Angiotensin II Activates the RhoA Exchange Factor Arhgef1 in Humans

Maria Luigia Carbone,* Jérémy Brégeon,* Nabila Devos, Gilliane Chadeuf, Anne Blanchard, Michel Azizi, Pierre Pacaud, Xavier Jeunemaître, Gervaise Loirand

Abstract—Although a causative role for RhoA-Rho kinase has been recognized in the development of human hypertension, the molecular mechanism(s) and the RhoA guanine exchange factor(s) responsible for the overactivation of RhoA remain unknown. Arhgef1 was identified as a RhoA guanine exchange factor involved in angiotensin II (Ang II)–mediated regulation of vascular tone and hypertension in mice. The aim of this study was to determine whether Arhgef1 is activated and involved in the activation of RhoA-Rho kinase signaling by Ang II in humans. In vitro stimulation of human coronary artery smooth muscle cells and human peripheral blood mononuclear cells by Ang II (0.1 μmol/L) induced activation of Arhgef1 attested by its increased tyrosine phosphorylation. Silencing of Arhgef1 expression by siRNA inhibited Ang II–induced activation of RhoA-Rho kinase signaling. In normotensive subjects, activation of the renin–angiotensin system by a low-salt diet for 7 days increased RhoA-Rho kinase signaling and stimulated Arhgef1 activity in peripheral blood mononuclear cells. In conclusion, our results strongly suggest that Arhgef1 mediates Ang II–induced RhoA activation in humans. Moreover, they show that measurement of RhoA guanine exchange factor activity in peripheral blood mononuclear cells might be a useful method to evaluate RhoA guanine exchange factor activity in humans. (Hypertension. 2015;65:00–00. DOI: 10.1161/HYPERTENSIONAHA.114.05065.)

Key Words: angiotensins ■ arteries ■ hypertension ■ leukocytes ■ Rho GTP-binding proteins ■ Rho guanine nucleotide exchange factors ■ signal transduction

The RhoA-Rho kinase signaling has been recognized as a major regulator of vascular tone and arterial blood pressure.1 Overactivation of this pathway has been suggested to participate to the pathogenesis of hypertension in several experimental models2–5 and in humans.6 RhoA acts as a molecular switch that cycles between an inactive GDP-bound form and an active GTP-bound form.7 The transition from the inactive to the active state requires a Rho guanine exchange factor (GEF) that promotes the release of GDP in exchange for GTP.8,9 In the active GTP-bound form, through the activation of its effector Rho kinase, the phosphorylation of the myosin phosphatase target (MYPT) subunit and the consequent inhibition of the myosin light chain phosphatase activity, RhoA is responsible for the Ca2+ sensitization of the contractile proteins that underlies the tonic component of vascular smooth muscle contraction.10 Functional and pharmacological analyses using Rho kinase inhibitors have further suggested that RhoA-dependent pathways are involved in the increased vascular resistance associated with hypertension.5,11 Although these observations suggest a possible pathophysiological role for RhoA-Rho kinase in the development of hypertension, the molecular mechanism(s) and the GEF(s) responsible for the overactivation of RhoA remain largely unknown. Several mouse models of RhoA GEF deletion have thus been developed during the few past years and used to address this question. Recent reports described that the RhoA GEF Arhgef12 (Larg) plays a key role in salt-induced high blood pressure, whereas Arhgef1 mediates the activation of RhoA by type 1 angiotensin II (Ang II) receptor (AT1R) activation and is essential for Ang II–dependent hypertension.12,13 Whether these GEFs are relevant to hypertension in humans remains to be proven.

RhoA–Rho kinase activity assessed in peripheral blood mononuclear cells (PBMCs) is increased in hypertensive patients and can be reduced by antihypertensive treatments.14 This method is proposed for assessing RhoA–Rho kinase activity in patients with hypertension and other cardiovascular diseases.15 In this study, we extended this concept to the analysis of GEF activity in human. We developed an in vitro approach to evaluate RhoA GEF activity and to identify...
whether Arhgef1 is activated by Ang II in humans. We show that Arhgef1 is activated by in vitro Ang II stimulation of human coronary artery smooth muscle cells (HCASMCs) and PBMC, and that Arhgef1 activity in PBMC is increased by in vivo stimulation of the renin–angiotensin system (RAS) by a low-salt diet.

**Methods**

**In Vitro Studies: HCASMC Culture and siRNA**

HCASMC (3 different lots; Promocell, Heidelberg, Germany) were cultured in smooth muscle cell (SMC) growth medium 2 (Promocell). The medium was changed every 2 or 3 days. When cells reached confluence, subculture was obtained by harvesting the cells with 0.2% ethylenediaminetetraacetic acid and 0.25% trypsin. Cells were used at passage 2 to 4 and were serum-starved for 24 hours before experiments. HCASMC were stimulated by Ang II (0.1 μmol/L) in the presence of PD123319 (1 μmol/L). Sigma-Aldrich, Saint-Quentin Fallavier, France) added 1 hour before Ang II stimulation to avoid potential type 2 Ang II receptor-mediated effects.

The sense strands of siRNAs (Eurogentec, Seraing, Belgium) used for Arhgef1 silencing was: 5′-GCAGCUCUGAGAACGGCAAGdTdT-3′. A scrambled, nontargeting siRNA has been used as negative control (5′-UUUCGGACCAUGGCUGCAGG-tdT-3′). The siRNA were introduced into HCASMCs with Jet PEI reagent (Polytransfection, Illkirch, France) according to the manufacturer’s recommendations. Cells were incubated with or without 1.2 μg of specific or scrambled siRNA for 48 hours before functional assays were conducted. The efficiency of siRNA-mediated gene silencing assessed by real-time quantitative polymerase chain reaction or by Western blot at 48 hours post-transfection was ≈50% (Figure 1), with no significant effect on other RGS-Rho GEFs (not shown). This assay has been performed in triplicates, in 4 independent experiments.

**Real-Time Quantitative Polymerase Chain Reaction**

siRNA-mediated Arhgef1 silencing was validated by real-time quantitative polymerase chain reaction performed on the iCycler iQ Detection System (Bio-Rad S.A., Marnes la Coquette, France) as previously described17 with the following primers for Arhgef1 (up: 5′-GCAGCUCUGAGAACGGCAAG-tdTdT-3′, down: 5′-TTGTTTGTCTTCCATTCTGTG-tdT-3′) and GAPDH (up: 5′-GACGACTAGCCGATCTTCTT-3′, down: 5′-AGTTAAAAGCAGCCTGGTGA-3′).

**Human Study: Subjects and Protocol**

We studied 47 normotensive (office blood pressure<140/90 mm Hg after 5 minutes rest in the supine position) healthy male nonsmoking subjects (mean age, 23.7±5.0 years; mean body mass index, 22.9±6.3 kg/m²). Informed consent for participation in the study was obtained from all subjects. The study protocol was performed in accordance with the Declaration of Helsinki guidelines and institutional guidelines, and was approved by the Comité Consultatif de Protection des Personnes se prêtant à des Recherches Biomédicales (Paris-Cochin, France). Each subject has received a low-sodium/high-potassium diet for 7 days (sodium, ≤20 mmol/d and potassium, >120 mmol/d). Plasma hormones, blood pressure, and heart rate were measured before (day 0) and at the end of the 7-day low-salt/high-potassium diet (day 7).

**Laboratory Analysis and Measurement of Blood Pressure**

The methods used for measuring blood pressure, collecting blood samples and for quantifying plasma active renin, total renin, and aldosterone were as described previously.18

**Isolation of PBMC**

Whole blood was collected into EDTA tubes. PBMC were isolated from whole blood under sterile conditions by density gradient centrifugation using Unisep tubes (Eurobio, Courtaboeuf, France). PBMC pellets were either stored at −80°C until used or immediately resuspended in RPMI and stimulated with Ang II, then processed for Western blot analyses.

**Western Blot Analysis**

Cells (HCASMCs or PBMC) were lysed in NETF buffer (NaCl 100 mmol/L, EGTA 2 mmol/L, Tris HCL 50 mmol/L, NaF 50 mmol/L, 1% NP-40, protease inhibitor and Ser/Thr phosphatase inhibitors (Sigma, cocktail I)). Cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes then incubated with specific antibodies. Immunoreactivity of phosphotyrosine protein was performed using the 4G10 antibody (Upstate Biotechnology; Millipore, Molsheim, France) Assessment of the phosphorylation level of the Rho kinase target MYPT-subunit 1 (P-MYPT) with a rabbit polyclonal antibody to phospho-MYPT (P-MYPT1; Santa Cruz Biotechnology, CliniSciences, Naterre, France). Antibody to Arhgef1 was purchased from Santa Cruz Biotechnology. Anti-MYPT1 was purchased from Cell Signaling. ATR1 antibody was from Santa Cruz Biotechnology. Immunoreactive proteins were detected by enhanced chemiluminescence detection procedure (Amersham Pharmacia Biotech, GE Healthcare, Velzey-Villacoublay, France) and quantified using QuantityOne (Bio-Rad). Equal loading was checked by reprobing the membrane with β-actin or α-tubulin antibody (Santa Cruz Biotechnologies).

**AT1R mRNA Expression**

Total RNA was extracted from PBMC using TRIzol reagent (Life technologies) according to the manufacturer’s instructions. RNA (1 μg) was reverse transcribed with moloney murine leukemia virus reverse transcriptase (Invitrogen) and deoxyribonucleic random hexamer as primers (Invitrogen). Real-time gene expression analysis of AT1 receptor was performed with predesigned TaqMan Gene Expression Assay (Hs00258938_m1; Life technologies) using the StepOnePlus Real-Time polymerase chain reaction System (Applied Biosystems). AT1 receptor mRNA expression was normalized to HPRT1 mRNA expression (Hs02800695_1) used as housekeeping control gene. Cycle threshold and relative quantification by the 2−Ct method values were determined using the StepOne Software v2.1.

**Statistical Analysis**

Results are presented as means±SEM. All reported P values were 2-sided, and a P<0.05 was considered statistically significant.
Nonparametric Mann–Whitney (for HCASMC data) and Wilcoxon signed-rank tests were used. Relations between variables were determined by Spearman correlation coefficients analysis. The data were processed using the software package GraphPad Prism 5.

Results

Arhgef1 Is Required for Ang II–Mediated RhoA-Rho Kinase Activation in HCASMC

Stimulation of control HCASMCs by Ang II (0.1 µmol/L) for 5 and 60 minutes induced RhoA-Rho kinase activation (Figure 2). Knockdown of Arhgef1 by siRNA prevented Ang II–induced RhoA-Rho kinase activation at both 5 and 60 minutes (Figure 2). This suggests that, as in rodent SMC, the RhoA exchange factor Arhgef1 is essential for the activation of RhoA signaling induced by Ang II in human SMC.

In Vitro Stimulation of Human PBMC by Ang II Induces Activation of Arhgef1

To validate whether PBMC can be used to monitor Arhgef1 activation by Ang II in humans, we first assessed whether Arhgef1 was activated by in vitro stimulation of PBMC by Ang II. Ang II activated Arhgef1 through Jak2-mediated Tyr738 phosphorylation.\(^{12}\) We, therefore, measured the activation of Arhgef1 in PBMC by Western blot analysis of its phosphorylation on Tyr residues. Stimulation of PBMC by Ang II (0.1 µmol/L) for 60 minutes increased both the phosphorylation of Arhgef1 on Tyr, attesting its activation, and the phosphorylation of MYPT indicating the activation of RhoA-Rho kinase signaling (Figure 3).

In Vivo Activation of RAS by Low-Sodium/High-Potassium Diet Induces Activation of Arhgef1 in Human PBMC

After 7 days of the low-sodium/high-potassium diet, as expected, the plasma total and active renin and aldosterone concentrations were markedly and significantly increased compared with those measured at day 0, thereby demonstrating the activation of the RAS (Table 1). Regression analysis confirmed that plasma aldosterone correlated with active renin level (Figure S1A in the online-only Data Supplement). This activation of RAS was associated with an increase in urinary Na\(^+\) excretion and a reduction of K\(^+\) excretion (Table 1). Systolic blood pressure slightly decreased at day 7 of the low-sodium/high-potassium diet (not shown). To analyze whether in vivo stimulation of the RAS activates Arhgef1, we next measured the activity of RhoA-Rho kinase signaling and the phosphorylation of Arhgef1 in PBMC collected at day 0 and at day 7 of the low-sodium/high-potassium diet. Activity of RhoA-Rho kinase was significantly increased after 7 days of low-sodium/high-potassium diet (Figure 4A). This is associated with an increased phosphorylation of Arhgef1 attesting its activation (Figure 4B). The low-sodium/high-potassium diet for 7 days induced a 1.7-fold increase in Arhgef1 phosphorylation from its initial level at day 0 (Figure S2A), without change neither in Arhgef1 expression (1.04±0.10 at day 7 normalized to day 0; Figure S2B and S2C) nor in AT1R protein and mRNA expression (1.14±0.32 and 1.34±0.34 at day 7 normalized to day 0, respectively; Figure S2D). Leukocyte Rho kinase activity and Arhgef1 phosphorylation did not correlate with plasma active renin or aldosterone levels (Figure S1B–S1E). Leukocyte Rho kinase activity did not correlate with Arhgef1 phosphorylation level, although the stimulation of Rho kinase activity and the increase in Arhgef1 phosphorylation induced by the low-sodium/high-potassium diet are weakly correlated (Figure S1F and S1G). These data thus provide evidence that activation of the RAS leads to increase the leukocytes activity of Arhgef1 and RhoA-Rho kinase signaling.

Discussion

Our study demonstrates that Ang II activates Arhgef1 in HCASMC and human PBMC in vitro and that in vivo physiological activation of the RAS by a low salt intake increases the activity of Arhgef1 in PBMC of normotensive subjects. The present data thus suggest a potential role of Arhgef1 in the regulation of blood pressure by Ang II in humans. Furthermore, our study provides evidence that measurement of Rho GEF activity in PBMC might be a useful method to evaluate Rho GEF activity in humans.

Recent works in both the rodent models of hypertension and transgenic mice have greatly increased our knowledge on the role of RhoA-Rho kinase signaling in blood pressure regulation and hypertension.\(^1\) In particular, the identification of GEFs responsible for the increased RhoA activity, such as Arhgef12 for salt-dependent hypertension and Arhgef1...
Therefore, RhoA GEFs represent new targets for antihypertensive therapies to selectively reverse the hyperactivity of RhoA occurring in different forms of hypertension, allowing adapted and targeted antihypertensive treatments. Translating results from rodents to human hypertension is, therefore, an important and promising challenge. In vitro experiments performed here confirm that, as previously described in these results from rodents to human hypertension, genetic analyses have defined RhoA GEFs as susceptibility genes for type 2 diabetes mellitus. From a pharmacological point of view, the first data suggesting a role of RhoA-Rho kinase in the pathogenesis of human hypertension were obtained by showing that the increase in forearm blood flow and the decrease in forearm vascular resistance produced by the Rho kinase inhibitor fasudil are significantly stronger in patients with essential hypertension than in the normotensive group of patients, suggesting that hypertension-associated vascular dysfunction depended, at least in part, on Rho kinase activation. Interestingly, Rho kinase activity in leukocytes correlates with the forearm blood flow response to Rho kinase inhibitors indicating that leukocyte Rho kinase activity is also an index of vascular RhoA-Rho kinase activity. Yet systolic blood pressure was not found to correlate with leukocyte Rho-associated kinase activity. Results from this recent prospective study in a large population, however, demonstrated that leukocyte Rho kinase activity is a potential biomarker for predicting cardiovascular events. The molecular mechanisms that regulate Rho kinase activity or those involved in the dysregulation of RhoA/Rho kinase signaling in human are not known. Only study in leukocytes from patients with Bartter and Gitelman syndromes has indirectly suggested a role of Arhgef1 and Rho kinase signaling pathway in the Ang II effect in humans.

Our results confirm that Arhgef1 is expressed in human PBMC and activated RhoA/Rho kinase signaling in response to Ang II stimulation in vitro. They further show that Arhgef1 activity and RhoA/Rho kinase signaling are increased by the physiological activation of the RAS. However, the increase in PBMC Arhgef1 activity and Rho kinase activity observed when the RAS was stimulated are only weakly correlated, and neither Arhgef1 activity nor Rho kinase activity correlated with plasma active renin and aldosterone. Several reasons could potentially explain this apparent discrepancy between in vitro and in vivo results. In vivo activation of the RAS induces multiple pathways as well as positive and negative feedback loops. These events lead to the generation of secondary circulating mediators, such as reactive oxygen species, chemokines, cytokines, or vasoactive mediators that can also positively or negatively regulate exchange factors and RhoA/Rho kinase signaling. In whole organism, the level of PBMC Arhgef1 and RhoA/Rho kinase activation could thus be not directly related to the level of RAS activity and AT1R activation, but may result from complex interactions of numerous upstream signals. Furthermore, even standardized protocols were used and phosphatase inhibitors included in the lysis buffer, the level of Arhgef1 and MYPT phosphorylation in human PBMC depends on cellular protein phosphatase activity that could potentially explain this apparent discrepancy between in vitro and in vivo results.

For Ang II–dependent hypertension, supports the concept that RhoA GEFs involved in the overactivation of RhoA are probably different according to the pathological context. Accordingly, RhoA GEFs represent new target for antihypertensive therapies to selectively reverse the hyperactivity of RhoA occurring in different forms of hypertension, allowing adapted and targeted antihypertensive treatments. Translating these results from rodents to human hypertension is, therefore, an important and promising challenge. In vitro experiments performed here confirm that, as previously described in rat and mouse SMCs, Arhgef1 is involved in Ang II–induced RhoA/Rho kinase activation in human arterial SMCs.

In humans, both genetic studies and pharmacological data indirectly support a potential role of Rho proteins in the generation of high blood pressure. Indeed, high basal blood pressure and increased systemic vascular resistance was shown to be associated with the nonsynonymous Thr431Asn Rock2 variant of the Rho kinase gene. More recently, a lower risk of high blood pressure was associated with a major haplotype block at the Rock2 locus in a recessive manner. About RhoA GEFs, although no study has been performed in the context of hypertension, genetic analyses have defined RhoA GEFs as susceptibility genes for other cardiovascular risk factors. Arhgef11 and Arhgef12 have been identified as candidate genes for type 2 diabetes mellitus. From a pharmacological point of view, the first data suggesting a role of RhoA-Rho kinase in the pathogenesis of human hypertension were obtained by showing that the increase in forearm blood flow and the decrease in forearm vascular resistance produced by the Rho kinase inhibitor fasudil are significantly stronger in patients with essential hypertension than in the normotensive group of patients, suggesting that hypertension-associated vascular dysfunction depended, at least in part, on Rho kinase activation. Interestingly, Rho kinase activity in leukocytes correlates with the forearm blood flow response to Rho kinase inhibitors indicating that leukocyte Rho kinase activity is also an index of vascular RhoA-Rho kinase activity. Yet systolic blood pressure was not found to correlate with leukocyte Rho-associated kinase activity. Results from this recent prospective study in a large population, however, demonstrated that leukocyte Rho kinase activity is a potential biomarker for predicting cardiovascular events. The molecular mechanisms that regulate Rho kinase activity or those involved in the dysregulation of RhoA/Rho kinase signaling in human are not known. Only study in leukocytes from patients with Bartter and Gitelman syndromes has indirectly suggested a role of Arhgef1 and Rho kinase signaling pathway in the Ang II effect in humans.

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**Table 1. Hormonal and Urine Parameters Before (Day 0) and After a 7-Day Low-Sodium/High-Potassium Diet (Day 7; n=47)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day 0</th>
<th>Day 7</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma active renin, pg/mL</td>
<td>27.4±1.9</td>
<td>68.3±3.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plasma total renin, pg/mL</td>
<td>330.5±14.1</td>
<td>433.1±18.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plasma aldosterone, pg/mL</td>
<td>69.9±5.3</td>
<td>331.2±19.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Natriuresis, mmol/24 h</td>
<td>169.1±12.6</td>
<td>22.9±1.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Kaliuresis, mmol/24 h</td>
<td>75.1±4.4</td>
<td>140.2±4.1</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
normalensive and hypertensive subjects would enable more specific confirmation of vascular GEFs activity. Nevertheless, we proposed that the use of PBMC can represent a relevant and useful functional assay of GEF activity in humans.

Acknowledgments

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Disclosures

None.

References


Figure 4. Activation of the renin–angiotensin system by low-sodium/high-potassium diet increases the activity of RhoA-Rho kinase (A) and Arhgef1 (B) in human peripheral blood mononuclear cells (PBMC). Graphs show individual changes in Rho kinase activity and Arhgef1 phosphorylation in PBMC collected at day 0 and day 7 of low-sodium/high-potassium diet. Rho kinase activity was assessed by the ratio of myosin phosphatase target (MYPT) phosphorylation to MYPT expression and Arhgef1 phosphorylation was measured as the ratio of Tyr phosphorylated Arhgef1 to total Arhgef1. Red symbols corresponded to mean. *P<0.05.


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Supplemental Data

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Running title: Ang II activates Arhgef1 in humans
**Figure S1.** Scatter plots showing the relationships between various parameters measured before (D0, empty circle) and after 7 days of low-sodium/high potassium diet (D7, black circle). Parameters analyzed are indicated above each graph. Rock activity is determined by the ratio P-MYPT/MYPT and Arhgef1 phosphorylation corresponds to the ratio P-Arhgef1/Arhgef1. ∆ Arhgef1 phosphorylation and ∆ Rho kinase activity are estimated by the ratio of Arhgef1 phosphorylation at D7 to Arhgef1 phosphorylation at D0 and the ratio of Rho kinase activity at D7 to Rho kinase activity at D0, respectively.
Figure S2. Arhgef1 phosphorylation, Arhgef1 and AT1 receptor expression in human PBMC before (D0) and after (D7) 7 days of low-sodium/high potassium diet. A. Arhgef1 phosphorylation. Individual values of Arhgef1 phosphorylation at D7 have been expressed relative to their level at day 0 set as 1 (*P < 0.05). B. Arhgef1 expression. Graphs show individual Arhgef1 expression in PBMC collected at D0 and D7 (left; Red symbols corresponded to means) and mean of individual values of Arhgef1 level at D7 expressed relative to their level at day 0 set as 1 (right). C. Representative blots of Arhgef1 phosphorylation and expression, and β-actin expression at D0 et D7 in PBMC from four different subjects. D. AT1R protein and mRNA expression. Graphs show mean of individual values of AT1R level at D7 expressed relative to their level at day 0 set as 1. Arhgef1 and ATR1 expression have been normalized to β-actin. AT1R mRNA expression has been normalized to HPRT1 mRNA expression.