Signal-Crosstalk Between Rho/ROCK and c-Jun NH$_2$-Terminal Kinase Mediates Migration of Vascular Smooth Muscle Cells Stimulated by Angiotensin II


Background—Rho and its effector Rho-kinase/ROCK mediate cytoskeletal reorganization as well as smooth muscle contraction. Recent studies indicate that Rho and ROCK are critically involved in vascular remodeling. Here, we tested the hypothesis that Rho/ROCK are critically involved in angiotensin II (Ang II)-induced migration of vascular smooth muscle cells (VSMCs) by mediating a specific signal cross-talk.

Methods and Results—Immunoblotting demonstrated that Ang II stimulated phosphorylation of a ROCK substrate, regulatory myosin phosphatase targeting subunit (MYPT)-1. Phosphorylation of MYPT-1 as well as migration of VSMCs induced by Ang II was inhibited by dominant-negative Rho (dnRho) or ROCK inhibitor, Y27632. Ang II–induced c-Jun NH$_2$-terminal kinase (JNK) activation, but extracellular signal-regulated kinase (ERK) activation was not mediated through Rho/ROCK. Thus, infection of adenovirus encoding dnJNK inhibited VSMC migration by Ang II. We have further demonstrated that the Rho/ROCK activation by Ang II requires protein kinase C-δ (PKCδ) and proline-rich tyrosine kinase 2 (PYK2) activation, but not epidermal growth factor receptor transactivation. Also, VSMCs express PDZ-Rho guanine nucleotide exchange factor (GEF) and Ang II stimulated PYK2 association with tyrosine phosphorylated PDZ-RhoGEF.

Conclusions—PKCδ/PYK2-dependent Rho/ROCK activation through PDZ-RhoGEF mediates Ang II–induced VSMC migration via JNK activation in VSMCs, providing a novel mechanistic role of the Rho/ROCK cascade that is involved in vascular remodeling. (Arterioscler Thromb Vasc Biol. 2005;25:1831-1836.)

Key Words: angiotensin II • Rho kinase/ROCK • c-jun NH$_2$-terminal kinase • vascular smooth muscle cells • migration

Angiotensin II (Ang II) has been implicated in various cardiovascular diseases such as hypertension, atherosclerosis, and restenosis after angioplasty. Therefore, there has been considerable interest in defining the functional significance of signaling pathways of the Ang II type 1 receptor (AT$_1$), which is dominantly expressed in vascular smooth muscle cells (VSMCs). Through this receptor, Ang II stimulates hypertrophy and hyperplasia of VSMCs. The AT$_1$ receptor primarily couples to G$_q$ leading to elevation of intracellular Ca$^{2+}$ and activation of protein kinase C (PKC). In addition, tyrosine kinase activation by Ang II is linked to downstream mitogen-activated protein kinase (MAPK) activation, thereby mediating the growth-promoting response in VSMCs. This in regard, several key tyrosine kinases have been identified that may contribute to the growth-promoting effects of the AT$_1$ receptor. These kinases include epidermal growth factor receptor (EGFR) and proline-rich tyrosine kinase 2 (PYK2).

In addition to its growth responses, VSMC migration by Ang II is strongly implicated in various cardiovascular diseases, whereas the detailed signaling mechanisms by which the AT$_1$ receptor mediates migration are insufficiently characterized. At least, a MAPK, ERK appears to be required for Ang II–induced migration of VSMCs. Recently, Zahn et al showed that 3 major MAPKs, ERK, p38MAPK, and c-Jun NH$_2$-terminal kinase (JNK), are all required for VSMC migration induced by platelet-derived growth factor (PDGF), suggesting that these MAPKs coordinately mediate VSMC migration.

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1831
A small G protein, Rho, and its effector Rho-kinase/ROCK, are involved in many aspects of cell motility, from smooth muscle contraction to cell migration. Selective ROCK inhibitors appear not only to reduce blood pressure but also prevent experimental restenosis, atherosclerosis, and vascular hypertrophy. In VSMCs, Rho is activated by Ang II, and activation of Rho and ROCK is implicated in migration stimulated by a G protein–coupled receptor (GPCR) agonist, thrombin. These data suggest a strong functional significance of Rho/ROCK activation in mediating a pathophysiological response of Ang II such as VSMC migration.

Based on the above information, we hypothesized that Rho and ROCK are critically involved in Ang II–induced VSMC migration through a specific signal transduction cascade. Here, we demonstrate several lines of evidence indicating that activation of Rho/ROCK through PKC and PYK2 activation is specifically required for Ang II–induced JNK activation and subsequent VSMC migration. These data will provide a novel role of the Rho/ROCK cascade that is involved in vascular remodeling associated with cardiovascular diseases.

Materials and Methods
The materials and methods used in this study are described in the online supplement (available at http://atvb.ahajournals.org).

Results
Activation of Rho/ROCK Is Required for Ang II–Stimulated Migration
ROCK on activation phosphorylates the substrate, myosin phosphatase target subunit-1 (MYPT-1), at Thr696. As shown in Figure 1A, Ang II induced a rapid and sustained phosphorylation of MYPT-1 at Thr696 in VSMCs. Pretreatment of VSMCs with a selective AT1 receptor antagonist, RNH6270, (10 μmol/L, 30 minutes) completely inhibited the MYPT-1 phosphorylation induced by Ang II (data not shown). Also, the Ang II–induced MYPT-1 phosphorylation was completely blocked by infection of adenovirus encoding dnRho (100 moi) or treatment of a ROCK inhibitor, Y27632 (Figure 1B). These results indicate that activation of the AT1 receptor by Ang II leads to Rho/ROCK-dependent Thr696-phosphorylation of MYPT-1 that could represent a selective marker of the Rho and ROCK activation in VSMCs.

Because Rho/ROCK has been implicated in VSMC migration induced by thrombin, we have examined whether Rho and ROCK are required for VSMC migration induced by Ang II. As shown in Figure 1D, migration of VSMCs induced by Ang II was completely blocked by dnRho or Y27632. These data suggest that activation of Rho and ROCK is required for VSMC migration induced by Ang II.

Signal Cross-Talk Between Rho/ROCK and JNK Mediates Ang II–Induced VSMC Migration
Ang II activates 3 major MAPKs in VSMCs, and activation of ERK and JNK is indispensable for VSMC migration stimulated by Ang II. Therefore, we have investigated the possible signaling cross-talk between Rho/ROCK activation and the MAPK family activation by Ang II. As shown in Figure 2A, Ang II–induced ERK and p38MAPK phosphorylations were not affected by dnRho. In contrast, Ang II–induced JNK phosphorylation was completely inhibited by dnRho (Figure 2B). Moreover, pretreatment of Y27632 completely blocked Ang II–induced JNK phosphorylation but not ERK phosphorylation (Figure 2C). In rat VSMCs, p54JNK phosphorylation by Ang II was dominantly observed and comigrated with the protein band recognized by anti-JNK2 antibody. Infection of adenovirus encoding dnJNK but not the control empty vector inhibited migration of VSMCs induced by Ang II (Figure 2D). These results suggest a
unique signaling crosstalk between Rho/ROCK and JNK in mediating migration of VSMCs induced by Ang II.

PYK2 and PDZ-RhoGEF Exist Upstream of Rho/ROCK Activation by Ang II

Some overlapping, as well as distinct signal upstreams involving a tyrosine kinase have been proposed for activation of MAPKs by Ang II in VSMCs.3,5 PYK2 has been shown to mediate ERK and JNK activation by Ang II.16,17 In contrast, we have reported that EGFR transactivation specifically mediates ERK and p38 MAPK activation by Ang II in VSMCs.14 Based on this previous background, we have examined whether Rho/ROCK activation requires PYK2 or EGFR activation. As shown in Figure 3A, infection of adenovirus encoding dnPYK2 prevented MYPT-1 phosphorylation induced by Ang II, whereas the infection did not affect Ang II–induced EGFR transactivation in VSMCs. In contrast, AG1478, a selective EGFR kinase inhibitor, did not

Figure 3. Ang II activates Rho/ROCK pathway through PYK2 but not EGFR. A, VSMCs infected with adenovirus encoding dnPYK2 (100 moi) or control empty vector (100 moi) for 48 hours were stimulated with Ang II (100 nmol/L) for 2 minutes. B, VSMCs pretreated with AG1478 (500 nmol/L) or with vehicle (0.1% DMSO) for 30 minutes were stimulated with Ang II (100 nmol/L) for 2 minutes. Cells lysates were analyzed by immunoblotting with antibodies as indicated. The extent of MYPT-1 phosphorylation was quantified by densitometry. Data shown are the mean±SEM from 3 independent experiments. *P<0.05 compared to basal control. †P<0.05 compared to the stimulated control. C, Equal amount of cell lysates from HEK293 and VSMCs were subjected to immunoblotting with antibodies as indicated. D, VSMCs were stimulated with Ang II (100 nmol/L) for indicated durations. Cell lysates were immunoprecipitated with anti-PYK2 antibody and immunoblotted with indicated antibodies. Upper and lower arrows denote tyrosine phosphorylated PDZ-RhoGEF and PYK2, respectively.
PKCδ is Required for Rho/ROCK-Dependent JNK Activation and Migration by Ang II

We have previously shown that PYK2 activation specifically requires the PKCδ isoform in VSMCs by using a PKCδ inhibitor, rottlerin, and adenovirus encoding dnPKCδ. Therefore, the role of PKCδ in Ang II–induced Rho/ROCK pathway activation was studied by using the PKCδ inhibitor, rottlerin (Figure I, available online at http://atvb.ahajournals.org). Rottlerin markedly attenuated MYPT phosphorylation induced by Ang II (Figure IA). Rottlerin blocked Ang II–induced JNK activation (Figure IB), whereas this inhibitor had no effect on ERK and p38MAPK activation by Ang II (Figure IC). Rottlerin inhibited Ang II–induced VSMC migration as well (Figure 1D). These pharmacological findings were further supported by the results obtained with adenovirus encoding dnPKCδ (Figure II, available online at http://atvb.ahajournals.org). As shown in Figure IIA, dnPKCδ inhibited MYPT phosphorylation induced by Ang II. Thus, dnPKCδ attenuated phosphorylation of JNK (Figure IIB) whereas dnPKCδ had no effect on ERK or p38MAPK phosphorylation by Ang II (Figure IIC). DnPKCδ also inhibited VSMC migration induced by Ang II (Figure IID). The specificity of the dominant-negative intervention was tested by a JNK activator, anisomycin. Overexpression of dn mutants used in this study did not affect JNK activation by anisomycin (Figure III, available online at http://atvb.ahajournals.org). Taken together, these findings provide a previously missing signaling link by which Ang II activates Rho/ROCK and suggests how Rho/ROCK specifically up-regulates VSMC migration through its selective cross-talk with JNK.

Discussion

The major findings presented in this study are that Rho/ROCK activation by Ang II specifically mediates JNK activation leading to migration of VSMCs, and that the upstream of Rho/ROCK activation by Ang II requires PYK2 and PKCδ activation. This is in line with previous findings that Ang II activates RhoA in cardiac myocytes and VSMCs. Thus, activation of Rho/ROCK represents one of the key signal transduction pathways originating from the VSMC AT1 receptor that mediates pathophysiological functions of Ang II such as contraction, atherogenesis, cardiovascular hypertrophy, and migration as presented here.

Limited information was available previously regarding the mechanistic insights by which Ang II activates Rho/ROCK. In general, GPCR agonists have been demonstrated to activate Rho through a Rho-specific GEF such as p115RhoGEF that interacts with G12 and G13. In addition to Gαs, Ang II receptors in VSMCs are able to couple G12 and G13. However, recent accumulating evidence suggest a G12βγ-dependent Rho activation as an alternative route by which some GPCRs activate Rho in non-muscle cells. This notion is supported by the recent finding of Ca2+ and calmodulin-dependent Rho activation in VSMCs. In the present study, we found a participation of PKCδ for Rho/ROCK activation in VSMCs. In addition, attenuation of mechanical stress-induced Rho activation was reported in VSMCs derived from PKCδ null mice. Therefore, a Ca2+–sensitive mechanism and Ca2+–insensitive PKCδ may both participate in Rho/ROCK activation in VSMCs in addition to G12βγ.
However, we could not detect JNK-dependent paxillin phosphorylation in VSMCs by using antiphosphoserine antibody. Alternatively, JNK may mediate Ang II–induced VSMC migration through c-Jun phosphorylation, because c-Jun as well as JNK is required for VSMC migration in response to PDGF.\textsuperscript{39,40} Also, because the ERK cascade activation operated through EGFR transactivation is required for Ang II–induced VSMC migration,\textsuperscript{40} it is likely that parallel ERK cascade activation together with the JNK cascade activation coordinately induce the migratory responses in VSMCs.

It has been established that the primary Rho family small G proteins involved in JNK activation are Rac and Cdc42 but not Rho.\textsuperscript{26} In this regard, PAK, which can interact with the active form of Rac or Cdc42, has been implicated in Ang II–induced JNK activation.\textsuperscript{41} However, in addition to our findings presented here, Rho-dependent JNK activation has been demonstrated in certain cell types.\textsuperscript{52,43} To exclude the possibility of nonspecific inhibition of other Rho family small G protein function by dnRho infection, we confirmed that Ang II–induced JNK activation was not affected by dnRac adenovirus (100 moi) in VSMCs (S. Eguchi, unpublished observation, 2005). Marinissen et al have recently shown that active ROCK is able to stimulate JNK and its direct upstream SEK1/MKK4 in HEK293 cells.\textsuperscript{44} Also, activation of MEKK1 by RhoA through direct interaction was reported.\textsuperscript{45} However, preliminarily, we could not detect this interaction in VSMCs on Ang II stimulation. Therefore, the precise mechanism of Rho/ROCK in JNK activation in VSMCs will require further investigation.

Results using the ROCK inhibitor, Y27632, should be interpreted carefully. The concentration used in this study may partially inhibit PKC\textsuperscript{80} and PKN activity.\textsuperscript{46} However, all of the inhibitory actions of Y27632 presented here were mimicked by dnRho in the present study, thus suggesting the inhibitory effect of Y27632 could be explained by its action on ROCK but not other kinases. In addition, several kinases other than ROCK could phosphorylate MYPT1 at Thr696.\textsuperscript{13,47} However, participation of these kinases on Ang II–induced MYPT phosphorylation is unlikely because they are minimally inhibited by Y27632.\textsuperscript{47} Finally, MYPT phosphorylation and the resultant inhibition of myosin phosphatase lead to enhanced phosphorylation of its substrates, such as myosin II, adducin, and moesin. These phosphorylations are implicated not only in cell contraction but also cell motility.\textsuperscript{13} Although we have used the MYPT phosphorylation as a readout of ROCK activation in VSMCs, it will be interesting to further investigate the role of MYPT in regulating signaling and function of Ang II in VSMCs.

Our findings presented here are limited within multi-passaged cultured VSMCs. Future studies are necessary to confirm the presence of the cascade in an in vivo condition or diseases associated with enhanced Ang II actions. In conclusion, the signal transduction of Rho/ROCK activation and its downstream signaling that mediate VSMC migration have been demonstrated in this study. VSMC migration stimulated by Ang II is considered as a potential mechanism of atherosclerosis and restenosis after vascular injury. Therefore, further clarification of this cascade could contribute to a better understanding of the molecular mechanism of cardiovascular diseases as well as to the development of better strategies for their treatment.

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References


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Materials and Methods

Reagents
AngII was purchased from Sigma. Anisomycin, Y27632, AG1478 and rottlerin were purchased from Calbiochem. Antibodies were purchased from the following sources: Thr^696/-phosphorylated MYPT-1 was from Upstate Biotechnology; MYPT-1 was from Covance; Tyr^204/- phosphorylated ERK1/2, ERK2, JNK2, p38MAPK, PKCδ, RhoA, p115 RhoGEF and LARG were from Santa Cruz Biotechnology; Thr^180/Tyr^182/-dually phosphorylated p38MAPK was from Cell Signaling; Thr^183/Tyr^185/-dually phosphorylated JNK2 was from Promega; Tyr^1068/-phosphorylated EGFR was from Biosource International; and PYK2 was from BD Transduction Laboratories. Antisera to PDZ-RhoGEF/GTRAP48 was generated as previously described.

Cell Culture
Rat aortic vascular smooth muscle cells (VSMCs) were maintained as described previously. Cells passage 3-12 at ~80% confluence in culture wells were made quiescent by incubation with serum-free medium for 2-3 days before stimulation. HEK293 cells were obtained from Dr. M. Autieri (Temple University).

Adenoviral Infection
Generation and characterization of replication-deficient adenovirus encoding a dominant-negative mutant of Rho (dnRho), pME18S-myc-N^19-RhoA, a dnJNK, pUC-K52R-JNK, a dnPYK2/CAKβ, pAxCA-K457A-PYK2/CAKβ, and a dnPKCδ, pAxCA-K376A-PKCaδ, were described in detail elsewhere. VSMCs were infected with adenoviruses at a multiplicity of infection (moi) of 100 as previously described.

Immunoprecipitation and Immunoblotting
Immunoprecipitation and immunoblotting were performed as previously described. The results were quantified by densitometry in the linear range of film exposure using CanoScan N670U (Canon) and Un-Scan-It Gel 4.3 software (Silk Scientific). Results were expressed as % increase in which the maximam response to AngII is defined as 100% because the basal signals are more varied depending on film exposure than the stimulated signals.
Migration Assay

Cell migration was performed using the Boyden chamber method as previously described. Cell counts for migrated VSMCs were performed in at least 8 randomly selected fields with persons who were blind to the experimental conditions.

Statistical Analysis

Data were analyzed by using the Student t test. The mean±SEM was determined with a significance level of p<0.05. The results shown in blots are representative of at least 3 separate experiments.

References


**Figure Legends**

Figure I. **Effect of a PKCδ inhibitor, rottlerin, on phosphorylations of MYPT-1 and MAPKs, and migration of VSMCs induced by AngII.** A, B and C, VSMCs pretreated with 10 µmol/L rottlerin or with vehicle (0.1% DMSO) for 30 min were stimulated with AngII (100 nM) for 2 min (A), 30 min (B) or 10 min (C). Cells lysates were analyzed by immunoblotting with antibodies as indicated. The extent phosphorylation of MYPT, JNK, ERK and p38MAPK was quantified by densitometry. Data shown are the mean±S.E.M. from three independent experiments. D, VSMCs pretreated with rottlerin (10 µmol/L) or with vehicle (0.1% DMSO) for 30 min were stimulated with AngII for 8 h. Migration activities were shown as percent changes relative to basal activity. n=3 in triplicate determination, means±S.E. *p<0.05 compare to basal control. †p<0.05 compare to the stimulated control.

Figure II. **dnPKCδ inhibited phosphorylation of MYPT-1 and JNK, and migration of VSMCs induced by AngII.** A, B and C, VSMCs infected with adenovirus encoding dnPKCδ (A 100 moi, B 25-100 moi and C 100 moi) or control empty vector (100 moi) for 48 hours were stimulated with AngII (100 nmol/L) for 2 min (A), 30 min (B) or 10 min (C). Cell lysates were analyzed by immunoblotting with antibodies as indicated. The extent phosphorylation of MYPT, JNK, ERK, and p38MAPK was quantified by densitometry. Data shown are the mean±S.E. from three independent experiments. D, VSMCs infected with adenovirus encoding dnPKCδ (100 moi) or control empty vector (100 moi) for 48 h were
stimulated with AngII (100 nmol/L) for 8 h. Migration activities were shown as fold increase relative to basal activity. n=3 in triplicate determination, means ± S.E., *p<0.05 compare to basal control. †p<0.05 compare to the stimulated control.

Figure III. Effect of adenovirus encoding the dominant negative mutants on JNK activation induced by AngII in VSMCs. VSMCs infected with adenovirus encoding control empty vector, dnRho, dnPKCδ, or dnPYK2 (100 moi) for 48 hours were stimulated with anisomycin (10 μg/mL) for 30 min. Cell lysates were immunoblotted with antibodies against Thr^{183}/Tyr^{185} dually phosphorylated JNK2 (JNK-p), total JNK2, RhoA, PKCδ and PYK2 as indicated. The extent phosphorylation of JNK was quantified by densitometry. Data shown are the mean ± S.E.M. from three independent experiments.
Fig. III

The figure shows a Western blot analysis of proteins under different conditions. The blots are labeled as follows:

- **IB:** JNK-p
- **IB:** JNK2
- **IB:** PKCδ
- **IB:** RhoA
- **IB:** JNK-p (vector, anisomycin)
- **IB:** JNK2 (vector, anisomycin)
- **IB:** PKCδ (vector, anisomycin)
- **IB:** PYK2 (dnPYK2, anisomycin)

The bar graph below the blot images compares the levels of JNK-p under different conditions:

- **vector**
- **dnRho**
- **dnPKCδ**
- **dnPYK2**

The y-axis represents the percentage of JNK-p, and the x-axis represents the treatment conditions with and without anisomycin.