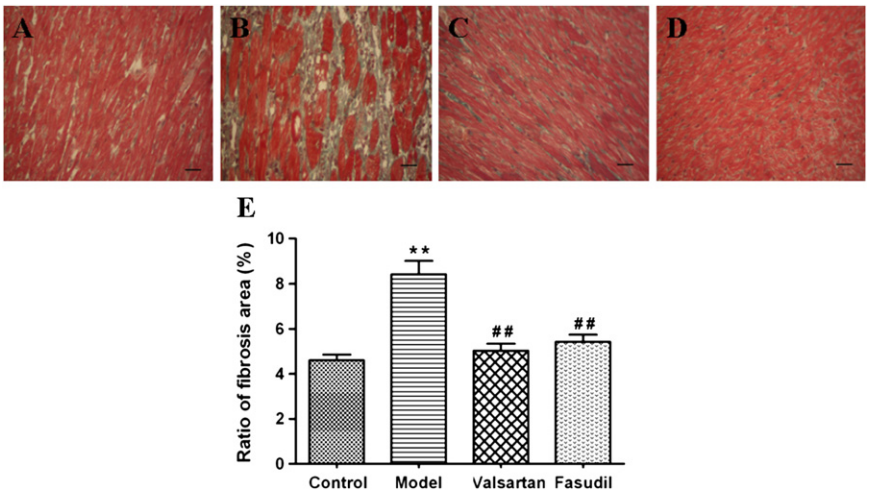


Figure 6 Masson's trichrome staining for heart in different groups (a bar indicates 25 μm). A, control group; B, model group; C, valsartan-treated group; D, fasudil-treated group; E, ratio of the total area of fibrosis to the total area of the image. Values are mean ± SEM, $n=3$. $^{**}P<0.01$ compared with the control group, $^{\#\#}P<0.01$ compared with the model group.

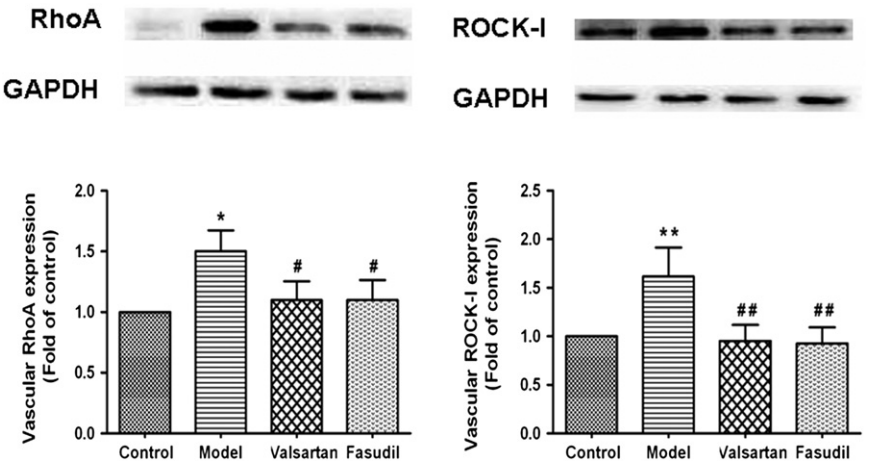


Figure 7 Expression of RhoA (left) and ROCK-I (right) in the aortas of L-NAME-treated hypertensive rats. Representative blots were obtained from Western blotting results. Each bar represents mean ± SEM of 4–6 tissue samples per group. $^{*}P<0.05$ and $^{**}P<0.01$ compared with the control group, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ compared with the model group.

significantly decreased in the aorta of NO-deficient rats compared to the control rats. Moreover, the vasoconstriction caused by KCl, Ang II and by NE in Ca^{2+}-free medium was also attenuated in the model group, though not significantly. Consequently, the vascular smooth muscle contractile function seemed globally reduced in response to exogenous vasoconstrictors.

Previously, Henrion and coworkers reported an improved contractile response to phenylephrine through treatment with angiotensin I-converting enzyme inhibitor (ACEI) quinapril in the aortic segments from L-NAME induced hypertensive rats. Here, we observed that a long-term administration of valsartan reversed the hyporeactivity of aorta to NE and Ang II in the NO-deficient rats. In contrast with the effect of valsartan on the vasoconstriction to NE, chronic fasudil treatment significantly improved the contractile response to NE in Ca^{2+}-free medium but showed limited benefits on sustained contraction to NE cumulatively added in K–H solution. Vascular smooth muscle may constrict physically with rapid and transient contraction in response to NE in Ca^{2+}-free medium, due to the intracellular Ca^{2+} release from sarcoplasmic reticulum triggered by inositol 1,4,5-triphosphate[14]. Previous observations indicate that the RhoA/ROCK pathway is implicated in the tonic phase of vasoconstriction with no evident role in the physic response[15]. The present findings thus further confirm the conclusion that inhibition of Rho-kinase interferes more with the force maintenance (tonic contraction) rather than the initial phase of force development (physic contraction).

Interestingly, we observed remarkably enhanced reactivity of the aorta to the protein kinase C (PKC) activator PDBu in chronic NOS inhibition rats compared to the control rats. The results from Bank and colleagues' research on the mechanism of vasoconstriction induced by long-term inhibition of NOS in rats indicate that the decreased production of NO resets the intrinsic vascular smooth muscle tone, which may be mediated by an increase in intracellular Ca^{2+} concentration or sensitivity[16]. Further study shows that NOS inhibition activates the L- and T-type Ca^{2+} channels in arterioles[17]. Additionally, the Ca^{2+} influx through T-type Ca^{2+} channels in arterial rings is reported to increase in the L-NAME induced hypertension[5].

Therefore, the aortic hyperactivity to PDBu in the model group might result from an excessive activation of Ca^{2+} channels. Both drug treatments decreased this hyperactivity, with a more pronounced reduction obtained in the fasudil-treated group, which may be ascribed to the important regulatory role of Rho-kinase in T-type Ca^{2+} channels[18].

Endothelium-dependent relaxative response to Ach was markedly blunted in the aorta of L-NAME induced hypertensive rats compared to the control ones in the present study and other similar experiment[4,5]. This endothelial dysfunction might result from a decreased production of NO and an increased release of vasoconstrictors in endothelial cells due to long-term inhibition of NOS[19]. In this work, the endothelium-dependent vasorelaxation was significantly augmented in the rats treated with fasudil, evidenced by a dramatically increased response to Ach in the fasudil-treated group in comparison to that of the model group. It has been reported that Ach fails to dilate the preconstricted aorta in quinapril-treated NO-deficient rats, implying that the antihypertensive drug such as ACEI may not enhance the endothelium-dependent vasorelaxation[4]. Similarly, we found that the cumulative concentration–response to Ach was slightly improved by valsartan therapy. These results suggested that the beneficial effect of Rho-kinase inhibition on endothelial function might be better than that of AT_1 receptor blockade. As the endothelium-intact aorta was studied in this experiment, the endothelium-denuded aorta should be used in further experiments in order to enhance the direct understanding of differential reactions between fasudil and valsartan on endothelial cells.

Western blotting results from RhoA/ROCK pathway in aorta gave more favorable substantiation to the alteration of vascular function and the beneficial effects of both valsartan and fasudil in the NO-deficiency rats. RhoA, one of the small GTP binding proteins, is known to govern a wide range of cellular functions through its downstream effector ROCK. RhoA/ROCK signaling pathway has been implicated in the inhibition of myosin phosphatase, accumulation of phosphorylated myosin light chain as well as the regulation of Ca^{2+} sensitivity and formation of stress fibers in vascular smooth muscle cells[8,9]. Because of the key role of RhoA and its effector ROCK in the contraction and proliferation of smooth muscle cells and the permeability of endothelial cells, this molecular pathway is now extensively accepted as an important target in various cardiovascular diseases, especially hypertension[9]. Recent findings have revealed an increased contribution of the RhoA/ROCK pathway to the basal and pathological tone of vasculature in different experimental hypertension animals, including chronic L-NAME treated rats[20], spontaneously hypertensive rats (SHRs)[20,21] and Ang II-induced hypertensive rats[22]. Additionally, the activation of RhoA/ROCK pathway is referred as a common molecular switch in downstream signaling and a pivotal component of hypertension[23]. In the present study, the expression of RhoA in aorta was significantly increased in the L-NAME-induced hypertensive rats compared to that of the control group, consistent with results of other work[20]. Moreover, the level of ROCK expression in the vascular tissues of the model group was much higher than that of control group, which indicated that the RhoA/ROCK signaling pathway was dramatically enhanced due to long-term NOS inhibition. Previous reports document that valsartan diminishes the expression level of membranous RhoA[24] and reduces the RhoA activation in SHRs[25]. Our results showed that the expressions of both RhoA and ROCK in the aortas of NO-deficient rats were significantly decreased by valsartan administration compared to those of control rats, further supporting the point that the antihypertensive effect of AT_1 receptor blocker may be mediated through the deactivation of RhoA/ROCK pathway. The data of the present study for the first time revealed that fasudil significantly decreased the expressions of RhoA and ROCK-I in the aortas of chronic NO-deficiency hypertensive rats. Combining with the earlier findings that endothelium-dependent dilation was markedly enhanced by Rho-kinase inhibition[26], it may give rise to the speculation that the hypotensive effect of fasudil is attributed to the improvement of endothelial dysfunction through a reduction in the activated RhoA/ROCK signaling pathway in the vasculature of hypertensive animals.

Apart from the attenuated vascular reactivity, the cardiac hypertrophy in the model group was also observed in the present study. Cardiac hypertrophy represents a remodeling process of heart due to diverse pathological stimuli[27], and previous reports have demonstrated that persistent hypertension induced by chronic L-NAME treatment results in hypertrophied hearts in rats[5,28].

Consistent with the finding that Ang II receptor antagonism ameliorates cardiac hypertrophy[5], the present study showed that administration of valsartan significantly reduced the heart weight and heart weight–body weight ratio, and markedly decreased the cardiomyocyte diameter and myocardial fibrosis. However, administration of fasudil significantly increases the left ventricle weight and left ventricle weight–body weight ratio in the L-NMAE-treated SHRs[29]. Recently, studies on transgenic animal models illustrate that cardiac-specific overexpression of RhoA does not cause ventricular hypertrophy[30] and haploinsufficiency of the ROCK1 gene fails to prevent the development of cardiac hypertrophy in response to Ang II or L-NAME[31], which suggests that the RhoA/ROCK pathway is not responsible for the development of cardiac hypertrophy. The results from the present study showed that fasudil therapy failed to reverse the increased heart index and the enlarged cardiomyocyte diameter, whereas fasudil treatment significantly decreased the area of myocardial fibrosis.

Accumulating evidence suggests that Rho/Rho-kinase pathway is substantially involved in the pathogenesis of various cardiovascular diseases[8,9]. Rho-kinase inhibitors are currently being developed as the next generation of therapeutic agents, with high potency and low side effect on heart rate, for certain cardiovascular pathological conditions, especially hypertension[32,33]. Fasudil, a selective Rho-kinase inhibitor, has been widely used for prevention and treatment of cerebral vasospasm after subarachnoid hemorrhage and pulmonary artery hypertension since a decade ago[8]. Our data demonstrate that fasudil shows antihypertensive effect on the long-term NO-deficient rats and beneficial effect on the endothelium-dependent vasorelaxation, whereas chronic fasudil treatment leads to a marked elevation in the heart rate. Therefore, fasudil therapy for the patients with compromised cardiac function should be pursued with caution at the clinical situation due to this unwanted adverse event.

In conclusion, these results suggest that chronic NOS inhibition not only induces high blood pressure and cardiac hypertrophy, but also impairs the vascular function. Both Ang II receptor antagonism and Rho-kinase inhibition exhibit an antihypertensive effect, while only Ang II receptor antagonist shows antihypertrophic potential on cardiomyocytes. The AT_1 receptor blocker improves the attenuated vasoconstriction, whereas the Rho-kinase inhibitor exerts greater beneficial effect on endothelium-dependent vasorelaxation than the

AT_1 receptor blocker. Our work provides the first evidence that both valsartan and fasudil decrease the augmented RhoA/ROCK signaling pathway in the vasculature of hypertensive rat, which may be one of the molecular mechanisms for their blood pressure-lowering activity.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (No. 30572182) and National Scientific & Technological Major Special Project- ''Significant Creation of New Drugs'' (Nos. 2009ZX09302-003 and 2009ZX09501-021). We are grateful for Dr. Xin Wang from the University of Manchester for revising this article, and we also would like to extend our thanks to Dr. Constantin Polychronakos from McGill University for editing our manuscript.

References

1. Versari D, Daghini E, Virdis A, Ghiadoni L, Taddei S. Endothelium-dependent contractions and endothelial dysfunction in human hypertension. *Br J Pharmacol* 2009;**157**:527–36.
2. Torok J. Participation of nitric oxide in different models of experimental hypertension. *Physiol Res* 2008;**57**:813–25.
3. Ribeiro MO, Antunes E, de Nucci G, Lovisolo SM, Zatz R. Chronic inhibition of nitric oxide synthesis. A new model of arterial hypertension. *Hypertension* 1992;**20**:298–303.
4. Henrion D, Dowell FJ, Levy BI, Michel JB. *In vitro* alteration of aortic vascular reactivity in hypertension induced by chronic N^G-nitro-*L*-arginine methyl ester. *Hypertension* 1996;**28**:361–6.
5. Kalliovalkama J, Jolma P, Tolvanen JP, Kähönen M, Hutri-Kähönen N, Wu X, et al. Arterial function in nitric oxide-deficient hypertension: influence of long-term angiotensin II receptor antagonism. *Cardiovasc Res* 1999;**42**:773–82.
6. De Gennaro CV, Fioretti S, Rigamonti A, Bonomo S, Manfredi B, Muller EE, et al. Angiotensin II type 1 receptor antagonism improves endothelial vasodilator function in *L*-NAME-induced hypertensive rats by a kinin-dependent mechanism. *J Hypertension* 2006;**24**:95–102.
7. Obst M, Gross V, Luft FC. Systemic hemodynamics in non-anesthetized *L*-NAME- and DOCA-salt-treated mice. *J Hypertension* 2004;**22**:1889–94.
8. Liao JK, Seto M, Noma K. Rho kinase (ROCK) inhibitors. *J Cardiovasc Pharmacol* 2007;**50**:17–24.
9. Lai A, Frishman WH. Rho-kinase inhibition in the therapy of cardiovascular disease. *Cardiol Rev* 2005;**13**:285–92.
10. Masumoto A, Hirooka Y, Shimokawa H, Hironaga K, Setoguchi S, Takeshita A. Possible involvement of Rho-kinase in the pathogenesis of hypertension in humans. *Hypertension* 2001;**38**:1307–10.
11. Moreau P, Takase H, Kung CF, Shaw S, Luscher TF. Blood pressure and vascular effects of endothelin blockade in chronic nitric oxide-deficient hypertension. *Hypertension* 1997;**29**:763–9.
12. Badejo Jr. AM, Dhaliwal JS, Casey DB, Gallen TB, Greco AJ, Kadowitz PJ. Analysis of pulmonary vasodilator responses to the Rho-kinase inhibitor fasudil in the anesthetized rat. *Am J Physiol Lung Cell Mol Physiol* 2008;**295**:828–36.
13. López RM, Ortíz CS, Ruíz A, Vélez JM, Castillo C, Castillo EF. Impairment of smooth muscle function of rat thoracic aorta in an endothelium-independent manner by long-term administration of N(G)-nitro-*L*-arginine methyl ester. *Fundam Clin Pharmacol* 2004;**18**:669–77.
14. Fang LH, Mu YM, Lin LL, Xiao PG, Du GH. Vasorelaxant effect of euxanthone in the rat thoracic aorta. *Vascul Pharmacol* 2006;**45**:96–101.
15. Swärd K, Mita M, Wilson DP, Deng JT, Susnjar M, Walsh MP. The role of RhoA and Rho-associated kinase in vascular smooth muscle contraction. *Curr Hypertens Rep* 2003;**5**:66–72.
16. Bank N, Aynedjian HS, Khan GA. Mechanism of vasoconstriction induced by chronic inhibition of nitric oxide in rats. *Hypertension* 1994;**24**:322–8.
17. Feng MG, Navar LG. Nitric oxide synthase inhibition activates L- and T-type Ca^{2+} channels in afferent and efferent arterioles. *Am J Physiol Renal Physiol* 2006;**290**:873–9.
18. Iftinca M, Hamid J, Chen L, Varela D, Tadayonnejad R, Altier C, et al. Regulation of T-type calcium channels by Rho-associated kinase. *Nat Neurosci* 2007;**10**:854–60.
19. Kung CF, Moreau P, Takase H, Luscher TF. *L*-NAME hypertension alters endothelial and smooth muscle function in rat aorta. Prevention by trandolapril and verapamil. *Hypertension* 1995;**26**:744–51.
20. Seasholtz TM, Zhang T, Morissette MR, Howes AL, Yang AH, Brown JH. Increased expression and activity of RhoA are associated with increased DNA synthesis and reduced p27(Kip1) expression in the vasculature of hypertensive rats. *Circ Res* 2001;**89**:488–95.
21. Mukai Y, Shimokawa H, Matoba T, Kandabashi T, Satoh S, Hiroki J, et al. Involvement of Rho-kinase in hypertensive vascular disease: a novel therapeutic target in hypertension. *FASEB J* 2001;**15**:1062–4.
22. Jin L, Ying Z, Hilgers RH, Yin J, Zhao X, Imig JD, et al. Increased RhoA/Rho-kinase signaling mediates spontaneous tone in aorta from angiotensin II-induced hypertensive rats. *J Pharmacol Exp Ther* 2006;**318**:288–95.
23. Seko T, Ito M, Kureishi Y, Okamoto R, Moriki N, Onishi K, et al. Activation of RhoA and inhibition of myosin phosphatase as important components in hypertension in vascular smooth muscle. *Circ Res* 2003;**92**:411–8.
24. Sagara Y, Hirooka Y, Nozoe M, Ito K, Kimura Y, Sunagawa K. Pressor response induced by central angiotensin II is mediated by activation of Rho/Rho-kinase pathway *via* AT1 receptors. *J Hypertension* 2007;**25**:399–406.
25. Moriki N, Ito M, Seko T, Kureishi Y, Okamoto R, Nakakuki T, et al. RhoA activation in vascular smooth muscle cells from stroke-prone spontaneously hypertensive rats. *Hypertens Res* 2004;**27**:263–70.
26. Chitaley K, Webb RC. Nitric oxide induces dilation of rat aorta *via* inhibition of Rho-kinase signaling. *Hypertension* 2002;**39**:438–42.
27. Huo R, Chen C, Chen Y, Li Z, Hou YL, Dong DL. 5-HT_3 receptor antagonists protect against pressure overload-induced cardiac hypertrophy in murine. *Acta Pharm Sin B* 2012;**2**:16–22.
28. Morton JJ, Beattie EC, Speirs A, Gulliver F. Persistent hypertension following inhibition of nitric oxide formation in the young Wistar rat: role of renin and vascular hypertrophy. *J Hypertens* 1993;**11**:1083–8.
29. Koshikawa S, Nishikimi T, Inaba C, Akimoto K, Matsuoka H. Fasudil. a Rho-kinase inhibitor, reverses *L*-NAME exacerbated severe nephrosclerosis in spontaneously hypertensive rats. *J Hypertens* 2008;**26**:1837–48.
30. Sah VP, Minamisawa S, Tam SP, Wu TH, Dorn GW 2nd, Ross Jr. J, et al. Cardiac-specific overexpression of RhoA results in sinus and atrioventricular nodal dysfunction and contractile failure. *J Clin Invest* 1999;**103**:1627–34.
31. Rikitake Y, Oyama N, Wang CC, Noma K, Satoh M, Kim H, et al. Decreased perivascular fibrosis but not cardiac hypertrophy in $ROCK1^{+/-}$ haploinsufficient mice. *Circulation* 2005;**112**:2959–65.
32. Kast R, Schirok H, Figueroa-Pérez S, Mittendorf J, Gnoth MJ, Apeler H, et al. Cardiovascular effects of a novel potent and highly selective azaindole-based inhibitor of Rho-kinase. *Br J Pharmacol* 2007;**152**:1070–80.
33. Löhn M, Plettenburg O, Ivashchenko Y, Kannt A, Hofmeister A, Kadereit D, et al. Pharmacological characterization of SAR407899, a novel rho-kinase inhibitor. *Hypertension* 2009;**54**:676–83.