Thiazide-Like Diuretics Attenuate Agonist-Induced Vasoconstriction by Calcium Desensitization Linked to Rho Kinase

Zhiming Zhu, Shanjun Zhu, Daoyan Liu, Tingbing Cao, Lijuan Wang, Martin Tepel

Abstract—Lowering blood pressure using thiazide-like diuretics, including chlorthalidone and hydrochlorothiazide, has been proven to be effective in clinical studies. However, the mechanisms by which thiazide-like diuretics lower blood pressure are still poorly understood. To evaluate whether thiazide-like diuretics cause calcium desensitization in smooth muscle cells, we measured their effects on agonist-induced increase of blood pressure in Wistar rats in vivo and on agonist-induced vasoconstriction of aortic rings, DNA synthesis, and protein synthesis, RhoA, Rho kinase, and intracellular calcium in vascular smooth muscle cells in vitro. Thiazide-like diuretics significantly attenuated angiotensin II–induced or norepinephrine-induced increase of systolic blood pressure in rats. Thiazide-like diuretics inhibited agonist-induced vasoconstriction of aortic rings in a concentration-dependent manner in the presence and absence of endothelium. The inhibitory effects of thiazide-like diuretics were similar to that of the specific Rho kinase inhibitor Y27632. RT-PCR and immunoblotting showed that RhoA and Rho kinase were significantly reduced in vascular smooth muscle cells after administration of thiazide-like diuretics. In contrast, thiazide-like diuretics did not affect protein tyrosine phosphatase-2 (SHP-2) expression. Agonist-induced changes of intracellular calcium were not affected by thiazide-like diuretics. The study indicates that thiazide-like diuretics inhibit agonist-induced vasoconstriction by calcium desensitization in smooth muscle cells linked to the Rho–Rho kinase pathway. (Hypertension. 2005; 45:233-239.)

Key Words: diuretics ■ kinase ■ vasoconstriction

Thiazide-like diuretics have been the cornerstone in hypertension management for several years. Recent data from the Anti-hypertensive and Lipid-Lowering to prevent Heart Attack Trial (ALLHAT) showed that blood pressure lowering using thiazide-like diuretics had beneficial effects, including fewer events for all cardiovascular diseases, stroke, and heart failure compared with an (ALLHAT) showed that blood pressure lowering using thiazide-like diuretics had beneficial effects, including fewer events for all cardiovascular diseases, stroke, and heart failure compared with an angiotensin-converting enzyme inhibitor.1 Thiazide-like diuretics, including chlorthalidone and hydrochlorothiazide, lower blood pressure by decreasing peripheral resistance rather than by their diuretic effect.2 Although it has been suggested that thiazide-induced vasoconstriction is mediated by opening of calcium-activated potassium channels or by inhibition of carbonic anhydrase,3–5 the precise mechanisms by which thiazide-like diuretics inhibit vasoconstriction and vascular growth are still unclear.

Rho is a member of the Ras family of small GTP-binding proteins and cycles between a GDP-bound inactive state and a GTP-bound active state. Rho is involved in regulation of actin/myosin-dependent contractility in smooth muscle cells.6 Smooth muscle myosin ATPases are activated after phosphorylation of regulatory myosin light chains by a calcium–calmodulin-dependent myosin light chain kinase, and they are inactivated after dephosphorylation by the calcium-dependent myosin light chain phosphatase. Activation of Rho and its effector Rho kinase inhibits the myosin light chain phosphatase by phosphorylation of the myosin phosphatase–targeting subunit, thereby decreasing activity of myosin phosphatase. Inhibition of the myosin light chain phosphatase causes calcium sensitization (ie, increasing the contraction of vascular smooth muscle cells in the absence of an increase of intracellular calcium concentration).7 Hence, long-term inhibition of the Rho–Rho kinase pathway may cause vasodilation and reduce blood pressure because it can be observed for thiazide-like diuretics.

The aim of the present study was to investigate whether thiazide-like diuretics cause calcium desensitization in smooth muscle cells. The study shows that thiazide-like diuretics inhibit agonist-induced vasoconstriction by calcium desensitization after affecting the Rho–Rho kinase pathway.

Methods

Hemodynamic Measurements

All experiments using male Wistar rats were performed as approved by the animal care and use committee. Rats were randomly administered chlorthalidone hydrochlorothiazide, Rho kinase inhibitor.
Hemodynamic measurements were done according to Symons et al.\textsuperscript{9} Vasoconstriction of aortic rings was measured as described previously.\textsuperscript{11,12} Vascular smooth muscle cells were obtained from thoracic aortas and cultured as described.\textsuperscript{13,14} DNA synthesis and protein synthesis were measured according to Touyz et al.\textsuperscript{15} Cytosolic calcium concentrations were measured using the fluorescent dye technique.\textsuperscript{16}

**RT-PCR and Immunoblotting of RhoA and Rho Kinase**

Expression of RhoA and Rho kinase mRNA was assessed by RT-PCR according to Hyvelin et al.\textsuperscript{17} Immunoblotting was performed using specific antibodies by standard procedures.\textsuperscript{16} Immunoblots of protein tyrosine phosphatase SHP-2 were performed as described by Wakino et al.\textsuperscript{18}

**Statistics**

All values reported are mean±SD. Comparisons between groups were analyzed using 1-way ANOVA with Bonferroni post hoc test. Two-sided P values <0.05 were considered to indicate statistical significance.

An expanded Materials and Methods section is available online at http://www.hypertensionaha.org.

**Results**

**Thiazide-Like Diuretics Attenuate Agonist-Induced Blood Pressure Increase in Rats**

Effects of thiazide-like diuretics on agonist-induced blood pressure increase were measured in Wistar rats that had been given placebo for control, chlorthalidone, or hydrochlorothiazide for 1 week. Under control conditions, angiotensin II administration significantly increased systolic blood pressure from 111±10 mm Hg to 137±20 mm Hg, whereas norepinephrine administration significantly increased systolic blood pressure from 111±10 mm Hg to 134±12 mm Hg (each n=5; P<0.05 by ANOVA). As shown in Figure 1, the angiotensin II–induced increase of systolic blood pressure was significantly attenuated from 26±17 mm Hg in the control group to 6±7 mm Hg in the chlorthalidone group or to 2±1 mm Hg in the hydrochlorothiazide group (each n=5; P<0.05 by ANOVA). The norepinephrine-induced increase of systolic blood pressure was also significantly attenuated from 24±8 mm Hg in the control group to 2±3 mm Hg in the chlorthalidone group or to 3±10 mm Hg in the hydrochlorothiazide group (each n=5; P<0.05 by ANOVA). In rats given the specific Rho kinase inhibitor Y27632 for 1 week, the angiotensin II– or norepinephrine-induced increase of systolic blood pressure was significantly attenuated to 2±7 mm Hg or 1±9 mm Hg, respectively (each n=5; P<0.05 by ANOVA). These data indicate that thiazide-like diuretics attenuate agonist-induced increase of blood pressure in vivo. The inhibitory effects of thiazide-like diuretics were similar to those observed after inhibition of the Rho–Rho kinase pathway.

Figure 2 shows the concentration-dependent reduction of angiotensin II–induced vasoconstriction by thiazide-like diuretics. In the presence of endothelium, incubation of segments from rat aorta with 1 µmol/L chlorthalidone or 1 µmol/L hydrochlorothiazide significantly reduced the angiotensin II–induced vasoconstriction to 22±11% or to 22±18%, respectively (each n=6; P<0.01 by ANOVA). Chlorthalidone or hydrochlorothiazide also significantly reduced the angiotensin II–induced vasoconstriction in the presence of the NO synthase inhibitor N\textsuperscript{G}-nitro-L-arginine-methyl ester to 45±9% or to 41±8%, respectively (each n=4; P<0.05 by ANOVA). Further, the inhibitory effect of thiazide-like diuretics could also be observed in the absence of endothelium, indicating that thiazide-like diuretics directly affect vascular smooth muscle cells.

The inhibitory effects of thiazide-like diuretics could also be observed when the vasoconstriction was induced by norepinephrine. In the presence of endothelium, incubation of segments from rat aorta with 1 µmol/L chlorthalidone or 1 µmol/L hydrochlorothiazide significantly reduced the norepinephrine-induced vasoconstriction to 67±4% or to 53±4%, respectively (each n=6; P<0.01 by ANOVA). In contrast, the initial vasoconstriction induced by potassium chloride was not significantly affected by chlorthalidone or hydrochlorothiazide (82±14% or 83±28%, respectively; each n=6; P=NS). Further, the barium-induced vasoconstriction was not significantly affected by chlorthalidone or hydrochlorothiazide (97±18% or 90±35%; each n=3; P=NS), probably because barium directly causes depolarization and hence vasoconstriction.\textsuperscript{19}

Next, the effects of thiazide-like diuretics were compared with those of the specific Rho kinase inhibitor Y27632. In the presence or absence of endothelium, incubation of segments from rat aorta with 1 µmol/L Y27632 significantly reduced the angiotensin II–induced vasoconstriction to 19±5% or 18±6% (each n=5; P<0.01 by ANOVA). In the presence or absence of endothelium, incubation of segments from rat aorta with 1 µmol/L Y27632 significantly reduced the norepinephrine-induced vasoconstriction to 36±6% or 46±8% (each n=5; P<0.01 by ANOVA). The dose-dependent relax-
ing effect of Y27632 on segments from aorta contracted with norepinephrine is shown in Figure 2E, indicating that the Rho–Rho kinase system is activated after norepinephrine administration. In the presence or absence of endothelium, incubation of segments from rat aorta with 1 μmol/L Y27632 did not significantly change the barium-induced vasoconstriction (98 ± 12% or 80 ± 25%; each n = 3; P = NS).

These data indicate that the angiotensin II– and norepinephrine-induced vasoconstriction in vitro is inhibited by thiazide-like diuretics similar to Rho kinase inhibition.

Effects of Thiazide-Like Diuretics on Angiotensin II–Induced DNA Synthesis, Protein Synthesis, and Intracellular Calcium in Vascular Smooth Muscle Cells

The effects of thiazide-like diuretics on angiotensin II–induced stimulation of DNA synthesis, measured by [3H] thymidine incorporation, and protein synthesis, measured by [3H] leucine incorporation, were investigated (Figure 3). Chlorthalidone and hydrochlorothiazide dose-dependently reduced angiotensin II–induced DNA synthesis and protein synthesis. In the presence of 100 μmol/L chlorthalidone or 100 μmol/L hydrochlorothiazide, the angiotensin II–induced DNA synthesis was reduced to 54 ± 6% or to 47 ± 5% (each n = 6; P<0.01 by ANOVA), respectively. In the presence of 100 μmol/L chlorthalidone or 100 μmol/L hydrochlorothia-

zide, the angiotensin II–induced protein synthesis was reduced to 81 ± 7% (n = 6) or to 73 ± 3% (each n = 6; P<0.01 by ANOVA), respectively.

Next, agonist-induced changes of cytosolic calcium were measured in vascular smooth muscle cells using the fluorescent dye technique. Chlorthalidone or hydrochlorothiazide did not significantly change the angiotensin II–induced calcium increase in vascular smooth muscle cells (chlorthalidone 89 ± 11% of control; hydrochlorothiazide 89 ± 35% of control; each n = 6; P = NS). Further, chlorthalidone or hydrochlorothiazide did not significantly change the norepinephrine-induced calcium increase in vascular smooth muscle cells (chlorthalidone 81 ± 16% of control; hydrochlorothiazide 85 ± 13% of control; each n = 6; P = NS).

Thiazide-Like Diuretics Affect the Rho–Rho Kinase Pathway

Because the agonist-induced vasoconstriction was significantly inhibited by thiazide-like diuretics, whereas calcium increase was not significantly changed, we hypothesized that calcium desensitization in smooth muscle cells might occur after administration of thiazide-like diuretics. Because a reduction of RhoA and Rho kinase is associated with calcium desensitization, we investigated the effects of thiazide-like diuretics on RhoA and Rho kinase using RT-PCR and
immunoblotting. Compared with control conditions, RhoA mRNA and Rho kinase mRNA were significantly reduced after administration of thiazide-like diuretics. Compared with control conditions, RhoA mRNA was significantly reduced in cultured vascular smooth muscle cells after incubation with chlorthalidone or hydrochlorothiazide to 20±12% or to 41±12%, respectively (each n=5; \( P<0.05 \) by ANOVA). The Rho kinase mRNA was also significantly reduced after incubation with chlorthalidone or hydrochlorothiazide to 63±18% or to 66±25%, respectively (each n=5; \( P<0.05 \) by ANOVA; Figure 4). Compared with control conditions, RhoA expression was significantly reduced in cultured vascular smooth muscle cells after incubation with chlorthalidone or hydrochlorothiazide to 41±15% or to 38±17%, respectively (each n=5; \( P<0.05 \) by ANOVA). The expression of Rho kinase was also significantly reduced after incubation with chlorthalidone or hydrochlorothiazide to 30±3% or to 36±3%, respectively (each n=5; \( P<0.05 \) by ANOVA; Figure 5). Next, measurements of membrane-associated and cytosolic fractions of RhoA in the absence and presence of thiazide-like diuretics were performed. In the presence of chlorthalidone or hydrochlorothiazide, the membrane-associated RhoA was slightly reduced to 78±9% or to 91±62%, respectively (each n=5; \( P=NS \)). On the other hand, in the presence of chlorthalidone or hydrochlorothiazide, the cytosolic RhoA was reduced to 29±4% or to 34±4% of control, respectively (each n=3; \( P<0.05 \) by ANOVA). Administration of hydrochlorothiazide did not significantly affect protein tyrosine phosphatase SHP-2 expression in vascular smooth muscle cells (78±89% or 112±116%; each n=5; \( P=NS \); Figure 5), respectively. These data indicate that the effects of thiazide-like diuretics on the Rho–Rho kinase pathway are not related to protein tyrosine phosphatase SHP-2.
The present study indicates that thiazide-like diuretics, including chlorthalidone and hydrochlorothiazide, attenuate agonist-induced vasoconstriction by calcium desensitization after affecting the Rho–Rho kinase pathway. In the present study, the reduced agonist-induced vasoconstriction in the presence of chlorthalidone or hydrochlorothiazide was similar to that observed in the presence of the specific Rho kinase inhibitor Y27632. In addition, preincubation with thiazide-like diuretics significantly reduced RhoA and Rho kinase in vascular smooth muscle cells. Reduced Rho kinase causes reduced phosphorylation of myosin phosphatase–targeting subunit at its inhibitory sites, thereby increasing myosin phosphatase activity. The increased myosin phosphatase activity while myosin light chain kinase activity remains unchanged leads to decreased phosphorylation of myosin regulatory light chain, causing calcium desensitization of the contractile apparatus and finally reduced contraction of smooth muscle cells.

Changes of Rho kinase–mediated regulation of vascular tone appears to be a likely cause of increased vascular resistance in hypertension. Vascular Rho kinase mRNA levels are increased in spontaneously hypertensive rats, stroke-prone spontaneously hypertensive rats, and angiotensin II–induced hypertensive rats. Moreover, inhibition of the Rho–Rho kinase pathway reduces blood pressure in several hypertensive animal models. Inhibition of the Rho–Rho kinase pathway also reduces vasoconstriction in human left internal mammary artery. Therefore, it can be assumed that the inhibitory effect of thiazide-like diuretics on the Rho–Rho kinase pathway observed in the present study mediates their well-known antihypertensive effects in humans. When activated, RhoA moves to the membrane fraction from the cytosolic fraction. We showed that membrane-associated fractions of RhoA were slightly reduced, whereas cytosolic fractions of RhoA were significantly reduced in the presence of thiazide-like diuretics. These findings may indicate that thiazide-like diuretics predominantly affect RhoA expression rather than translocation of RhoA to the membrane. It has been reported recently that peroxisome proliferator-activated receptor γ ligands inhibit the Rho–Rho kinase pathway by inducing protein tyrosine phosphatase SHP-2. In the present study, we showed that thiazide-like diuretics did not affect protein tyrosine phosphatase SHP-2 expression. The effects of thiazide-like diuretics and peroxisome proliferator-activated receptor γ ligands on the Rho–Rho kinase pathway are apparently mediated by different mechanisms.

Chlorthalidone showed a dose-dependent inhibition of angiotensin II–induced vasoconstriction of aortic rings. A chlorthalidone concentration of 1 μmol/L significantly reduced angiotensin II–induced vasoconstriction. In addition, a chlorthalidone concentration of 100 μmol/L significantly reduced DNA synthesis, protein synthesis, and Rho kinase expression. The concentrations used in the present study can be achieved clinically in patients. Mean serum concentrations of chlorthalidone in patients were ~40 μmol/L, as reported in the literature. These findings indicate that the inhibitory effects of thiazide-like diuretics on vasoconstriction attributable to calcium desensitization linked to the Rho–Rho kinase induced aortic rings. A chlorthalidone concentration of 1 μmol/L significantly reduced angiotensin II–induced vasoconstriction. In addition, a chlorthalidone concentration of 100 μmol/L significantly reduced DNA synthesis, protein synthesis, and Rho kinase expression. The concentrations used in the present study can be achieved clinically in patients. Mean serum concentrations of chlorthalidone in patients were ~40 μmol/L, as reported in the literature. These findings indicate that the inhibitory effects of thiazide-like diuretics on vasoconstriction attributable to calcium desensitization linked to the Rho–Rho kinase pathway.
pathway may also be an important mechanism in hypertensive patients. In support of that assumption, Pickers et al showed that hydrochlorothiazide increases human forearm blood flow.4 Furthermore, the Rho kinase inhibitor fasudil increased forearm blood flow and decreased forearm vascular resistance in hypertensive patients stronger than in normotensive control subjects, probably because of increased Rho kinase expression in hypertensive patients.29 These findings are supported by the fact that the antihypertensive action of diuretics is related to initial blood pressure.27

In the kidney, chlorothalidone and hydrochlorothiazide exert their diuretic action by binding to the sodium–chloride cotransporter (NCC) in the distal convoluted tubule.29 NCC is selectively expressed in the kidney and apparently does not play a significant role for vessel wall function.29,30 Furthermore, it is unlikely that the effects of thiazide-like diuretics on vasoconstriction are also mediated by NCC because NCC knockout mice show normal blood pressure.31 In addition, the hydrochlorothiazide-induced vasodilation in humans could also be observed in patients with Gitelman syndrome, indicating that the absence of NCC does not alter the vasodilatory effect of hydrochlorothiazide.4

Activation of RhoA and Rho kinase has been shown to play a role in angiotensin II–induced hypertrophic changes in vascular smooth muscle cells.32 Moreover, oral treatment with the Rho kinase inhibitor fasudil blocked the angiotensin–induced hypertrophic changes of vascular smooth muscle cells.33 Taking these results into consideration, the findings of the present study that chlorothalidone and hydrochlorothiazide reduce expression of RhoA and Rho kinase indicate that thiazide-like diuretics reduce vasaconstriction and vascular hypertrophy by affecting the Rho–Rho kinase pathway.

Perspectives

The present study showed a novel mechanism by which thiazide-like diuretics inhibit vasaconstriction and vascular growth. Thiazide-like diuretics attenuated agonist-induced increase of blood pressure and vasoconstriction by calcium desensitization after affecting the Rho–Rho kinase pathway. Further research based on thiazide-like diuretics or structurally related substances may help identify more specific inhibitors of the Rho–Rho kinase system to establish novel and clinically relevant antihypertensive drugs.

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ONLINE SUPPLEMENT

Thiazide-like diuretics attenuate agonist-induced vasoconstriction by calcium desensitization linked to Rho kinase

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Expanded Materials and Methods

Animals, hemodynamic measurements, and preparation of aortic rings

Male 3-month-old Wistar rats weighing 220-250g were housed under a 12h/12h day/night cycle. All experiments were performed as approved by the Animal Care and Use Committee. Rats were randomly divided into 4 groups, each group consisting of 5 rats. Rats were administered chlorthalidone (benzene-sulfonamide, 2-chloro-5-2,3-dihydro-1-hydroxy-3-oxo-1H-isouindol-1-yl; Sigma-Aldrich; 0.38 mg/kg/day), hydrochlorothiazide (6-Chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide; Sigma-Aldrich; 0.18 mg/kg/day), specific Rho kinase inhibitor, Y27632 (R-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide hydrochloride; Merck Biosciences; 1 mg/kg/day), or placebo for control through gavage for one week. Hemodynamic measurements were done according to Symons et al. Rats were anesthetized with pentobarbital sodium (60 mg/kg i.p.). While rats were breathing spontaneously the right carotid artery was cannulated for measurements of arterial blood pressure with a pressure transducer (model MLT 1030, Power Lab, Australia). The right jugular vein was cannulated for intravenous infusion of angiotensin II (24 µg/kg/h) or norepinephrine (24 µg/kg/h). In a subset of animals the thoracic aorta was dissected, carefully freed from connective tissue, and isometric force was measured according to established methods using a force transducer connected with a polygraph (model AD Instrument, Power Lab). The aortic rings were equilibrated for 60 minutes. The resting tension was set to 2g. Aortic rings were treated with increasing concentrations of chlorthalidone or hydrochlorothiazide for 30 minutes. Then 100 nmol/L angiotensin II, norepinephrine, potassium, or barium chloride were added and the contractions of the rings were measured. Additional experiments were done in the absence of endothelium, in the presence of nitric oxide synthase inhibitor, G-Nitro-L-arginine methylester (NAME), or in the presence of the specific Rho kinase inhibitor, Y27632.

Culture of vascular smooth muscle cells, measurements of DNA synthesis, protein synthesis and cytosolic calcium

Vascular smooth muscle cells were obtained from thoracic aortas and cultured by tissue explant method as described. DNA synthesis or protein synthesis was measured in quiescent vascular smooth muscle cells using [³H] thymidine or [³H] leucine (Amersham Pharmacia Biotech) according to Touyz et al. Cytosolic calcium concentrations were measured in cultured vascular smooth muscle cells loaded with the fluorescent dye fura2 according to previously published techniques.
RT-PCR of RhoA and Rho kinase
Expression of RhoA and Rho kinase mRNA was assessed by RT-PCR according to Hyvelin et al. Quiescent vascular smooth muscle cells were incubated in the absence and presence of 1 μmol/L chlorthalidone or hydrochlorothiazide for 24 hours. RNA was extracted from vascular smooth muscle cells by Trip pure reagent (Roche). Two μg of total RNA was reverse-transcribed with a reverse transcriptional system (Promega, Madison, USA). RhoA and Rho kinase cDNA were amplified in PCR reactions using the following primers: sense 5'-ACC AGT TCC CAG AGG TTT ATGT-3' and antisense 5'-TTT GGT CTT TGC TGA ACACT-3' for Rho A; and sense 5'- GCA CAT GTA TGA AAA TGG ATG AAAC-3' and antisense 5'- CAT AAT TTT GCT GTA GGT TCC TAC AAGT-3' for Rho kinase. The single stranded cDNA was amplified by PCR using taq DNA polymerase (Boehringer, Mannheim, Germany). Twenty-six cycles were performed under the following conditions: 94°C for 40 seconds; 55°C for 40 seconds; 72°C for 50 seconds. The same sample was used for GAPDH cDNA amplification to confirm that equal amounts of RNA were reverse transcribed. The primers used were sense 5'-ACG GCA AAT TCA ACG GCA CAG TCA-3' and antisense 5'-TGG GGG CAT CGG CAG AAG G-3'. The PCR products were size fractionated on 2% agarose gel, and DNA was visualized by ethidium bromide staining.

Immunoblotting for RhoA and Rho kinase
Quiescent vascular smooth muscle cells, grown on culture plates, were incubated in the absence or presence of 1 μmol/L chlorthalidone or hydrochlorothiazide. Vascular smooth muscle cells were homogenized in high-salt buffer containing NaCl 600 mmol/L, MOPS 40 mmol/L, EGTA 5 mmol/L, DTT 1 mmol/L, leupeptin 1 µg/mL, aprotinin 1 µg/mL, phenylmethylsulfonyl fluoride 50 mmol/L. Cells were scraped off, transferred to Eppendorf tubes, and sonicated for 5 seconds. The protein supernatant was separated by centrifugation, and protein concentrations were determined with Bio-Rad protein assay reagent (Bio-Rad Laboratories). Membrane and cytosolic fractions were separated by ultracentrifugation. Proteins were separated by using 10% SDS-polyacrylamide gel and transferred to polyscreen PVDF transfer membranes (Perkin Elmer, Boston, USA) at 95mA for 1 hour. Membranes were blocked with blocking buffer containing Tris-buffered saline and 0.1% Tween-20 with 5% wt/vol non-fat dry milk and incubated for 4 hours at room temperature Membranes were incubated with mouse monoclonal anti-RhoA or anti-Rho kinase antibodies (Santa Cruz Biotechnology, Santa Cruz, USA) diluted 1:1000 for 24 hours at 4°C. They were then
washed, incubated with a goat anti-mouse horseradish peroxidase-conjugated antibody (Dako Corporation, Carpinteria, CA) diluted 1:2000 for 12 hours at 4°C, and washed extensively. After incubation with the secondary antibodies for 1 hour, the proteins were detected by enhanced chemiluminescence (Amersham Bioscience, Buckinghamshire, UK) and quantified by densitometry. Each sample was processed 3 times. Immunoblots of protein tyrosine phosphatase SHP-2 were performed as described by Wakino et al.

**Statistics**

All values reported are mean ± SD. Comparisons between groups were analyzed using one-way ANOVA with Bonferroni post-hoc test. Two-sided p values below 0.05 were considered to indicate statistical significance.

References of the Online Supplement


