Ste20-Related Kinase SLK Phosphorylates Ser188 of RhoA to Induce Vasodilation in Response to Angiotensin II Type 2 Receptor Activation

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Abstract—The small G protein Rho signaling pathways are recognized as major regulators of cardiovascular functions, and activation of Rho proteins appears to be a common component for the pathogenesis of hypertension and vascular proliferative disorders. Recent evidence suggests that modulation of Rho protein signaling by phosphorylation of Rho proteins provides an additional simple mechanism for coordinating Rho protein functions. Phosphorylation of RhoA by cAMP- or cGMP-activated kinase on Ser188 induces cytosolic sequestration of RhoA through increased interaction with guanine dissociation inhibitor, thereby resulting in inhibition of RhoA-dependent functions. Here we show that stimulation of angiotensin II (Ang II) type 2 receptor (AT2R) in vascular smooth muscle cells induces Ser188 phosphorylation of RhoA independently of cAMP- or cGMP-activated kinase. We identify the Ser/Thr kinase Ste20-related kinase SLK as a new kinase phosphorylating RhoA on Ser188. Activation of the signaling cascade involving Src homology 2 domain–containing protein-tyrosine phosphatase 1, casein kinase II and SLK is responsible for RhoA phosphorylation and inhibition of RhoA-mediated arterial contraction induced by AT2R activation. These results thus identify the molecular mechanism linking AT2R to RhoA inhibition and vasodilation. (Circ Res. 2008;102:1265-1274.)

Key Words: Rho ■ signal transduction ■ phosphorylation ■ angiotensin II ■ vascular smooth muscle

RhoA is a member of the Rho protein family that has been identified as an essential regulator of vascular smooth muscle cell functions. Through the activation of its target Rho kinase, RhoA is the major regulator of the tonic component of vascular smooth muscle cell contraction and plays a critical role in the control of vascular smooth muscle differentiation, proliferation, and migration.1 Subsequent studies have demonstrated the participation of the RhoA/Rho kinase signaling pathway in several vascular pathologies, including hypertension, coronary artery spasm, effort angina, atherosclerosis, and restenosis.1,2 Indeed, although basal RhoA activity is required for homeostatic functions in physiological conditions, its sustained overactivation has pathological consequences in the vascular system, particularly in vascular smooth muscle cells. Activation of RhoA-dependent pathways is involved in excessive contraction, and thereby increases blood pressure but also in excessive cell growth and migration that participate in pathological cardiovascular remodeling.1

RhoA acts as a molecular switch. In the inactive GDP-bound form, RhoA is locked in the cytosol by guanine dissociation inhibitors (GDIs). In the active GTP-bound form released from GDI, RhoA translocates to plasma membrane where it interacts with effectors to transduce the signal downstream. GTPase-activating proteins then turn off activation. In addition to this regulation, we and others have demonstrated that phosphorylation/dephosphorylation cycle also controls RhoA activity.3 Cyclic GMP-dependent protein kinase (PKG) or cAMP-dependent protein kinase A (PKA) phosphorylate Ser188 of RhoA.4,5 Both in vitro and in vivo experiments indicated that Ser188 phosphorylation of RhoA induces increased association to GDI, leading to cytosolic accumulation of RhoA,4 and inhibition of RhoA-mediated functions.5 RhoA phosphorylation thus appears as a simple mechanism that could be used to control the dynamics of RhoA protein actions and to permit specific termination of RhoA protein signals.

It has been recently shown that inactivation of RhoA/Rho kinase signaling pathway may play a role in the vasodilation induced by angiotensin II (Ang II) type 2 receptor (AT2R) activation.7 Although most physiological responses triggered by Ang II such as vascular smooth muscle cell contraction, growth and inflammation have been ascribed to Ang II type 1 receptor (AT1R) activation, accumulating evidence indi-
cates that AT1R antagonizes the effects of the AT2R, especially by inducing vasodilation, antagrowth and antiinflammatory actions.9 Coupling of AT1R to intracellular signaling pathways is less well understood than that of AT2R and seems to depend on the cell type. Three major cascades can be activated following AT1R stimulation including: (1) activation of protein phosphatases, in particular Src homology 2 domain–containing protein-tyrosine phosphatase 1 (SHP-1); (2) regulation of the nitric oxide/cGMP system; and (3) stimulation of PLA2 and release of arachidonic acid.9

Here we directly analyze the effect of Ang II on serine phosphorylation of RhoA both in vitro in vascular smooth muscle cells and in/ex vivo in artery samples. We describe the SHP-1/caseine kinase II (CK2)/Ste20-related kinase (SLK) pathway as a new signaling cascade activated following AT1R stimulation and identify SLK as a novel kinase phosphorylating RhoA on Ser188. SHP-1–dependent SLK–mediated RhoA phosphorylation is responsible for AT1R–induced vasodilation and may contribute to the antihypertensive effects of AT1R inhibitors.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Cell Culture and Transfection

Rat aortic smooth muscle cells were isolated by enzymatic dissociation. The different constructions used and small interfering (si)RNA were transfected by electroporation (Nucleofector, Amaxa). Protein extracts form cell cultures were used for coimmunoprecipitation and Western blot analysis.

Recombinant Protein Expression and Kinase Assays

GST-WT-RhoA and GST-S188A-RhoA were expressed in Escherichia coli and purified as previously described.5 Phosphorylation of recombinant RhoA was determined using immunoprecipitated SLK and SLK mutants. SLK activity was assessed by measuring the extent of SLK autophosphorylation.

Measurement of RhoA/Rho Kinase Activity

Activation of the RhoA/Rho kinase pathway was monitored by Western blot analysis of the phosphorylation level of the Rho kinase target myosin phosphatase target subunit 1 (MYPT).

Contraction Measurement

Thoracic aorta rings were transfected with siRNA for 48 hours, then suspended under isometric conditions and connected to a force transducer (Pioden Controls Ltd).

Animal Model

Male WKY rats and SHR (250 g) were treated for 2 weeks with placebo or AT1R antagonist (candesartan; 2 mg/kg per day) in their drinking water. All experiments were conducted in accordance with institutional guidelines for the care and use of laboratory animals.

Statistics

All results are expressed as the means±SD of sample size n. Significance was tested by ANOVA or Student t test.

Results

AT1R Stimulation Induces Serine Phosphorylation of RhoA

RhoA phosphorylation, association to GDI, activation, and expression were analyzed in rat aortic smooth muscle cells stimulated with Ang II (0.1 μmol/L) for 1 to 360 minutes. Figure 1A shows that serine-phosphorylated RhoA was detected under control condition. Ang II induced a gradual increase in the amount of serine-phosphorylated RhoA, associated with an increased association to GDI (7.3±0.2 fold over control at 360 minutes, n=3), indicating cytosolic relocation of RhoA. Analysis of MYPT phosphorylation indicates that Ang II also induced activation of RhoA/Rho kinase, with 2 peaks of activation at 5 (2.8±0.1 fold over control, n=3) and 60 to 120 minutes (3.0±0.1 fold over control, n=3).

In the presence of the AT1R inhibitor losartan, Ang II–induced increase in MYPT phosphorylation was totally inhibited, but the Ang II–induced phosphorylation of RhoA and increased GDI association were still present and even more pronounced for short Ang II applications (Figure 1A and 1B). The increase in RhoA phosphorylation occurred in a concentration-dependent manner (additional data, Figure 1). Ang II–mediated increase in RhoA phosphorylation and association to GDI was abolished in the presence of the AT2R antagonist PD123319 (Figure 1C) and was mimicked by stimulation with the AT2R agonist CGP42112A (Figure 1D). In cells stimulated with CGP42112A for 360 minutes, the amount of serine-phosphorylated was increased by 7.6±0.2-fold over control (n=3), and the association to GDI enhanced by 7.2±0.2-fold over control (n=3). In contrast, CGP42112A did not stimulate MYPT phosphorylation and Ang II–mediated increase in MYPT phosphorylation was still observed in the presence of PD123319 (Figure 1C and 1D). This pharmacological analysis thus suggests that whereas Ang II activates the RhoA/Rho kinase pathway through its interaction with AT1R, stimulation of AT2R increases RhoA phosphorylation and its interaction with cytosolic GDI. This conclusion is supported by Western blot analysis and quantitative RT-PCR experiments, demonstrating the expression of both AT1R and AT2R in cultured smooth muscle cells (Figure 1E).

To further confirm this result, we used specific siRNA to selectively knockdown AT1R and AT2R. In cells treated with AT1R siRNA, a strong increase in RhoA phosphorylation was still produced by Ang II (Figure 1F). In contrast, siRNA-mediated AT2R silencing completely prevented Ang II–induced RhoA phosphorylation (Figure 1F). Therefore, in addition to pharmacological data, specific genetic manipulation of AT1R and AT2R demonstrates that Ang II–induced RhoA phosphorylation is mediated by AT2R. This role of AT2R was also confirmed in CHO cells that overexpressed AT1R but did not express AT2R (additional data, Figure II).

Ang II Signaling Regulates RhoA Phosphorylation In Vivo

Chronic pharmacological inhibition of AT1R is associated with Ang II and AT1R upregulation.10 If AT1R–mediated serine phosphorylation of RhoA is a physiological process regulating RhoA-dependent function in vivo, it is thus expected that the amount of phosphorylated RhoA is increased in animals treated with AT1R inhibitor. To assess this hypothesis, we analyzed RhoA phosphorylation and RhoA binding to GDI in pulmonary artery and aorta of candesartan-
treated normotensive (WKY) and spontaneously hypertensive rats (SHR). Serine-phosphorylated RhoA is detected under control condition both in WKY and SHR (Figure 2A). The amount of serine-phosphorylated RhoA immunoprecipitated from pulmonary artery and aorta of candesartan-treated SHR and WKY rats and its association to GDI was strongly increased compared to their controls (Figure 2A). These results thus provide evidence that phosphorylation and cyto-

Figure 1. AT_2R stimulation induces RhoA phosphorylation. A through D, RhoA phosphorylation, RhoA/GDI interaction, RhoA, MYPT, and MYPT phosphorylation in aortic smooth muscle cells stimulated for 1 to 360 minutes by Ang II (0.1 μmol/L) under control condition (A), in the presence of the AT_1R inhibitor losartan (1 μmol/L) (B), in the presence of the AT_2R inhibitor PD123319 (1 μmol/L) (C), and in cells stimulated by the AT_2R agonist CGP42112 (0.1 μmol/L, 1 to 360 minutes) (D). E, Western blot for AT_1R and AT_2R expression in 2 different batches of aortic smooth muscle cells and relative mRNA expression of AT_1aR, AT_1bR, and AT_2R determined by quantitative RT-PCR. F, Western blot for RhoA phosphorylation and RhoA level, AT_1R and AT_2R expression in cells treated with scramble siRNA, AT_1R siRNA, and AT_2R siRNA, stimulated by Ang II (1 μmol/L; 5 to 60 minutes). RhoA phosphorylation was expressed relative to the phosphorylation level obtained in the absence of agonist stimulation in each experimental condition. The data presented are representative of 3 independent experiments. #P<0.01, *P<0.001.
solic localization of RhoA in arteries are regulated by Ang II signaling pathway in vivo.

AT2R Stimulation Induces Phosphorylation of RhoA on Ser188

The only serine residue shown to be subjected to phosphorylation in the RhoA sequence is the Ser188.4,5 We thus analyzed whether the Ser188 was the site for AT2R-mediated RhoA phosphorylation by expressing hemagglutinin (HA)-tagged wild-type RhoA (WT-RhoA) or Ser188 phospho-resistant (S188A-RhoA) or Ser188 phospho-mimetic (S188E-RhoA) RhoA mutants in aortic smooth muscle cells. As expected, WT-RhoA was phosphorylated on serine by Ang II, in association with its increased interaction with cytosolic GDI (Figure 2B). Under basal conditions, S188E-RhoA showed an enhanced association to GDI, but neither its binding to GDI nor its serine phosphorylation was increased by Ang II stimulation (Figure 2B). In agreement with this observation, the level of serine phosphorylation and interaction with GDI of S188A-RhoA was not changed in presence of Ang II. These data indicate that AT2R stimulation induces phosphorylation of RhoA on Ser188.

AT2R-Induced Ser188 Phosphorylation of RhoA Does Not Need NO Synthase, PKA, or PKG Activity

The involvement of NO synthase (NOS) and PKG activity in Ang II–mediated Ser188 RhoA phosphorylation has been assessed by the use of their respective inhibitors L-N-g-nitro arginine (L-NNA) and Rp-8-Br-PET-cGMPs. The increase in RhoA phosphorylation induced by Ang II for was not affected by NOS or PKG inhibition (5.0±0.3-fold over control versus 5.4±0.2 in control, n=4, P>0.5; Figure 3A). Western blot using an anti-PKG antibody on immunoprecipitated RhoA showed that PKG, associated with RhoA under basal condition, was released by AT2R stimulation (Figure 3B). In similar experiments, the use of H-89 revealed that inhibition of PKA did not modify AT2R stimulation–induced RhoA phosphorylation (Figure 3C), and the same observation was made in the presence of both PKG and PKA inhibitors (data not shown). Examination of PKA/RhoA interaction indicated that PKA interacted with RhoA under control condition but AT2R stimulation abolished this interaction (Figure 3D). The concentration of PKG and PKA inhibitors used efficiently inhibited PKG and PKA activity (additional data, Figure III). These results thus provide evidence that AT2R-induced increase in RhoA phosphorylation is not mediated through the activation of NOS, PKG, or PKA. The involvement of other kinases such as phosphatidylinositol 3-kinase, MAPK/ERK kinase (MEK1/2), and protein kinase C has also been ruled out by the use of LY 294006, PD98059, and U0126, and GF109203X, respectively (Figure 3E). In our experimental conditions, the efficiency of LY294006 has been checked by measuring Akt phosphorylation, PD98059 and U0126 by measuring ERK1/2 phosphorylation and GF109203X, by measuring CPI-17 phosphorylation (data not shown).

The involvement of serine phosphatase activity in AT2R-induced Ser188 phosphorylation of RhoA revealed that AT2R-induced serine phosphorylation of RhoA could not result from reduction of phosphatase activities, which, in fact, are increased by AT2R stimulation (Figure IV in the online data supplement).

SLK Associates to RhoA

SLK is a serine/threonine kinase ubiquitously expressed in adult tissues. Expression of active SLK induced loss of actin stress fibers and, therefore, mimicked the effects of RhoA phosphorylation.4,11 This observation thus prompted us to select SLK as a potential RhoA-phosphorylating kinase. Western blot analysis indicated that SLK was present in vascular smooth muscle cells (Figure 4A). Specificity of the immunoreactive band has been checked (supplemental Figure V). Coimmunoprecipitation experiments further revealed that SLK interacted with RhoA in vascular smooth muscle cells, and the amount of SLK that coimmunoprecipitated with RhoA was not significantly modified by AT2R stimulation (Figure 4B and supplemental data Figure VI).
SLK Phosphorylates RhoA on Ser188

An in vitro kinase assay was performed using recombinant RhoA as substrate. WT-SLK and the active 1-373-SLK but not the kinase-dead mutant K63R-SLK induced phosphorylation of RhoA, indicating that RhoA was indeed a substrate for SLK (Figure 4C). The use of S188A-RhoA mutant prevented phosphorylation of RhoA by SLK and 1-373-SLK, indicating that SLK-mediated RhoA phosphorylation occurred on Ser188 (Figure 4C).

To investigate whether the phosphorylation of RhoA following AT1R stimulation was mediated by SLK, phosphorylation of endogenous RhoA has been analyzed in smooth muscle cells expressing WT-SLK and SLK mutants (Figure 4D). AT1R stimulation induced RhoA phosphorylation in cells expressing WT-SLK but not in cells expressing the inactive K63R-SLK mutant. On the opposite, expression of the active 1-373-SLK strongly increased the basal level of RhoA phosphorylation, which was not further increased on
AT₂R stimulation. A similar result was obtained with the SS/AA-SLK mutant. Serine phosphorylation of SLK at position 347/348 downregulates its kinase activity and the phosphorylation-resistant SS/AA-SLK mutant displayed a high kinase activity. These in vitro and in-cell results thus suggest that AT₂R-induced Ser188 phosphorylation of RhoA is mediated by SLK.

**AT₂R Stimulation Induces Upregulation of SLK Kinase Activity**

To further confirm the role of SLK in AT₂R-induced RhoA phosphorylation, we directly measured the effect of AT₂R stimulation on SLK activity by performing SLK kinase assays. SLK kinase activity was increased by 1.5- to 3-fold in cells stimulated with Ang II (Figure 4E). This effect was inhibited in the presence of PD123319, indicating that the stimulatory action of Ang II on SLK activity is mediated by AT₂R stimulation.

**AT₂R Stimulation Induces Decrease of SLK Phosphorylation**

The observation that the phosphorylation resistant mutant SS/AA-SLK increased RhoA phosphorylation and prevented further action of AT₂R stimulation on RhoA phosphorylation prompted us to hypothesize that AT₂R-induced stimulation of SLK activity could result from its dephosphorylation. To address this hypothesis, we examined the level of serine phosphorylation of immunoprecipitated SLK and SLK mutants. Under basal conditions, SLK and K63R-SLK mutant, but not the SS/AA-SLK mutant, are phosphorylated on serine residues, indicating that the phosphorylated serine residues recognized by the anti-phosphoserine antibody are the serine residues at position 347/348 and that these residues are basally phosphorylated. Stimulation of AT₂R produced a 5- to 8-fold decrease in the level of SLK and K63R-SLK phosphorylation (Figure 5A). No change was observed for the SS/AA-SLK mutant (Figure 5A). These results indicate that SLK is basally phosphorylated on serine at position 347/348 in vascular smooth muscle cells and that stimulation of AT₂R decreased phosphorylation of these serine residues, thus suggesting that AT₂R-induced stimulation of SLK kinase activity results from the release of the inhibitory action of the phosphoserine at position 347/348.

**AT₂R Stimulation–Induced RhoA Phosphorylation Involves CK2**

CK2 has been identified as the kinase that phosphorylates SLK on serine residues 347 and 348. If CK2 is the kinase responsible for the basal phosphorylation of SLK in vascular smooth muscle cells, it is expected that inhibition of CK2 would lead to increase SLK kinase activity and, in turn, increase RhoA phosphorylation. The CK2 inhibitor I induced a 4- to 7-fold increase in RhoA phosphorylation in cells expressing SLK (Figure 5B). Under this condition, AT₂R stimulation was no longer able to induce further increase in...
RhoA phosphorylation (data not shown). This effect was not observed in cells expressing the inactive K63R-SLK mutant, indicating that the increased RhoA phosphorylation induced by CK2 inhibition was attributable to stimulatory effect on SLK activity. This result has been confirmed by the use of siRNA targeting CK2 that produced a 4-fold increase in RhoA phosphorylation in cells expressing SLK but not in cells expressing the K63R-SLK mutant (supplemental Figure VI).

AT2R Stimulation–Induced SHP-1 Activation Mediates Dephosphorylation of CK2
As CK2 inhibition mimicked the effect of AT2R stimulation on RhoA phosphorylation, it could be envisaged that decreased phosphorylation and activation of SLK induced by CK2 inhibition was attributable to stimulatory effect on SLK activity. This result has been confirmed by the use of siRNA targeting CK2 that produced a 4-fold increase in RhoA phosphorylation in cells expressing SLK but not in cells expressing the K63R-SLK mutant (supplemental Figure VI).

AT2R Stimulation–Induced RhoA Phosphorylation Depends on SHP-1 Activity
We then directly analyzed the involvement of SHP-1 in the mechanism coupling AT2R stimulation to SLK-mediated RhoA phosphorylation. As expected, if SLK activity were indirectly upregulated by AT2R activation through SHP-1–mediated CK2 dephosphorylation, siRNA-mediated SHP-1 silencing abolished AT2R stimulation–induced RhoA phosphorylation (Figure 5E). This result was further confirmed by expression of catalytically inactive C453S-SHP-1 and D419A-SHP-1 mutants that completely inhibited AT2R stimulation–induced RhoA phosphorylation (Figure 5F). These results thus suggest that AT2R-induced dephosphorylation of CK2 through SHP-1.

Essential Role of RhoA in AT2R Stimulation-Induced Vasodilation
To assess the functional consequence of the SHP-1–dependent SLK-mediated RhoA phosphorylation induced by AT2R stimulation, we performed contraction measurements in aorta rings from candesartan-treated SHR rats (Figure 6). As previously reported, increasing concentrations of Ang II—
induced relaxation of aorta rings precontracted by noradrenaline (NA) (Figure 6A). This effect was abolished in the presence of the AT2R inhibitor PD123319 (Figure 6A) and in rings pretreated with AT2R siRNA (supplemental Figure VIII). NA-induced contraction resulted of both a calcium-dependent component, activated by a rise in intracellular calcium concentration, and a RhoA-dependent component, corresponding to the calcium sensitization of contractile protein. To identify which component was inhibited by AT2R stimulation, we selectively suppressed the RhoA-dependent component of the NA-induced contraction by a 48-hour treatment of aorta rings with siRNA targeting RhoA. Western blot analysis confirmed that siRNA efficiently produced 80% to 100% inhibition of RhoA expression (Figure 6B). Under these conditions, the amplitude of the NA-induced contraction was reduced by 40% to 50%, and the remaining contraction was not modified in the presence of the Rho kinase inhibitor Y-27632 (10 μmol/L, not shown). This calcium-dependent NA-induced contraction was not relaxed by AT2R stimulation, indicating that AT2R stimulation-induced relaxation completely depended on inhibition of the RhoA-dependent component of the contraction (Figure 6B). To further assess the involvement of the SHP-1/SLK signaling pathway, similar experiments were performed after treatment of aortic rings with siRNA targeting SHP-1 and SLK. Treatment with SHP-1 siRNA and SLK siRNA did not modify the amplitude of the NA-induced contraction but completely prevented the relaxing effect of AT2R stimulation (Figure 6C and 6D). These results thus support the essential role of SHP-1–dependent SLK-mediated RhoA phosphorylation in the relaxing effect of AT2R stimulation.

Discussion

Our work identifies SLK as a new serine-threonine kinase that phosphorlylates Ser188 of RhoA and establishes a novel signaling cascade downstream to AT2R stimulation. In vascular smooth muscle cells, AT2R stimulation by Ang II activates SLK through a coupling mechanism involving SHP-1 and CK2. This signaling pathway is responsible for AT2R stimulation–mediated vasodilation (Figure 7).

Although it is widely accepted that AT2R accounts for a large part of the cardiovascular effects induced by Ang II, an increasing body of evidence indicates that AT2R also contributes to the regulation of blood pressure and renal function.9 Stimulation of AT2R undoubtly induces relaxation in several vascular territories.15 Studies in animal models of hypertension have revealed that AT2R is upregulated and mediates vasodilation only when AT1Rs are blocked, suggesting that AT1R participates in the mechanisms, whereby Ang II receptor antagonism lowers blood pressure.7 Recent study confirms these data in human by showing that AT1R-Rs are upregulated and contribute to Ang II–induced vasodilation in resistance arteries of hypertensive diabetic patients treated with AT1R blockers.16 Although the intracellular signaling pathways downstream to AT2R are now fully defined, several mechanisms have been proposed to participate to AT2R-mediated relaxation/vasodilation.15 Both endothelium-independent and endothelium-dependent AT2R-mediated vasodilations have been described, the latter being associated with stimulation of NO/cGMP pathway, either directly or indirectly through the increased release of bradykinin.15,17 It has been proposed that AT2R stimulation blocked the Na+-H+ exchanger, promoting intracellular acidosis, which, in turn, activates kininogenases in endothelial and smooth muscle cells to cleave bradykinin from intracellularly stored kininogens.18 Recently, the inhibition of the RhoA/Rho kinase signaling pathway has been suggested to play a role in AT2R-induced vasodilation.7 In conditions associated with AT2R upregulation (valsartan treated-hypertensive rats), Ang II–induced vasorelaxation is associated with reduced RhoA/Rho kinase activation that could result from phosphorylation of RhoA on Ser188.19 Stimulation of NOS activity and activation of PKG, the expression of which is increased in hypertensive rats treated with AT1R blockers, have been proposed to mediate AT1R-induced downregulation of RhoA/Rho kinase pathway.20 However, neither the level of RhoA phosphorylation nor the involvement of PKG have been directly assessed in these studies.

By using arteries from a similar experimental model, our results obtained by siRNA-mediated RhoA silencing confirm that AT2R-induced relaxation is attributable to inhibition of the RhoA-dependent component of the contraction.7 Our results show that AT2R stimulation indeed induced RhoA phosphorylation in vascular smooth muscle cells. Through this mechanism, AT1Rs counteract the activation of RhoA/Rho kinase mediated by vasoconstrictors, in particular by AT1R stimulation. On the other hand, AT2R seems to oppose to AT2R-mediated RhoA phosphorylation because RhoA phosphorylation induced by Ang II in the presence of losartan, or by the AT1 agonist CGP42112, is increased and occurred earlier. The effect of Ang II stimulation on RhoA phosphorylation and RhoA/Rho kinase activation is therefore
likely to reflect the balance of AT$_1$R/AT$_2$R expression in given artery and condition.

In this study, we show that RhoA phosphorylation is responsible for the AT$_2$R-induced relaxation. However, as the RhoA/Rho kinase pathways is also involved in a wide range of cellular processes, it could be supposed that AT$_2$R-mediated inhibition of the RhoA/Rho kinase pathway also participates to other AT$_2$R-mediated actions. Interestingly, among the numerous targets of the RhoA/Rho kinase pathway is the Na$^+$--H$^+$ exchanger NHE1, which is activated by Rho kinase. The inhibition of RhoA/Rho kinase, resulting from AT$_2$R-mediated RhoA phosphorylation may therefore participate to the inhibition of the Na$^+$--H$^+$ exchanger observed in response to AT$_2$R stimulation.

Our analysis of the molecular mechanisms linking AT$_1$R to RhoA in vascular smooth muscle cells did not confirm the role of NO and PKG previously suggested in AT$_2$R-induced RhoA phosphorylation. Here, we demonstrate that inhibition of the RhoA-dependent contraction through SHP-1-- and SLK-dependent mechanism is responsible for the relaxing effect of AT$_2$R stimulation. This observation thus supports the major role of SHP-1--dependent SLK-mediated RhoA phosphorylation and consequent RhoA/Rho kinase inhibition in the vasodilator action of AT$_2$R.

SHP-1 has been identified as an early transducer in AT$_2$R signaling. SHP-1 is involved in AT$_2$R-mediated MAPK cascades inhibition; however, its contribution to AT$_1$R-dependent processes that are independent of MAPK pathways remained questioned. By showing that SHP-1 is a key mediator of AT$_2$R-mediated RhoA phosphorylation and relaxation, our results reinforce the large importance of SHP-1 as general signal transducer of AT$_2$R and suggest that other upstream signals that lead to SHP-1 activation may also modulate RhoA phosphorylation. Stimulation of AT$_2$R promotes apoptosis of cultured vascular smooth muscle cells and SHP-1 has been shown to play pivotal role in the proapoptotic effect of AT$_2$R stimulation. SLK was also found to induce apoptosis in fibroblasts. Our observation that AT$_2$R stimulation--mediated SLK activation depended on SHP-1 may suggests that SLK could also be involved in the proapoptotic effect of AT$_2$R activation in vascular smooth muscle cells.

In conclusion, we identify SLK as a new kinase that regulates RhoA signaling and vascular smooth muscle contraction and describe a novel signaling pathway that negatively controls RhoA-dependent functions by phosphorylation of RhoA on Ser188. This signaling pathway involving SHP-1, CK2, and SLK is responsible for AT$_2$R-induced vasodilation by inhibiting RhoA/Rho kinase-dependent contraction. Because blockade of AT$_2$R increases both the plasma level of Ang II and the expression of AT$_1$R, treatment with AT$_2$R inhibitors presently used in clinical practice may lead to AT$_2$R stimulation. By showing that AT$_2$R activation induces Rho kinase inhibition, our results contribute to a better understanding of mechanisms whereby AT$_1$R Rs add to the antihypertensive effects of AT$_2$R inhibitors. Our results also suggest that directly targeting the AT$_2$R in cardiovascular disease would be useful.

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Disclosures

None.

References


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Ste20-related kinase SLK phosphorylates Ser 188 of RhoA to induce vasodilation in response to Angiotensin II type 2 receptor activation

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Materials and Methods

Cell Culture and transfection

Rat aortic smooth muscle cells were isolated by enzymatic dissociation. Cells were cultured in DMEM with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Smooth muscle cells at passage 2 or 3 were used in this study. The different constructions used were transfected by electroporation (nucleofector, Amaxa) according to the manufacturer's instructions, leading to transfection efficiency of 60-80%. Twenty four hours after transfection, the culture medium is replaced by fresh culture medium and cells are treated for different time, harvested and analyzed by Western Blotting. For RhoA, DNA vectors corresponding to pKH3-HA-RhoA (WT-RhoA), pKH3-HA-RhoA-S188A (S188A-RhoA) and pKH3-HA-RhoA-S188A (S188E-RhoA) were used. For transfection of wild type and mutant forms of SLK, pcDNA3-Myc-SLK (WT-SLK), pcDNA3-Myc-SLK (K63R-SLK, kinase dead SLK), pcDNA3-Myc-1-373-SLK (1-373-SLK, activated SLK) and pcDNA3-Myc-S347A/S348A-SLK (SS/AA-SLK, phosphorylation resistant SLK) expression vectors were used. SHP-1 plasmids used were pSVL-SHP-1 for wild-type SHP-1 (WT-SHP-1), and two dominant negative, phosphatase-inactive SHP-1 mutants: pSVL-D415A-SHP-1 (D419A-SHP-1) and pSVL-C453S-SHP-1 (C453S-SHP-1). The C453S-SHP-1 mutant is characterized as a substrate trapping SHP-1 mutant. These SHP-1 constructs were kindly
siRNA

The siRNA were introduced into vascular smooth muscle cells by electroporation (Nucleofector, Amaxa) according to the manufacturer's instructions. For ex-vivo experiments in arterial rings, siRNA were introduced using cationic polymer transfection reagent for 48 hours (Jet Pei Polyplustransfection, Ozyme, France). The sense and anti-sense strands of siRNAs (Eurogentec, Seraing, Belgium) used were: AT1R, sense 5'-GCGUGAGCUUCAACCUCUAdTdT-3', antisense 5'-UAGAGGUUGAAGCUCACGCdTdT-3'; AT2R, sense 5'-AUGCCAACACAACAGCAGCdTdT-3', anti-sense 5'-GCUGCUGUUGUGUUGGCAUdTdT-3'; RhoA, sense 5'-GAAGUCAAGCAUUUCUGUCdTdT-3', anti-sense 5'-GACAGAAUUGCUUGACUUCdTdT-3'; SHP-1, sense 5'-CAGAGCGUGUGGAGUAUUAdTdT-3', anti-sense 5'-UAUUUGUGAUAUUGAUGGCdTdT-3'; CK2, sense 5'-GCAUCAAAUAUCACAAAAdTdT-3', anti-sense 5'-UAUUUGUGAUAUUGAUGGCdTdT-3' and scrambled, sense 5'-GCCUGUGAUGACUACAGACdTdT-3', anti-sense 5'-GUCUGUAGUCAUCACAGGCdTdT-3'.
Coimmunoprecipitation and Western Blot Analysis

The use of anti-Ser antibody to detect phosphoSer RhoA in immunoprecipitated RhoA is the sole method allowing the observation of the phosphorylation of endogenously expressed RhoA. The commercially available anti-phospho-Ser188-RhoA antibody does not detect the native prenylated form of RhoA. Cells or tissues were harvested in NETF buffer (100 mmol/L NaCl, 2 mmol/L EGTA, 50 mmol/L Tris-Cl pH 7.4 and 50 mmol/L NaF) containing 1% NP-40, 2 mmol/L orthovanadate, protease inhibitors and phosphatase inhibitor cocktail (Sigma). Samples were precleared with 40 µl of protein G-sepharose beads, and immunoprecipitations were carried out with monoclonal anti-HA antibody, or monoclonal anti-RhoA antibody preadsorbed on protein G-sepharose beads. The protein G-sepharose-bound immune complexes were washed twice in NETF buffer containing NP-40 (1% w/v) and once in NETF without detergent. Total protein samples and pellets from the immunoprecipitations were heated at 95 °C for 5 min in Laemmli sample buffer for SDS-polyacrylamide gel electrophoresis and analyzed by immunoblot for phosphoserine, RhoA, RhoGDI and/or HA. Signals from immunoreactive bands were detected by ECL (Amersham) and quantified using QuantityOne (BioRad).

RNA isolation and real-time RT-PCR

Total RNA was extracted using TRIzol Reagent (Life Technologies SARL, Cergy Pontoise, France) and genomic DNA contamination was eliminated with ribonuclease-free deoxyribonuclease (DNase) I (Life Technologies). Total RNA samples (1 µg) were reverse transcribed into cDNA by the M-MLV reverse transcriptase (Life Technologies). The resulting cDNA was subjected to real-time PCR on the iCycler iQ™ Detection System (Bio-Rad S.A., Marnes la Coquette, France) using SYBR green as fluorescent probe (Molecular Probes, Eugene, OR, USA). Amplifications were performed in triplicate with specific primers.
for rat AT1aR, AT1bR, AT2R and GAPDH mRNA. The equivalent amplification efficiency of each primer pair has been checked. The sequence of the primers used are: AT1aR: sense 5’-TCTTACCGGCCTTCGGATAACA-3’, antisense 5’-CAGGGTGAATGGTCCTTTGGTC-3’; AT1bR sense 5’-TGCCCTGGCTGATTTATGCTTT-3’, antisense 5’-CAGGTAGCGATCGATGCTGAGA-3’; AT2R sense 5’-CCGGCAGATAAGCATTGGAAG-3’, antisense 5’-CACAGGTCCAAAGAGGCCAGTCA-3’; GAPDH sense 5’-AGTTAAAAGCAGCCCTGGTGA-3’, anti-sense-5’-GACAGTCAGCCGATCCTTCTT-3’. The expression level of GAPDH was used as a reference value to normalize Ang II receptor gene expression. Relative quantitative gene expression was calculated following the ??Ct-method and results were expressed by setting the AT1aR expression as 1.

Recombinant protein expression and In vitro kinase assay

GST-WT-RhoA and GST-S188A-RhoA were expressed in Escherichia coli and purified as previously described. Phosphorylation of recombinant RhoA was determined using SLK and SLK mutants that have been immunoprecipitated with anti-Myc antibody from cells transfected with pcDNA3-Myc plasmid encoding SLK, the active 1-373-SLK and the kinase dead K63R-SLK. The reaction was carried out in a phosphorylation buffer (50 mmol/L Tris, 10 mmol/L MgCl₂, 1 mmol/L DTT, 20 µmol/L ATP) and with 500 ng of RhoA substrate for 30 min at 30°C. The reaction was stopped by addition of cold phosphorylation buffer and samples were boiled in Laemmli buffer. Proteins were separated by in SDS-PAGE and phosphorylation was visualized by blotting with anti-phosphoserine antibody.
SLK kinase assays

SLK activity was assessed by measuring the extent of SLK autophosphorylation, as described previously. Total lysate from control cells or cells stimulated with Ang II in the presence of losartan (1 µmol/L), in the absence or presence of PD 123319 (1 µmol/L) was immunoprecipitated using 2 µg of anti-SLK antibody and 20 µl protein A-sepharose for 4 h at 4°C. Immunoprecipitates were washed three times with NETN (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA and 0.1% Nonidet P-40) and once with SLK kinase buffer (20 mmol/L Tris-HCl, pH 7.5, 15 mmol/L MgCl₂, 10 mmol/L NaF, 10 mmol/L β-glycerophosphate, and 1 mmol/L orthovanadate). Kinase reactions were initiated by the addition of 5 µCi of [γ-³²P]ATP. After a 30-min incubation at 30°C with agitation, the reaction mixtures were spotted onto Whatman P81 paper. The paper was washed three times for 5 min with 0.75% phosphoric acid. ³²P incorporation into substrate was then determined by Cerenkov counting.

Measurement of RhoA/Rho kinase activity

Activation of the RhoA/Rho kinase pathway has been monitored by western blot analysis of the phosphorylation level of the Rho kinase target myosin phosphatase target subunit 1 (MYPT1) on Thr-696 using a rabbit polyclonal anti-phospho-MYPT1 (Thr696).

Contraction measurement

Desendothelialized thoracic aorta rings from candesartan treated SHR were transfected with siRNA for 48 hours, then suspended under isometric conditions and connected to a force transducer (Pioden Controls Ltd). Dose-response curves to Ang II in the presence of losartan with or without PD123319 were performed on noradrenaline (NA, 1 µmol/L) precontracted aorta rings.
Animal model

Male WKY rats and SHR (250 g) were separated into 4 groups receiving for 2 weeks placebo or AT1R antagonist (candesartan; 2 mg/kg per day) in their drinking water. At completion of this time, the main pulmonary and the aorta were taken out and dissected under binocular. Tissues or aortic rings were then prepared as indicated for Western blot analysis, immunoprecipitations or contraction measurement.

Chemicals and Drugs

Mouse monoclonal anti-RhoA antibody (26C4), rabbit polyclonal anti-AT1R, anti-AT2R, anti-SHP-1 and rabbit polyclonal anti-phospho-MYPT1 (sc-17556) were purchased from Santa Cruz Biotechnology, Inc. (CA, USA). Mouse monoclonal anti-HA (HA.11) was purchased from Berkeley Antibody Co. (BabCO, Richmond, CA, USA). Rabbit monoclonal anti-phospho tyrosine (4G10) and rabbit polyclonal anti-RhoGDI antibodies were purchased from Upstate Biotechnology (Euromedex, Munolsheim, France). Rabbit polyclonal anti-phosphoserine was purchased from Zymed (InVitrogen, France). SLK was detected using an anti-SLK rabbit polyclonal antibody. Rabbit polyclonal anti-CK2 alpha was purchased from Cell Signaling (Ozyme, France). Candesartan was provided by AstraZeneca (Sweden). H-89 (N-[2-((p-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide was purchased from Calbiochem (Darmstadt, Germany). Rp-8-Br-PET-cGMPS was purchased from Biolog Life Science Institute (Bremen, Germany). All other reagents were purchased from Sigma (Saint-Quentin Fallavier, France).

Statistics
All results are expressed as the means±SD of sample size \( n \). Significance was tested by ANOVA or Student's \( t \) test.

**Supplemental results**

1. **Ang II does not induce RhoA phosphorylation in CHO cells lacking AT2R**

To further confirm the role of AT2R in Ang II-induced RhoA phosphorylation, we used CHO cells that over-expressed AT1R. Western blot analysis clearly demonstrated the expression of AT1R, while AT2R is not expressed in these cells (Additional data, Figure 1). Stimulation with Ang II induced activation of RhoA/Rho kinase, attested by the increased phosphorylation of MYPT. This effect was inhibited in the presence of losartan. No change in RhoA phosphorylation was observed in response to Ang II, in the absence or presence of losartan (Additional data, Figure 1). These observations are thus in agreement with those made in vascular smooth muscle cells and confirm that Ang II-induced RhoA activation is mediated by AT1R while Ang II-induced RhoA phosphorylation results from AT2R activation.

2. **Phosphatase activity**

*AT2R-induced Ser 188 phosphorylation of RhoA does not depend on phosphatase activity*

To assess whether the stimulation of RhoA phosphorylation induced by AT2R activation could be related to inhibition of serine phosphatase activity, we measured the global serine/threonine phosphatase activity in lysates from cells stimulated for 0-60 min by Ang II in the presence of losartan (Additional data, Figure 2). Ang II induced increase in serine/threonine phosphatase activity that was maximal after 10-30 min and then decreased (Additional data, Figure 2A). This effect was completely abolished in the presence of a non selective inhibitor of PP1, PP2A and PP2B protein phosphatase. The effect of protein phosphatase inhibitors was further addressed on Ang II-induced RhoA phosphorylation.
Inhibition of protein phosphatases PP1 and PP2A by okadaic acid (250 or 500 nM) induced a rise in the basal level of RhoA phosphorylation (1.4±0.1 and 1.5±0.1 fold over control, n=3, with 250 and 500 nM okadaic acid, respectively) (Additional data, Figure 2B). However, AT2R stimulation in the presence of okadaic acid was still able to further increase RhoA phosphorylation (3.4±0.2 and 3.8±0.2 fold over control in the presence of 250 and 500 nM okadaic acid, respectively, P<0.01, n=3). The PP2B inhibitor cyclosporin A also increased the resting level of RhoA phosphorylation (2.1±0.2 % and 2.3±0.1 % of control, n=3, with 10 and 100 µM cyclosporin A, respectively; Additional data, Figure 2B) and stimulation of the cells with Ang II induced additional increase in serine phosphorylation of RhoA (4.2±0.2 and 4.0±0.2 fold over control in the presence of 10 and 100 µM cyclosporin A, respectively, P<0.001, n=3; Additional data, Figure 2B). This result suggests that AT2R-induced serine phosphorylation of RhoA could not result from reduction of phosphatase activities which, in fact are increased by AT2R stimulation.

**Material and Methods for Measurement of serine/threonine phosphatase activity**

Cells were stimulated with 0.1 µM Ang II in the presence of losartan in the presence of losartan 1 µM with or without phosphatase cocktail inhibitor 1 (Sigma) and harvested with NETF buffer containing 1% NP-40. Samples were centrifuged at 7500 g for 10 min at 4°C and phosphatase activity assay (IQ phosphatase assay, Pierce, Perbio science, France) was performed with 50 µg of protein. Fluorescence was measured (victor2, PerkinElmer) to analyze the phosphatase activity.

3. Legends

**Additional data, Figure 1.** Concentration-response curve of Ang II-induced RhoA phosphorylation (Ang II: 1 nmol/L-0.1 µmol/L, 20 min; losartan 1 µmol/L). Maximal RhoA
phosphorylation is obtained in response to 0.1 μmol/L Ang II and the concentration producing the half-maximal effect is close to 3 nmol/L.

**Additional data, Figure 2.** Western blots showing AT1R and AT2R expression and MYPT phosphorylation in lysates, and phosphorylation of RhoA immunoprecipitated from CHO cells overexpressing AT1R but lacking AT2R. Cells were stimulated or not with Ang II (0.1 μmol/L) in the absence or presence of 1 μmol/L losartan. Ang II stimulation did not increase RhoA phosphorylation in the absence or in the presence of losartan, while it induced a losartan-sensitive increase in MYPT phosphorylation. Equal loading was confirmed by Western blot with anti-β-actin antibody in lysates, and anti-RhoA antibody in immunoprecipitates.

**Additional data, Figure 3.** Rp-8-Br-PET-cGMPS and H-89 inhibit RhoA phosphorylation induced by DETA NONOate and forskolin (FSK), respectively. Western blot showing RhoA phosphorylation in control and in cells stimulated with 100 μM/L DETA NONOate or 25 μM/L FSK, in the absence or in the presence of Rp-8-Br-PET-cGMPS (100 nmol/L) or H-89 (3.5 μM/L), respectively. The data presented are representative of at least three independent experiments.

**Additional data, Figure 4.** AT2R-induced Ser 188 phosphorylation of RhoA did not depend on phosphatase activity. A. Total serine/threonine phosphatase activity of aortic smooth muscle cells stimulated with 0.1 μmol/L Ang II for 0-60 min in the presence of 1 μmol/L losartan, in the absence (square) and presence of non-selective PP1, PP2A and PP2B protein phosphatase inhibitor (circle). B. Western blot showing RhoA phosphorylation in control and in cells stimulated with Ang II in the presence of losartan (1 μmol/L), in the absence and presence of okadaic acid (250 and 500 nmol/L) and cyclosporine A (10 and 100 nmol/L) Equal amount of RhoA was confirmed by Western blot with anti-RhoA antibody.
Additional data, Figure 5. Western blotting with anti-SLK antibody of lysate from COS cells used as negative control, and aortic smooth muscle cells (ASMC) treated with scramble or SLK siRNA.

Additional data, Figure 6. Western blotting with anti-SLK antibody of immunoprecipitated RhoA and total lysates from control cells and cells stimulated with Ang II (0.1 µmol/L; 1 µmol/L losartan). Taking into account the amount of lysate used for RhoA immunoprecipitation and the amounts loaded, quantification of the immunoreactive SLK bands in the immunoprecipitated and in lysates indicates that approximately 20% of SLK were bound to RhoA.

Additional data, Figure 7. Phosphorylation of endogenous RhoA in rat aortic smooth muscle cells expressing WT-SLK and K63R-SLK, treated with scramble or CK2 siRNA. Equal amount of RhoA was confirmed by Western blot with anti-RhoA antibody. Efficiency of siRNA-mediated CK2 silencing was verified by western blot with anti-CK2 antibody and equal loading was checked by western blot with anti-β-actin antibody in lysates.

Additional data, Figure 8. Relaxing effect of AT2R receptor stimulation. Concentration-response curves for the effect of increasing concentrations of Ang II in de-endothelialized aorta rings of candesartan-treated SHR pre-contacted by 1 µmol/L NA in control condition and after 48 h treatment with scramble and AT2R siRNA. Tension was expressed as a percentage of maximal tension recorded in response to NA before addition of Ang II. Western blot analysis was performed to check the efficiency of siRNA treatment under each condition. Each point represents a mean of 4–8 experiments.

4. References


Additional data Figure 1

Additional data Figure 2

Additional data Figure 3
Additional data Figure 4

Additional data Figure 5