*Original Article*

# Possible Involvement of Rho-Kinase in Aldosterone-Induced Vascular Smooth Muscle Cell Remodeling

Kayoko MIYATA[1)], Hirofumi HITOMI[2),3)], Peng GUO[2)], Guo-Xing ZHANG[4)], Shoji KIMURA[4)], Hideyasu KIYOMOTO[2),3)], Naohisa HOSOMI[2)], Shoji KAGAMI[5)], Masakazu KOHNO[2),3)], and Akira NISHIYAMA[3),4)]

**There is increasing evidence supporting potential roles of aldosterone in the pathogenesis of vascular injury. The present study aimed to determine the involvement of Rho-kinase in aldosterone-induced vascular smooth muscle cell (VSMC) remodeling. In cultured rat VSMC, the effects of aldosterone on Rho-kinase activity, the reorganization of the cytoskeleton and cellular migration were examined. Aldosterone (1 nmol/L) significantly increased phosphorylation of myosin phosphate target subunit-1 (MYPT1), a marker of Rho-kinase activity, and the amount of GTP-Rho with a peak at 90 min in VSMC. Aldosterone also stimulated VSMC stress fiber formation and migration. These effects of aldosterone were markedly attenuated by pretreatment with eplerenone (10 μmol/L), a selective mineralocorticoid receptor antagonist, or Y27632 (10 μmol/L), a specific Rho-kinase inhibitor. These findings indicate that Rho-kinase is involved in the pathogenesis of aldosterone-induced VSMC remodeling. (*Hypertens Res* 2008; 31: 1407–1413)**

***Key Words*: aldosterone, Rho-kinase, mineralocorticoid receptor, vascular smooth muscle cells, eplerenone**

## Introduction

A growing body of clinical and pre-clinical evidence suggests that aldosterone plays an important role in the pathogenesis of hypertension and cardiovascular disease. Patients with primary aldosteronism have a higher incidence of cardiovascular complications than do those with essential hypertension (*1*). Furthermore, the Randomized Aldactone Evaluation Study (RALES) (*2*) and the Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficiency and Survival Study (EPHESUS) (*3*) demonstrated that adding mineralocorticoid receptor (MR) antagonists to standard therapies, including angiotensin converting enzyme inhibitors, loop diuretics and digoxin, significantly reduces morbidity and mortality in patients with heart failure. In recent years, attention has also focused on aldosterone-dependent vascular remodeling (*4–11*). Patients with primary aldosteronism showed a greater relationship between the media-to-lumen ratio and relative wall thickness than those with essential hypertension (*6*). Vascular wall hypertrophy (*7*) and stiffness (*8*) were also observed in rats chronically treated with aldosterone and salt. In salt-loaded stroke-prone spontaneously hypertensive rats, treatment with MR antagonists attenuated remodeling of the mesenteric (*9*), brain (*10*) and renal (*10*) arteries. Ward *et al.* (*11*) showed that eplerenone, a selective MR antagonist,

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From the [1)]Life Sciences Research Center, [2)]Department of Cardiorenal and Cerebrovascular Internal Medicine, [3)]Hypertension and Kidney Disease Research Center, and [4)]Department of Pharmacology, Kagawa University Medical School, Kagawa, Japan; and [5)]Department of Pediatrics, The University of Tokushima School of Medicine, Tokushima, Japan.

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Address for Reprints: Akira Nishiyama, M.D., Ph.D., Department of Pharmacology, Kagawa University Medical School, 1750–1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761–0793, Japan. E-mail: akira@kms.ac.jp

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markedly attenuates coronary arterial remodeling after angioplasty in pigs. Collectively, the reported data indicate the potential contribution of aldosterone to the progression of vascular remodeling. However, the precise mechanisms responsible for aldosterone-induced vascular remodeling have remained undetermined.

Rho-kinase, an effector of the small G protein Rho, is activated by several stimuli and mediates various cellular functions including smooth muscle contraction, actin cytoskeleton organization and migration (*12–14*), all of which might be involved in the pathogenesis of vascular remodeling. It has been demonstrated that treatment with Rho-kinase inhibitors attenuates the progression of vascular remodeling under several pathological conditions, independent of blood pressure changes (*15–17*). Recent studies have also shown that in aldosterone-induced hypertensive rats, increased collagen gene expression of left ventricular tissues (*18*) and renal fibrosis (*19*) were associated with activation of Rho-kinase. Furthermore, treatment with specific Rho-kinase inhibitors attenuated aldosterone-induced tissue injury without changing blood pressure, suggesting the possible participation of the aldosterone/Rho-kinase–dependent pathway in cardiovascular injury (*18*, *19*). However, to the best of our knowledge, the contribution of Rho-kinase to aldosterone-induced vascular remodeling has not been convincingly demonstrated.

The present study was thus designed to define the involvement of Rho-kinase in the pathogenesis of aldosterone-induced vascular smooth muscle cell (VSMC) remodeling. Hence, we examined the effects of aldosterone on Rho-kinase activity in cultured VSMC. Since rearrangements of the VSMC cytoskeleton and associated cell migration are potential key events that contribute to the progression of vascular remodeling (*20*), we also investigated whether aldosterone stimulates VSMC stress fiber formation and migration through the activation of Rho-kinase.

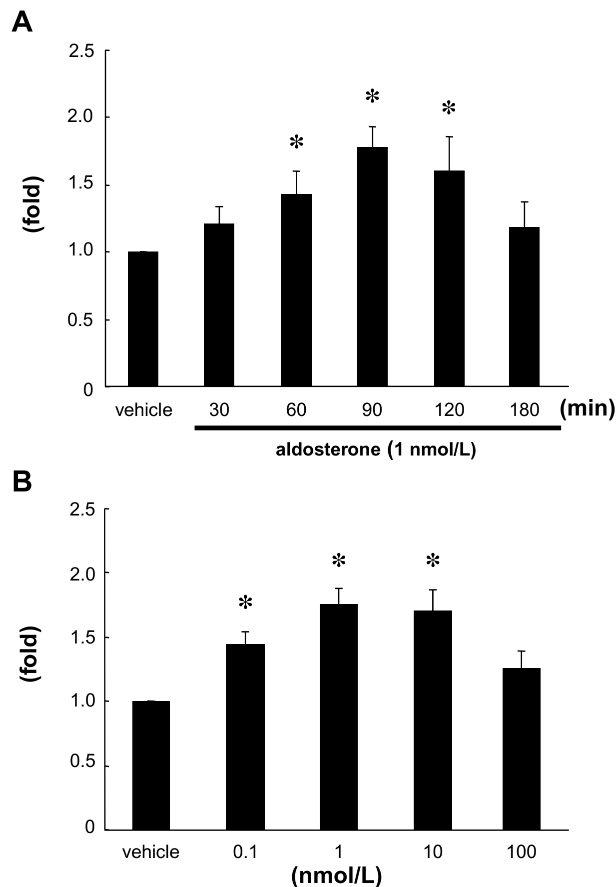


**Fig. 1.** *A: Time course of aldosterone-stimulated myosin phosphate target subunit-1 (MYPT1) phosphorylation in vascular smooth muscle cells (VSMC). VSMC were stimulated with 1 nmol/L aldosterone for the indicated times (30–180 min). B: Aldosterone stimulates VSMC MYPT1 phosphorylation in a concentration-dependent manner. VSMC were stimulated with the indicated concentrations of aldosterone (0.1–100 nmol/L) for 90 min.* **$p < 0.05$ vs. vehicle (0.01% ethanol).*

## Methods

### Cell Culture

VSMC were isolated from male Sprague-Dawley rats and maintained according to published methods (*21*, *22*). Control solutions always contained the appropriate amount of vehicle: ethanol for aldosterone (Across Organics, Geel, Belgium) or DMSO for eplerenone (Pfizer Inc., New York, USA) and Y27632 (Calbiochem, La Jolla, USA) (less than 1:1,000 for each).

### Measurement of Rho-Kinase Activity

Since Rho-kinase inhibits myosin phosphatase by phosphorylating its myosin-binding subunit, myosin phosphate target subunit-1 (MYPT1) (*13*, *14*), phosphorylated levels of MYPT1 were used as a marker of Rho-kinase activity (*13*, *14*, *17*, *19*). To evaluate the phosphorylated levels of MYPT1 in VSMC, we used two different approaches: ELISA and immunoblotting with phospho-specific antibody against phospho-MYPT1. In brief, phosphorylated levels of MYPT1 were determined using an ELISA kit with a phospho-specific monoclonal antibody against phospho-MYPT1 at Thr-696 (Cyclex, Nagano, Japan) (*23*). Immunoblotting was also performed with an antibody against phospho-MYPT1 (at Thr-696; Upstate Biochemistry, Lake Placid, USA) (*19*). To check for equal loading, membranes were re-probed with an antibody against β-actin (Sigma Chemical Co., St. Louis, USA). Data are expressed as the relative differences after normalization to β-actin protein expression.

We also measured Rho activity by pull-down experiments using a GST-Rhotekin fusion protein bound to glutathione-agarose beads (Cytoskeleton, Denver, USA), as previously described (*24*). VSMC were washed with PBS, lysed and

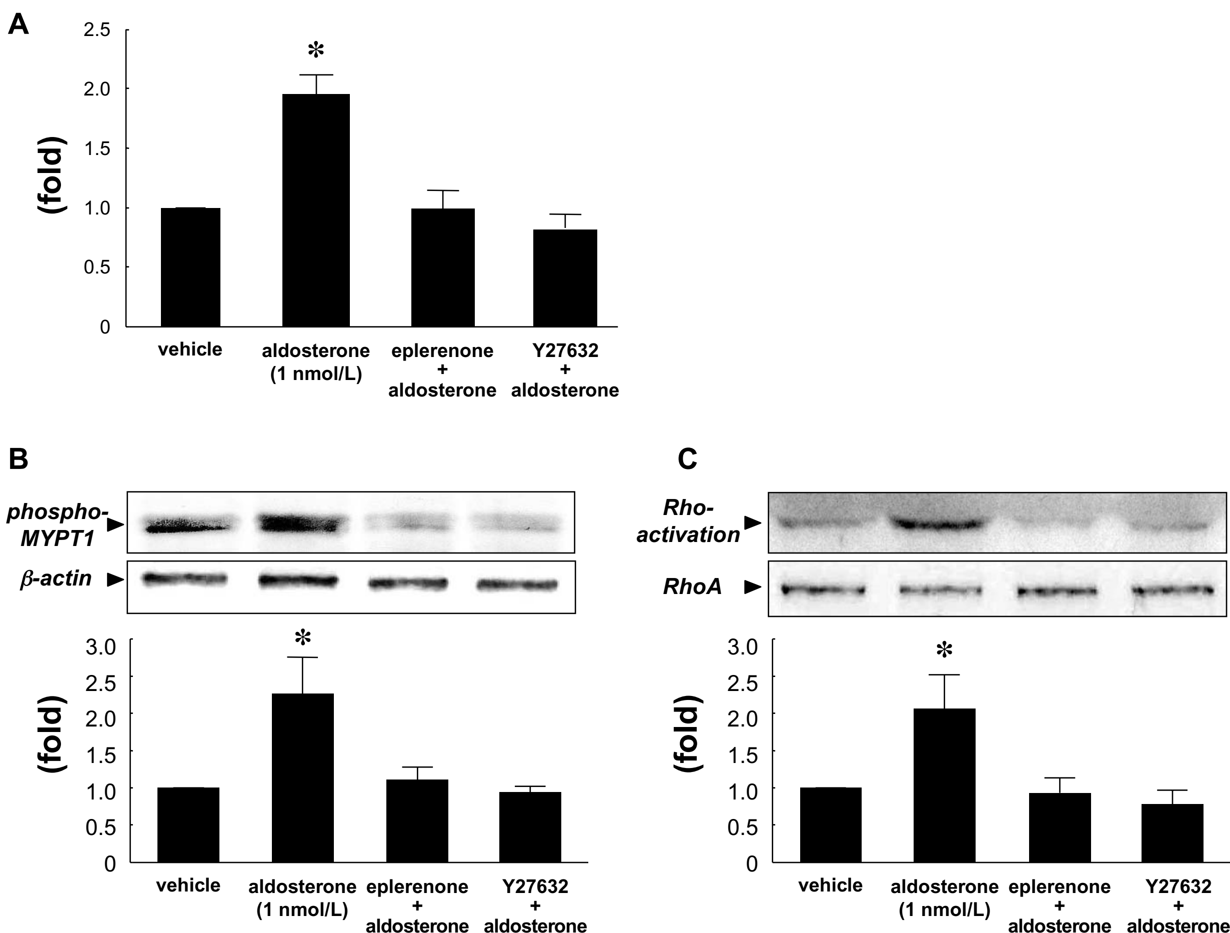


**Fig. 2.** *Effects of eplerenone and Y27632 on aldosterone-induced myosin phosphate target subunit-1 (MYPT1) phosphorylation and Rho activation in vascular smooth muscle cells (VSMC). To evaluate phosphorylated levels of MYPT1 in VSMC, we used two different approaches: ELISA (A) and immunoblotting (B) with an antibody against phospho-MYPT1, as described in the Methods. Rho activity was also measured by a pull-down assay using the GST-Rhotekin fusion protein bound to glutathione-agarose beads (C). Pre-incubation with eplerenone (10 μmol/L) or Y27632 (10 μmol/L) attenuated aldosterone-induced MYPT1 phosphorylation and Rho activation. *$p < 0.05$ vs. vehicle.*

incubated with Rhotekin-RBD beads and Rho was detected by Western blotting using monoclonal Rho specific antibody. To determine equal protein loading, 50 μg of total cell lysates were loaded in each lane and protein levels of RhoA were evaluated by Western blot (*24*). Data are expressed as the relative differences after normalization to RhoA protein expression.

## Fluorescence Microscopy of VSMC Actin Filaments

VSMC were fixed with 4.0% formaldehyde in phosphate buffered saline (PBS) and treated with PBS containing 0.4% Triton-X. After washing with PBS, filamentous-actin (F-actin) filaments of VSMC were stained with Alexa-Fluor488-phalloidin (Molecular Probes, Eugene, USA), as previously described (*25*). Stained F-actin was imaged using a laser scanning confocal microscope system (Bio-Rad Laboratories, Hercules, USA) in 7–9 different experiments.

## VSMC Migration Assay

Migration assay was performed using 24-well Transwell chambers with an 8.0 μm pore polycarbonate membrane (Corning Inc., Acton, USA) according to a previously described assay (*22*) with modifications. In brief, the underside of the membrane was coated with rat type I collagen (50 μg/mL; Sigma Chemical Co.). Suspended growth-arrested VSMC in medium were added to the upper chamber at $3 \times 10^5$ cells/well. Medium containing 0.1% bovine serum albumin and platelet-derived growth factor (10 ng/mL; Sigma Chemical Co.) was added to the lower chamber. After removing nonmigrating VSMC from the top of the membrane by gentle scrapping, the membrane was fixed with ethanol and stained

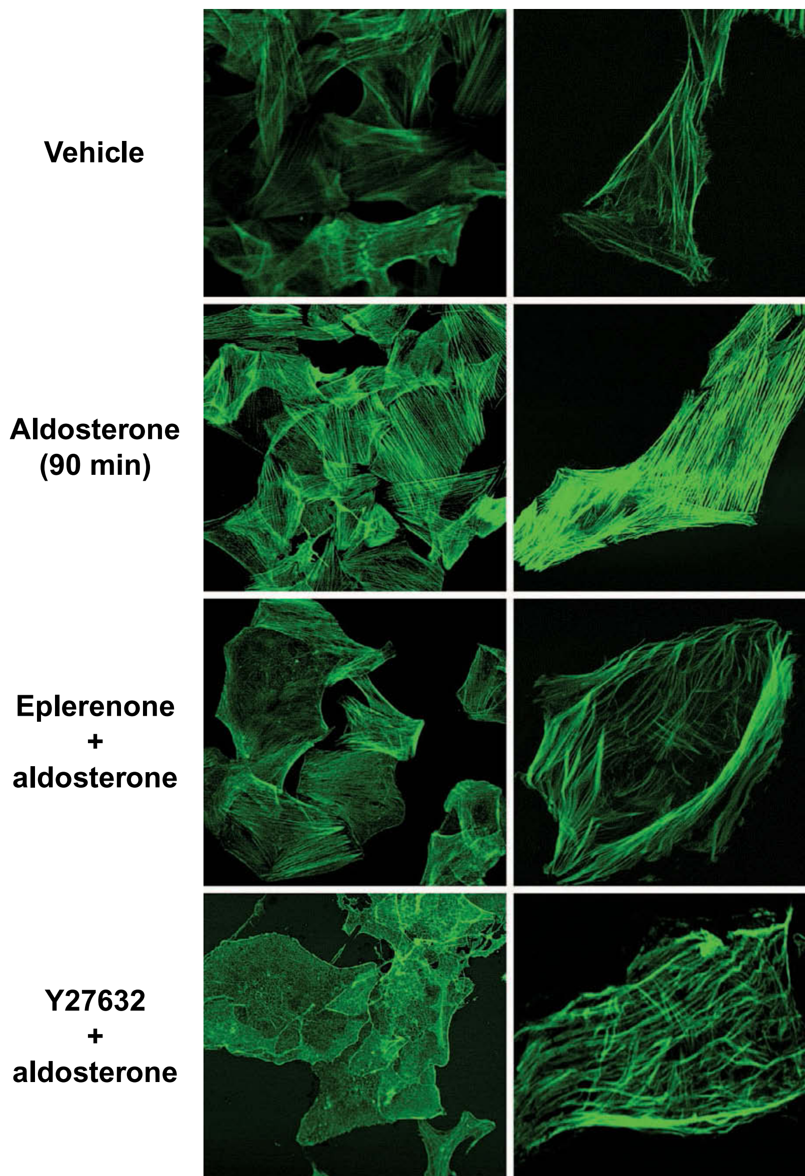


**Fig. 3.** *Effect of aldosterone on the reorganization of the vascular smooth muscle cells (VSMC) actin cytoskeleton. Filamentous-actin (F-actin) filaments were stained with Alexa-Fluor488-phalloidin as described in the Methods. Aldosterone treatment for 90 min led to cytoskeletal reorganization with increased actin stress fiber formation in VSMC. Aldosterone-induced stress fiber formation was prevented by preincubation with eplerenone or Y27632 in VSMC. Representative images from one of 7–9 independent experiments are shown. Original magnification, ×800 (left) and ×1,600 (right).*

with Diff-Quick (Kokusai-Shiyaku Co., Kobe, Japan). The number of cells migrating to the lower surface was evaluated by microscopy by counting the number of stained nuclei. All assays were performed in triplicate, and each sample was counted randomly in 10 different areas in the center of the membrane.

### Statistical Analysis

Values are presented as the means±SEM. Statistical comparisons of differences were performed using one- or two-way analysis of variance combined with Newman-Keuls post hoc test. $p<0.05$ was considered statistically significant.

## Results

### Effects of Aldosterone on MYPT1 Phosphorylation in VSMC

Figure 1A shows the time course of aldosterone-stimulated

MYPT1 phosphorylation measured by an ELISA ($n$=7–9 for each). Aldosterone (1 nmol/L)-induced phosphorylation of MYPT1 peaked at 90 min (1.76±0.13-fold). Figure 1B shows the concentration-dependent effects of aldosterone treatment (90 min) on MYPT1 phosphorylation ($n$=7–9 for each). Aldosterone-stimulated MYPT1 phosphorylation was maximal at 1 nmol/L (1.76±0.10-fold).

### Effects of an MR Antagonist and Rho-Kinase Inhibitor on Aldosterone-Induced MYPT1 Phosphorylation and Rho Activation in VSMC

To investigate the role of MR and Rho-kinase in aldosterone-induced MYPT1 phosphorylation, the effects of eplerenone, a selective MR antagonist (*26*), and Y27632, a specific Rho-kinase inhibitor (*27*), on MYPT1 phosphorylation were examined by an ELISA ($n$=7–11 for each). VSMC were pretreated with eplerenone (10 μmol/L) or Y27632 (10 μmol/L) for 60 and 30 min, respectively, prior to stimulation with aldosterone (1 nmol/L). As shown in Fig. 2A, aldosterone-induced MYPT1 phosphorylation was markedly attenuated by pretreatment with eplerenone in VSMC. Furthermore, aldosterone-induced MYPT1 phosphorylation was prevented by pretreatment with Y27632, suggesting that MYPT1 phosphorylation reflects Rho-kinase activity. We also performed immunoblotting with a specific antibody against phospho-MYPT1 in VSMC ($n$=7–9 for each). Similar to the data obtained in the ELISA experiments (Fig. 2A), immunoblotting analysis showed that the aldosterone-induced MYPT1 phosphorylation was markedly attenuated by pretreatment with eplerenone or Y27632 in VSMC. On the other hand, no significant differences in the amounts of β-actin were observed among the samples (Fig. 2B).

Similar to the changes in MYPT1 phosphorylation, aldosterone (1 nmol/L) significantly increased GTP-bound Rho levels, as shown by the pull-down assays (*24*). Furthermore, aldosterone-induced increases in GTP-bound Rho levels were diminished by pretreatment with eplerenone or Y27632 in VSMC (Fig. 2C; $n$=5–7 for each).

### Effects of Aldosterone on Actin Reorganization and Migration in VSMC

To examine the effects of aldosterone on the reorganization of the VSMC actin cytoskeleton, F-actin filaments were stained with Alexa-Fluor488-phalloidin (*25*) ($n$=7–9 for each). As shown in Fig. 3, exposure of VSMC to 1 nmol/L aldosterone for 90 min led to cytoskeletal reorganization with increased actin stress fiber formation. Aldosterone-stimulated stress fiber formation was prevented by preincubation with eplerenone (10 μmol/L for 60 min) or Y27632 (10 μmol/L for 30 min) in VSMC.

We also examined the effects of aldosterone on VSMC migration ($n$=9–11 for each). As shown in Fig. 4, aldosterone (1 nmo/L) treatment for 5 h increased the number of migrating VSMC by approximately 2-fold. We previously demonstrated that proliferation of cultured rat VSMC in response to aldosterone did not start before 24 h (*21*). Pretreatment with eplerenone (10 μmol/L for 60 min) or Y27632 (10 μmol/L for 30 min) markedly attenuated the aldosterone-induced increases in migrating VSMC (Fig. 4).

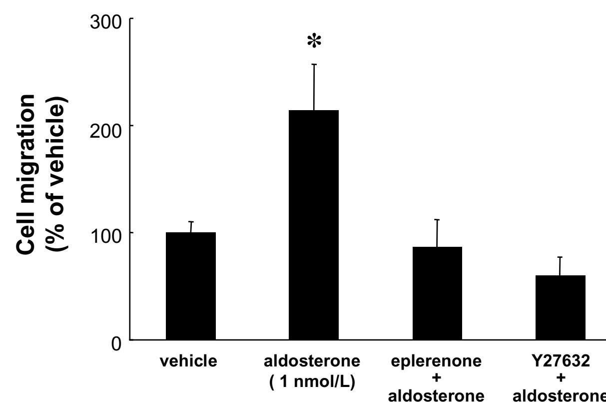


**Fig. 4.** *Effect of aldosterone on vascular smooth muscle cells (VSMC) migration. Aldosterone treatment for 5 h significantly increased the number of migrating VSMC. Pretreatment with eplerenone or Y27632 markedly attenuated aldosterone-induced VSMC migration. *$p < 0.05$ vs. vehicle.*

## Discussion

Vascular remodeling is an independent marker of cardiovascular risk (*28*). The objective of the present study was to investigate the role of Rho-kinase in aldosterone-dependent VSMC remodeling. In this study, we have provided evidence that aldosterone activates an Rho-kinase–dependent pathway and stimulates stress fiber formation and migration in cultured VSMC. In addition, aldosterone-induced Rho-kinase activation as well as stress fiber formation and migration were markedly attenuated by treatment with an MR antagonist or a Rho-kinase inhibitor. These data suggest the potential involvement of Rho-kinase in the pathogenesis of aldosterone-induced VSMC remodeling.

In VSMC, the cytoskeleton is a filamentous network consisting largely of F-actin, which provides a scaffold to alter the mechanical properties of cells (*20*). It has also been documented that the reorganization of the VSMC cytoskeleton is an early event associated with vascular remodeling (*20*). Rho-kinase, a key mediator of the Rho signaling pathway, is known to regulate the reorganization of the actin cytoskeleton in a variety of cell types, including VSMC (*12*, *13*). Mack *et al.* (*29*) demonstrated that the Rho-kinase–dependent pathway regulates actin stress fiber formation. Our results provide evidence that aldosterone stimulates both Rho-kinase activity and F-actin stress fiber formation in VSMC. Further, treatment with Y-27632, a specific inhibitor of Rho-kinase (*27*),

markedly attenuated the aldosterone-induced stress fiber formation. Collectively, these data indicate that aldosterone reorganizes the VSMC cytoskeleton by activating Rho-kinase, which may play a role in aldosterone-induced VSMC remodeling. We previously demonstrated that MR is abundantly expressed in cultured rat VSMC and mediates cellular responses to aldosterone (*21*). Since aldosterone-induced VSMC Rho-kinase activation and stress fiber formation were attenuated by pretreatment with eplerenone, these effects of aldosterone may be mediated through activation of MR.

The assembly and reorganization of the actin cytoskeleton cause a change in the shape of VSMC and lead to cell migration (*20*). It has also been well recognized that VSMC migration contributes to the pathogenesis of vascular remodeling (*20*, *30*). Our results provide evidence that aldosterone stimulates VSMC migration. We also found that aldosterone-induced VSMC migration was associated with an increase in Rho-kinase activity. Although conflicting results have been reported with regard to the role of Rho-kinase in cell migration (*13*), many reports have demonstrated that Rho-kinase pathway activation increases VSMC migration (*13*, *31–36*). For example, Y27632 has been shown to attenuate the migration of VSMC induced by platelet-derived growth factor (*31*, *32*), urokinase plasminogen activator (*33*), thrombin (*33*, *34*), serum (*35*) or 5-hydroxytriptamine (*36*). In the present study, we observed that Y27632 prevented VSMC Rho-kinase activation and migration induced by aldosterone. These data suggest that an Rho-kinase–dependent pathway plays a role in aldosterone-induced VSMC migration, which may contribute to the pathogenesis of vascular remodeling, at least in part.

In the present study, aldosterone increased Rho-kinase activity, which peaked at 1 nmol/L in cultured VSMC. This aldosterone concentration would be a little higher than normal rat plasma levels. However, several studies have reported that rat plasma aldosterone levels increased up to nanomolar ranges under some pathological conditions (*37*, *38*). Since aldosterone can bind to not only MR but also glucocorticoid receptors (*39*), we cannot rule out the possibility that some of the effects of aldosterone observed in the present study might have been mediated *via* activation of glucocorticoid receptors. However, the present results show that the highly selective MR antagonist, eplerenone (*26*), significantly attenuated aldosterone-induced Rho-kinase activation, stress fiber formation and cell migration. Thus, it seems likely that MR played a predominant role in these effects of aldosterone. Nevertheless, it remains undetermined whether these effects of aldosterone are dependent or independent of transcription and translation. Further studies are needed to address these issues.

In conclusion, the present study indicates that Rho-kinase is involved in aldosterone-induced VSMC remodeling. We will perform further experiments to determine the precise molecular mechanisms by which aldosterone stimulates VSMC stress fiber formation *via* Rho-kinase–dependent pathways. In addition, a future *in vivo* study will be performed to investigate the role of Rho-kinase in aldosterone-dependent vascular injury.

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