Phosphorylation of Smooth Muscle 22α Facilitates Angiotensin II–Induced ROS Production Via Activation of the PKCδ-P47phox Axis Through Release of PKCδ and Actin Dynamics and Is Associated With Hypertrophy and Hyperplasia of Vascular Smooth Muscle Cells In Vitro and In Vivo

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Rationale: We have demonstrated that smooth muscle (SM) 22α inhibits cell proliferation via blocking Ras-ERK1/2 signaling in vascular smooth muscle cells (VSMCs) and in injured arteries. The recent study indicates that SM22α disruption can independently promote arterial inflammation through activation of reactive oxygen species (ROS)-mediated NF-κB pathways. However, the mechanisms by which SM22α controls ROS production have not been characterized.

Objective: To investigate how SM22α disruption promotes ROS production and to characterize the underlying mechanisms.

Methods and Results: ROS level was measured by dihydroethidium staining for superoxide and TBA assay for malondialdehyde, respectively. We showed that downregulation and phosphorylation of SM22α were associated with angiotensin (Ang) II–induced increase in ROS production in VSMCs of rats and human. Ang II induced the phosphorylation of SM22α at Serine 181 in an Ang II type 1 receptor–PKCδ pathway–dependent manner. Phosphorylated SM22α activated the protein kinase C (PKCδ-p47phox) axis via 2 distinct pathways: (1) disassociation of PKCδ from SM22α, and in turn binding to p47phox, in the early stage of Ang II stimulation; and (2) acceleration of SM22α degradation through ubiquitin-proteasome, enhancing PKCδ membrane translocation via induction of actin cytoskeletal dynamics in later oxidative stress. Inhibition of SM22α phosphorylation abolished the Ang II–activated PKCδ-p47phox axis and inhibited the hypertrophy and hyperplasia of VSMCs in vitro and in vivo, accompanied with reduction of ROS generation.

Conclusions: These findings indicate that the disruption of SM22α plays pivotal roles in vascular oxidative stress. PKCδ-mediated SM22α phosphorylation is a novel link between actin cytoskeletal remodeling and oxidative stress and may be a potential target for the development of new therapeutics for cardiovascular diseases. (Circ Res. 2012;111:697-707.)

Key Words: angiotensin II ■ VSMC ■ ROS ■ SM22α ■ Ang II ■ PKCδ

The renin-angiotensin system plays an essential role in regulating the physiological processes of the cardiovascular system. As the primary effector molecule of this system, angiotensin II (Ang II) has multiple pathophysiological effects on vascular smooth muscle cells (VSMCs), including increase in arterial pressure, induction of VSMC hypertrophy, and the expression of multiple vasoactive and inflammatory substances.1 A significant body of evidence indicates that the reactive oxygen species (ROS) are involved in mediating the signal transduction of Ang II2-3 and potentiates Ang II–induced hypertension.4-7 The major source of intracellular ROS in vascular cells is NADPH oxidase, a multisubunit
enzymatic complex, which is regulated by cytoplasmic subunits such as p47phox. Multiple recent reports indicate that the p47phox subunit of NADPH oxidase is the pivot factor in Ang II–stimulated ROS production in vascular cells.8–10 Ang II activates NADPH oxidases in VSMCs by inducing phosphorylation and membrane translocation of p47phox and by de novo protein synthesis of p47phox and other NADPH oxidase subunits.11–13 This produces ROS and activates a myriad of other signaling events and transcription factors such as NF-κB and AP-1.13

The molecular and cellular actions of Ang II in cardiovascular diseases are almost exclusively mediated by Ang II type 1 receptors (AT1R).1 The protein kinase C (PKC), as downstream of the AT1 receptor, is shown to be essential in Ang II–accelerated ROS production via promoting phosphorylation and membrane translocation of p47phox. PKCδ is one of the main isoforms in rat VSMCs.14 However, no information is available regarding a role of PKCδ in Ang II–mediated ROS production.

Despite increasing knowledge about the regulation of oxidative stress, the exact molecular mechanisms of functional protein governing ROS production in VSMCs have not yet been fully defined. The latest study has revealed that the disruption of smooth muscle (SM) 22α, an actin-associated protein, induces vascular inflammation through activation of ROS-mediated NF-κB pathways.15 SM22α is a member of the calponin family, containing a calponin homology domain conserved from yeast to human.16,17 The known function of SM22α is to bundle and stabilize actin filaments, which is involved in actin filament assembly and cytoskeletal rearrangements.18,19 Although this protein is not required for the development and basal homeostatic function of SMCs in the developing mouse,19 the role of SM22α may not be compensated under pathological conditions. Indeed, loss of SM22α in apolipoprotein E knockout (ApoE−/−) mice results in increased atherosclerotic lesions with prominent macrophage infiltration, a marker of enhanced inflammation.20 Our recent studies reveal that high expression of SM22α inhibits cell proliferation via blockade of the Ras-ERK1/2 signaling pathway in VSMCs and in injured arteries.21 Because SM22α is an actin-associated protein, and NADPH oxidase activation may be affected by change in actin cytoskeleton organization,22 cytoskeleton remodeling induced by disruption of SM22α in VSMCs may activate multiple ROS production machineries.15 The precise mechanism of SM22α in controlling oxidative stress remains to be identified.

In this study, we sought to determine how SM22α disruption would activate ROS generation in VSMCs and characterize the molecular pathways involved. We show that both downregulation and phosphorylation of SM22α at serine 181 activate the PKCδ–p47phox axis via its dissociation with PKCδ and actin dynamics and mediate ROS production, which is associated with hypertrophy and hyperplasia of VSMCs in vitro and in vivo.

Methods

An expanded Materials and Methods section is available in the Online Data Supplement.

Rat cDNA of SM22α and its phosphorylation site mutants, S181D or S181A, was cloned into Ad/CMV/V5-DEST vectors (Invitrogen) to obtain the replication-defective adenovirus according to the manufacturer’s instructions, named Ad-GFP-SM22α, Ad-GFP-SM22α, Ad-GFP-S181D, and Ad-GFP-S181A, respectively.

VSMCs were infected for 24 hours with the above adenovirus constructs, using a multiplicity of infection of 100. The hypertrophy and hyperplasia of VSMCs were examined by 5-bromo-2-deoxyuridine (BrdU) incorporation and protein synthesis, respectively. In rats, 28% pluronic F-127 gel (Sigma) containing the above adenovirus at a concentration of 10⁷ pfu/mL was spread evenly around the outside of the left carotid arteries. After 2 days, the rats were reanesthetized and osmotic minipumps (Alzet 2ML4) were implanted subcutaneously in the midscapular region for either vehicle (saline) or Ang II at a rate of 750 μg/kg per day.

Systolic blood pressure was measured on days 3 and 0 (basal) before pump implantation and every 3 to 6 days after implantation using a standard tail cuff (BP-2010A System, Sofitron). ROS level was measured by dihydroethidium (DHE) staining for superoxide and TBA assay for malondialdehyde (MDA), respectively.

Data are presented as mean±SEM. A statistical analysis was performed using the Student t test. A value of P<0.05 was considered to be significant.

Results

Downregulation of SM22α Expression Increases Oxidative Stress in Ang II–Induced VSMCs

Over the past decade, experiments have demonstrated that Ang II is a potent stimulus for ROS generation and cell growth.23 We showed that chronic treatment with Ang II (10⁻⁷ mol/L) decreased the SM22α and SM α-actin levels and elevated expression of proliferating cell nuclear antigen protein (a marker of cell growth) in a time-dependent manner (Online Figure IA), suggesting that Ang II induces the phenotypic remodeling from contractile to synthetic state and cell proliferation in VSMCs. Meantime, the results from DHE staining for superoxide and TBA assay for MDA, respectively, showed that ROS production was increased on Ang II stimulation, with 2 peaks at 0.5 to 1 hour and 24 hours, respectively, showing that ROS production was increased on Ang II stimulation, with 2 peaks at 0.5 to 1 hour and 24 hours, respectively (Figure 1A and Online Figure IB), accompanied by de novo protein synthesis of p47phox and other NADPH oxidase subunits.11–13 This produces ROS and activates a myriad of other signaling events and transcription factors such as NF-κB and AP-1.13
Figure 1. Downregulation of SM22α expression increases oxidative stress in Ang II–induced VSMCs. A, ROS levels were measured using DHE staining and TBA assay in VSMCs treated with Ang II (10^{-7} mol/L) for 0 to 48 hours, respectively. Bar graphs show mean±SEM values from 3 independent experiments (n=3). *P<0.05, **P<0.01 versus 0-hour control. B, Real-time RT-PCR for the relative SM22α mRNA level in VSMCs. Bar graphs show mean±SEM values from 3 independent experiments (n=3). *P<0.05 versus siCon. C, ROS generation in VSMCs was measured using DHE staining and TBA assay, respectively, and SM22α expression was determined by Western blot. Bar graphs show mean±SEM values from 3 independent experiments (n=3). *P<0.05 versus siCon; #P<0.05 versus Ad-GFP.

To test this hypothesis, specific SM22α small interference RNA (siSM22α) was used to silence the expression of endogenous SM22α (Figure 1B and 1C). We showed that the knockdown of SM22α but not calponin enhanced ROS generation (Figure 1C and Online Figure IC), whereas overexpression of SM22α by infection with Ad-GFP-SM22α reduced it in VSMCs (Figure 1C). These results support our speculation that the downregulation of SM22α may be involved in Ang II–induced oxidative stress.

Disruption of SM22α Is Required for the Phosphorylation of P47^{phox} and ROS Production Induced by Ang II in VSMCs

It is well known that short-time stimulation of Ang II activates NADPH oxidases, mainly the p47^{phox} subunit, which generates ROS in VSMCs.24 In the present study, we also demonstrated that the phosphorylation of p47^{phox} by Ang II was maximal at 10 minutes, and this effect was maintained over a 20-minute period (Figure 2A) in a manner similar to the change of ROS production. Also, the membrane translocation of p47^{phox} to the cell cortex on Ang II stimulation was discovered after subcellular fragmentation by Western blot and immunofluorescence, respectively (Online Figure II A and B). On the basis of the establishment of a correlation between SM22α and ROS production (described above), we next investigated whether disruption of SM22α is required for p47^{phox} activation, using transfection with siSM22α. The knockdown of SM22α significantly enhanced Ang II–induced phosphorylation of p47^{phox} and ROS generation (Figure 2B and Online Figure IIC), whereas overexpression of SM22α by infection with Ad-GFP-SM22α decreased it (Figure 2C and Online Figure IID). These data suggest that disruption of SM22α may be responsible for activation of p47^{phox} and ROS production in VSMCs, as previously shown.15

Phosphorylation of SM22α at Ser181 Mediates P47^{phox} Activation and ROS Production Via Promoting Interaction of PKCδ With P47^{phox}

It has been known that Ang II–induced ROS production occurs in early and later 2 phases.5,26 To confirm the possible roles of SM22α in early oxidative stress, based on 3 potential phosphorylation sites in SM22α,16 an immunoprecipitation assay was performed to examine the phosphorylation of SM22α. The results showed that phosphorylated SM22α accumulated in the short-time Ang II–stimulated VSMCs without affecting its overall expression (Figure 3A). Similar to the time course of Ang II–induced p47^{phox} phosphorylation, the phosphorylation of SM22α was maximal at 10 to 30 minutes and declined by 60 minutes after Ang II stimulation.

To determine the correlation between phosphorylation of SM22α and activation of p47^{phox}, VSMCs were infected by SM22α Ser181 site-mutant adenovirus, Ad-GFP–S181D (substitution of serine 181 to aspartic acid to mimic serine phosphorylation) and Ad-GFP–S181A (mutation 181 residue from serine to alanine to inhibit SM22α phosphorylation). Immunoprecipitation results showed that wild-type (WT) SM22α and S181A mutant significantly suppressed p47^{phox} phosphorylation induced by Ang II (Figure 3B). Similarly, the results from Western blot showed that the increased p47^{phox} in the membrane fraction on Ang II stimulation was abolished by overexpression of SM22α and S181A mutant, respectively (Figure 3B). These data indicate that phosphorylation of SM22α at Ser181 may facilitate p47^{phox} activation.

Previous studies found that both SM22α and p47^{phox} could be phosphorylated by PKC in vitro and in vivo.16,24 To further elucidate the molecular mechanism by which the phosphorylation of SM22α mediates the activation of p47^{phox}, we focused on the relationship between SM22α and the signaling molecules p47^{phox} and PKCδ. Using coimmunoprecipitation, we discovered that PKCδ was associated with SM22α but not p47^{phox} in quiescent VSMCs (Figure 3C). However, on Ang II stimulation for 10 minutes, PKCδ was dissociated from SM22α and in turn interacted with p47^{phox}, which revealed an increased PKCδ and p47^{phox} in the membrane fraction, with a concomitant increase in SM22α phosphorylation and...
interaction of SM22α cells (Online Figure IIIB). To confirm that the decreased WT- and S181A-expressed cells but not in S181D-transfected VSMCs were transfected by siCon or siSM22α, then stimulated by Ang II (10⁻⁷ mol/L) for 10 minutes or not, and p47phox phosphorylation was determined by IP and WB. Bar graphs show mean±SEM values from 3 independent experiments (n=3). *P<0.05, **P<0.01 versus 0 minute control.

To verify the involvement of SM22α phosphorylation in direct activation of the PKCδ-p47phox axis, low endogenous SM22α expressing cell, 293A cells were transfected with GFP-S181D, GFP-S181A, and WT constructs, respectively. PKCδ was markedly associated with S181A and weakly bound to S181D (Online Figure IIIA). The interaction of PKCδ with p47phox induced by Ang II was decreased in both WT- and S181A-expressed cells but not in S181D-transfected cells (Online Figure IIIB). To confirm that the decreased interaction of SM22α with PKCδ was due to SM22α phosphorylation, we used the AT1R antagonist valsartan to block Ang II–induced AT1R activation. Valsartan significantly reduced Ang II–induced SM22α phosphorylation (Figure 4A). One of the key pathways linked to AT1R in VSMCs is the activation of PKCs, which activate multiple biological responses, including ROS production, inflammation, and cell growth. It is known that SM22α Ser 181 is a potential phosphorylation site of PKC. To verify the involvement of PKC in SM22α phosphorylation, VSMCs were stimulated with the PKC agonist phorbol myristate acetate (PMA) to activate PKC. We observed the increased SM22α phosphorylation in PMA-induced VSMCs (Figure 4B). We next examined whether the AT1R-PKC signaling pathway is associated with SM22α phosphorylation using selective inhibitors. We found that preincubation with Rottlerin, a PKCδ-selective inhibitor, significantly attenuated Ang II–induced SM22α phosphorylation (Figure 4C). Moreover, Staurosporine, a potent broad inhibitor of PKCs, displayed an inhibitory effect similar to that in Rottlerin (Figure 4C). Alternately, knockdown of PKCδ by small interference RNA (siRNA) also abolished Ang II–induced SM22α phosphorylation but did not affect AT1R phosphorylation (Figure 4D).

Figure 2. Disruption of SM22α is required for the phosphorylation of p47phox and ROS production induced by Ang II in VSMCs. A. Immunoprecipitation (IP) and Western blot (WB) for p47phox phosphorylation after stimulation by Ang II (10⁻⁷ mol/L) for indicated times. Bar graphs show mean±SEM values from 3 independent experiments (n=3). *P<0.05, **P<0.01 versus 0 minute control. B, VSMCs were transfected by siCon or siSM22α, then stimulated by Ang II (10⁻⁷ mol/L) for 10 minutes or not, and p47phox phosphorylation was determined by IP and WB. Bar graphs show mean±SEM values from 3 independent experiments (n=3). *P<0.05, **P<0.01 versus siCon. C, Ad-GFP–infected or Ad-GFP–SM22α–infected VSMCs were treated with Ang II (10⁻⁷ mol/L) for 10 minutes or not. Bar graphs show mean±SEM values from 3 independent experiments (n=3). *P<0.05 versus Ad-GFP.
Downregulation of SM22α May Be Responsible for the Long-Term Effects of Ang II on Oxidative Stress via Increasing Actin Dynamics in VSMCs

The actin cytoskeleton not only controls cell shape and motility but also plays an important role in many signaling pathways. It has only been demonstrated that PKC phosphorylation on Ser181 reduces SM22α binding to actin in vitro. In the present study, we demonstrated that such phosphorylation actually occurs in VSMCs. In eukaryocyte, protein ubiquitination is generally dependent on its phosphorylation, which triggers the activity of E3 ubiquitin ligase. For this, we first examined whether SM22α phosphorylation modulates its degradation. The results showed that Ang II induced SM22α ubiquitination with a peak at 4 hours in VSMCs, which occurred before downregulation of SM22α (Figure 5A). To determine whether the increased SM22α ubiquitination is dependent on its phosphorylation, VSMCs preincubated with the PKC-selective inhibitor Rottlerin were then stimulated by Ang II. Rottlerin inhibited Ang II–induced SM22α ubiquitination (Figure 5B). We also found...
that the ubiquitylation of SM22α significantly increased in VSMCs infected with Ad-GFP–S181D on Ang II stimulation, compared with Ad-GFP–S181A (Figure 5C). To verify this finding, 293 A cells were transfected with the indicated constructs and then treated with Ang II. We showed that S181D mutant but not S181A had an increase in ubiquitylation (Online Figure IVA). These results suggest that Ang II–induced phosphorylation facilitates SM22α degradation via the ubiquitin-proteasome pathway.

Our studies and others have demonstrated that SM22α plays critical role in regulating and stabilizing the actin cytoskeleton.18,33,34 Next, we investigated the relationships between downregulation of SM22α mediating actin dynamics and ROS production. VSMCs were treated with Ang II for 12, 24, and 48 hours, respectively, and the distribution of actin in the cytosolic soluble (Sol, G-actin) and cytoskeletal fractions (Csk, F-actin) was tested by Western blot. The interaction between p47phox and PKCα markedly increased after Ang II treatment for 24 hours (Figure 3C), parallel with the decreased Csk/Sol-actin ratio (Online Figure IVB), suggesting that depolymerization of F-actin may facilitate the activation of the PKCα-p47phox pathway. To further confirm this argument, cytochalasin B (CB) was used to destroy the actin cytoskeleton. The depolymerization of the actin cytoskeleton resulted in the increased interaction of PKCα with p47phox in CB-pretreated VSMCs (Figure 5D and Online Figure IVC). In contrast, the actin-stabilizing toxin jasplakinolide inhibited it (Online Figure IVD and E). The knockdown of SM22α also increased interaction of PKCα with p47phox, accompanied with decreasing of the Csk/Sol-actin ratio (Online Figure IVF). Taken together, Ang II–induced actin dynamics may be required for long-term effects of oxidative stress via degradation of SM22α by the ubiquitin-proteasome pathway.

Phosphorylation of SM22α Is Involved in Both Hypertrophy and Hyperplasia of VSMCs Via Increased ROS Generation In Vitro and In Vivo

To investigate the role of SM22α phosphorylation mediating oxidative stress in hypertrophy and proliferation of VSMCs in vitro and in vivo, we first tested BrdU incorporation and total protein amount per cell in VSMCs stimulated with Ang II. The BrdU incorporation and the total protein amount per cell increased after Ang II stimulation for 24 and 72 hours, respectively, which was decreased by overexpression of SM22α and S181A mutant (Figure 6A).

Furthermore, we directly examined the effects of SM22α phosphorylation on vascular hypertrophy and blood pressures under basal conditions and after treatment with Ang II (750 μg/kg per day), after infection of the adenovirus constructs expressing WT and mutants of SM22α. Systolic blood pressure was significantly increased in rats receiving Ang II plus the adenovirus constructs, compared with vehicle (Figure 6B and Online Table I). The medial thickness and medial...
cross-sectional area of the carotid arteries were increased after Ang II infusion for 14 days, were significantly enhanced at 28 days in Ang II plus GFP and S181D overexpression, respectively, consistent with increasing of ROS generation after Ang II infusion for 14 days (Figure 6C and 6D and Online Figure VA through C). Using the balloon injury model of rat carotid arteries, we found the same trend that the phosphorylation of SM22α enhanced ROS generation and neointimal hyperplasia induced by injury, accompanied with increasing of BrdU incorporation (Figure 6E and Online Figure VD through F). Taken together, these results suggest that SM22α may play key roles in cellular hypertrophy and hyperplasia of VSMCs via modulating oxidative stress in vitro and in vivo. Thus, SM22α Ser 181 site may be an effective molecular target for prevention of hypertension and restenosis after angioplasty.

**Discussion**

SM22α directly binds to the actin cytoskeleton and induces actin bundling to facilitate the formation of cytoskeletal structure such as stress fibers. However, Sm22α−/− mice are viable, fertile, and exhibit no obvious phenotypic abnormalities.19 For many years, its function was unknown.

In the present study, we demonstrated that the downregulation and mainly phosphorylation of SM22α were associated with Ang II–induced ROS production in VSMCs. Ang II induced the phosphorylation of SM22α at Ser 181 in an AT1R-PKCδ pathway–dependent manner. Phosphorylated SM22α activated the PKCδ-p47phox axis and promoted ROS generation via 2 distinct pathways. First, in early stimulation, the phosphorylation of SM22α at Ser 181 resulted in its dissociation from PKCδ, which promoted binding of p47phox to PKCδ and subsequently p47phox activation. Second, the phosphorylation-accelerated SM22α ubiquitin-proteasome degradation mediated actin cytoskeletal remodeling, which may facilitate the translocation of the PKCδ and PKCδ-p47phox axis activation, ROS production, VSMC proliferation, and hypertrophy in a later stimulation. In addition, Ang II–induced medial hypertrophy and balloon injury–induced neointima formation were significantly suppressed by infection of SM22α S181A mutant in vivo, indicating that PKCδ–mediated phosphorylation of SM22α is involved in oxidative stress and vascular remodeling.

The present study verified that Ang II induced ROS production with 2 peaks: for 0.5 to 1 hour, and for 24 hours, inconsistent with the previous description.25,26 The possible explanation for such inconsistent findings may be due to the application of different methods to detect ROS production, because ROS could be generated by NADPH oxidases, xanthine oxidase, uncoupled nitric oxide synthase, and mitochondria in the vessel wall.55 Our findings suggest that these 2 peaks are regulated by phosphorylated SM22α in different manners. Sequence analysis reveals that there are 3 potential phosphorylation sites in SM22α structure such as stress fibers. However, SM22α−/− mice are viable, fertile, and exhibit no obvious phenotypic abnormalities.20,21 For many years, its function was unknown.

In the present study, importantly, we found that SM22α may play key roles in cellular hypertrophy and hyperplasia of VSMCs via modulating oxidative stress in vitro and in vivo. Thus, SM22α Ser 181 site may be an effective molecular target for prevention of hypertension and restenosis after angioplasty.

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response to Ang II, PKCδ phosphorylates SM22α at Ser 181, and the phosphorylated SM22α detached from PKCδ and lost its inhibiting PKCδ activity. The interactions of PKC with other proteins play an important role in the functions of PKC itself and the other proteins with which it interacts. In this study, we also showed that phosphorylation of SM22α Ser181 is required for dissociation of SM22α from PKCδ.

In VSMCs, Ang II induces rapid translocation of p47phox to the membrane, resulting in increased NADPH oxidase activity via activation of PKC through multiple pathways. Recent evidence suggests that inhibition of the PKCβ isoform blocks Ang II–induced production of ROS in VSMCs. Thus far, it is rarely reported that PKCδ phosphorylates p47phox in Ang II–induced ROS production. To elucidate the significance of dissociation of phosphorylated SM22α from PKCδ in Ang II–induced p47phox activation, we examined the correlation between this dissociation and PKCδ-p47phox axis activation. Unexpectedly, we found that PKCδ was a main kinase for p47phox activity in Ang II–stimulated VSMCs, and the phosphorylation of SM22α facilitated this axis activity via release of PKCδ. Interestingly, the nonphosphorylated mutant of SM22α, S181A, inhibited PKCδ translocation and p47phox activation, although PKCδ was activated by Ang II via AT1R. Studies from other laboratories have indicated that PKC activity is related to its subcellular localization. We speculated that the rate-limiting step in activation of p47phox may be represented by the dissociation of phosphorylated SM22α from PKCδ, which then becomes phosphorylated and starts ROS production. PKCδ-mediated SM22α phosphorylation regulates the activity of itself, as well as its subcellular localization. Taken together, in vitro and in vivo data indicate that phosphorylation of SM22α by PKCδ promotes activation of the PKCδ-p47phox pathway. We therefore discovered a novel signaling pathway that governs ROS production down-
stream of PKC. In addition, we also demonstrated that Ang II–induced phosphorylation of SM22α and ROS generation were AT1R-dependent, consistent with a previous report.45 Our findings suggest a novel role of SM22α in rapid activation of p47phox induced by Ang II. Thus, SM22α is regulated by PKCα and regulates PKCα activity as an adaptor protein that affects the oxidative stress of VSMCs.

SM22α directly binds to the actin cytoskeleton and induces actin bundling to facilitate the formation of cytoskeletal structure such as stress fibers.16,17,41 The actin-bundling effect of SM22α may lead to a stable cytoskeleton.8,22 Our data demonstrated that depolymerization of the actin cytoskeleton by cytochalasin B resulted in the increased interaction of PKCα with p47phox in VSMCs. It seems plausible that the enhance of PKCα-p47phox activity by SM22α downregulation would be due, at least in part, to decreased actin cytoskeleton stability. In support of this possibility, we demonstrated an attenuated PKCα-p47phox activity in SM22α-overexpressed VSMCs. The recent report indicates that there is correlation between NADPH oxidase activation and diminished stress fiber formation in PAC1 cells after Sm22α knockdown.15 Activation of NADPH oxidase requires the membrane assembly of cytosolic p47phox, p67phox, p40phox, and Rac2.22,46 It was reported that the actin cytoskeleton and associated proteins may affect this process.22 Our study thus suggests that SM22α downregulation mediating actin dynamics is important for the later ROS production induced by Ang II in VSMCs.

We demonstrated for the first time that the delivery of SM22α and S181A significantly inhibited cross-sectional area with suppression of ROS production by Ang II in the carotid arteries, suggesting that SM22α is causally involved in medial hypertrophy in vivo, acting independently of changes in blood pressure. Ang II not only mediates hypertension and vascular hypertrophy but also is implicated in VSMC proliferation. In the current study, we showed that SM22α and mutant S181A inhibited Ang II–induced VSMC proliferation in vitro and in injury-induced neointimal hyperplasia to some extent in vivo. Taken together, the findings suggest that SM22α phosphorylation plays an important role in hypertrophy and proliferation of VSMCs.

In summary, phosphorylation of SM22α has dual roles in the regulation of PKCα-p47phox activity. In addition to its release of PKCα, thus allowing p47phox binding and catalysis, phosphorylated SM22α is dissociated from actin, leading to an unstable actin cytoskeleton, which is essential for PKCα translocation and p47phox activation in Ang II–induced ROS production of VSMCs (Figure 7). SM22α is a novel PKCα-regulating and PKCα-regulated adaptor protein that affects hypertrophy and hyperplasia of VSMCs via modulating oxidative stress. Thus, our results provide the molecular evidence for previous reports indicating an important role for SM22α in oxidative stress of VSMCs during the development of hypertension and neointimal hyperplasia.

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Disclosures
None.

References


What Is Known?

- Oxidative stress is a crucial early event in the development of vascular smooth muscle cells (VSMCs) hypertrophy and hyperplasia.
- SM22α, an actin-binding cytoskeleton protein, has been reported to exert functions that antagonize oxidative stress, inflammation and proliferation in VSMCs.

What New Information Does This Article Contribute?

- Phosphorylation of SM22α facilitates Ang II–induced ROS production through release of PKCδ from SM22α and binding to p47phox, which leads to activation of the PKCδ–p47phox axis. These changes are associated with hypertrophy and hyperplasia of VSMCs in vitro and in vivo.
- Ang II induces phosphorylation of SM22α at Serine 181 in an AT1R-PKCδ-dependent manner in VSMCs.
- Phosphorylation of SM22α also accelerates degradation of SM22α through the ubiquitin-proteasome system, which enhances PKCδ membrane translocation and activation of the PKCδ–p47phox axis on induction of actin cytoskeletal dynamics in the later stage of oxidative stress.
- SM22α is a novel PKCδ-regulating and PKCδ-regulated adaptor protein that affects oxidative stress of VSMCs.
- PKCδ-mediated phosphorylation of SM22α might be involved in development of hypertension and restenosis through facilitating oxidative stress.

Reactive oxygen species (ROS) are involved in signaling pathways of Ang II. Excess ROS generation initiates vascular diseases, which could be repressed by SM22α overexpression. Our present work provides evidence that SM22α phosphorylation acts as a new mechanism linking the ROS production and VSMC hypertrophy and hyperplasia. This finding expands our knowledge about the function of SM22α in addition to its roles in stabilizing actin cytoskeleton, in inhibiting hypertrophy and proliferation. Blockade of SM22α phosphorylation represents a potential novel antioxidation strategy for intervention in vascular diseases.
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SUPPLEMENTAL MATERIAL

Phosphorylation of Smooth Muscle 22 Alpha Facilitates Angiotensin II-Induced ROS Production via Activation of PKCδ-P47phox Axis through Release of PKCδ and Actin Dynamics and Is Associated with Hypertrophy and Hyperplasia of Vascular Smooth Muscle Cells in Vitro and in Vivo

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Detailed Methods

Cell culture and treatment

VSMCs were isolated from the thoracic aorta of 80-100 g male Sprague-Dawley rats as previously described. VSMCs were grown in low glucose Dulbecco’s-modified Eagle’s medium (DMEM) (Invitrogen) with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. Human VSMCs were isolated from umbilical artery, and grown in high glucose DMEM with 30% FBS. The VSMCs were maintained at 37°C in a humidified atmosphere containing 5% CO₂, and only passages 3 to 5 cells at 70-80% confluence were used in the experiments, except if stated otherwise. The polyethylene glycol (PEG)-SOD (100 U/mL) was used to scavenge superoxide. To inhibit the activation of AT₁R, PKC, PKCδ or PKCα and β, the cells were starved for 24 hours and then incubated with the selective inhibitors valsartan (10 µmol/L), Staurosporine (1 µmol/L), Rottlerin (10 µmol/L) or Gö6976 (2 µmol/L) for 2 hours and then stimulated with Ang II (10⁻⁷ mol/L, Sigma). PMA (10⁻⁷ mol/L) was used to activate PKC. To reduce the degradation of ubiquitin, VSMCs were pretreated with MG132 (10 µmol/L) for 2 hours before stimulated by Ang II; Cytochalasin B (CB, 2 µmol/L) and jasplakinolide (JPK, 50 µmol/L) were used to disrupt and stabilize the actin cytoskeleton. This study was performed via a protocol approved by the Institutional Animal Care and Use Committee of Hebei Medical University, in accordance with the Guide for the Care and Use of Laboratory Animals, and the Hebei Medical University Clinical Research Ethics Committee.

Site-directed mutagenesis of SM22α

Full-length cDNA of rat SM22α was subcloned into the pEGFP-C2 vector to generate
GFP-SM22α, which is the template of site-directed mutagenesis. Site-directed mutation of SM22α was carried out by PCR using oligonucleotide primers that coded for the appropriate point substitutions of amino acids. The reactions were carried out using a QuikChange site-directed mutagenesis kit (Stratagene). Each mutation was verified by DNA sequence analysis. PCR primers used in the site-directed mutagenesis: GFP-S181D (181S to D): 5'GGCCT TCAAA TGGGC GGCAA CAGAG GGGCC TCACA G-3' and 5'-GGGCT TCAAA TGGGC GACAA CAGAG GGGCC TCACA G-3', GFP-S181A (181S to A): 5'GGCCT TCAAA TGGGC GACAA CAGAG GGGCC TCACA G-3' and 5'-GGGCT TCAAA TGGGC GCCAA CAGAG GGGCC TCACA G-3'.

Adenovirus packaging and infection

Full-length cDNA of rat SM22α was cloned into pEGFP-C2, a mammalian expression vector and encoding a red-shifted variant of wild-type GFP, to generate pEGFP-SM22α, so SM22α was expressed as fusions to the C terminus of GFP. Then the fusions of SM22α plus GFP were subcloned into pAd/CMV/V5-DEST Gateway Vector (Invitrogen) to make the GFP tagged SM22α adenovirus Ad-GFP-SM22α, according to the manufacturer’s protocol. Ad-GFP, Ad-GFP-S181D and Ad-GFP-S181A were obtained the same to the above. All of these clones were verified by sequencing. The VSMCs were infected with the above adenovirus (5×10⁹ pfu/mL) for 24 hours, washed and incubated in serum-free medium without adenovirus for 24 hours, then stimulated by Ang II.

SiRNA transfection

The cultured VSMCs were grown to 50-60% confluence, and then transfected with specific duplex siRNA, siSM22α (5'-GCU AGU GGA GUG GAU UGU ATT-3' and 5'-UAC AAU CCA CUC CAC UAG CTT-3'), siCalponin-1 (5'-UAG ACU GAU A GU UGC CUG AUC CAG GTT-3' and 5'-CCU GGA UCA GGC AAC UAU CAG UCU ATT-3'), siPKCδ (5'-UGC GAA GUA AAG AAG UCU CUG UUC UUG AUA GGU CUG UUC ATT-3') or non-specific scrambled siRNA, siCon (5'-GCU AGA GUA GCC GUG AUA UCG TT-3' and 5'-CGA AUU CAC CGC UAC UCU AGC TT-3') using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. At 6-12 hours after transfection, VSMCs were treated with Ang II as mentioned.

Cell Proliferation assays

VSMC proliferation was performed with the BrdU Cell Proliferation kit (Millipore cat. no. 2750) according to manufacturer’s recommendations. Cells were cultured for 12 hours and then labeled for 12-24 hours with BrdU. OD readings were done at 450 nm and normalized to Ad-GFP (-) treated cells.

Western blot and immunoprecipitation
Lysates from VSMCs were prepared with RIPA lysis buffer. The membrane and cytosolic fraction was separated by ultracentrifugation. The proteins were separated by 10% SDS-PAGE, and electro-transferred to a PVDF membrane. Membranes were blocked with 5% milk in TBST for 2 hours at room temperature, and incubated with primary antibodies against SM22α (1:1000, cat. no. ab14106, Abcam), PKCδ (1:500, cat. no.610309, BD), Phospho-PKCδ (1: 800, cat. no.2055S, Cell Signaling), SM α-actin (1 :1000, cat. no.11841-1, Epitomics), p47phox (1:500, cat. no. B1171, Bioworld), GFP (1 : 1000, cat. no. sc-9996, Santa Cruz Biotechnology Inc.), AT1R (1:200, sc-1173, Santa Cruz Biotechnology Inc.), ub (1:500, cat. no. sc9133, Santa Cruz Biotechnology Inc.), calponin-1 (1:1000, cat. no.1806-1, Epitomics) or β-actin (1 : 1000, sc-47778, Santa Cruz Biotechnology Inc.) at 4°C overnight, and then incubated with the horseradish peroxidase-conjugated secondary antibody (1:10000, Santa Cruz Biotechnology) for 2 hours. The blot was detected using the Chemiluminescence plus Western blot analysis kit (Santa Cruz Biotechnology). Alternatively, membranes incubated with IRDye800® conjugated secondary antibody (1:20000, Rockland) for 1 hour, following scanning with the Odyssey Infrared Imaging System (LI-COR Biosciences), then the integrated intensity for each detected band was determined with the Odyssey Imager software (v3.0). The experiments were replicated at least three times.

Immunoprecipitation was performed as described previously. Briefly, cell extracts were first precleared with 20 µL of protein A-agarose (50% v/v). The supernatants were immunoprecipitated with indicated antibodies for 1 hour with rocking at 4°C, followed by incubation with protein A-agarose overnight at 4°C . Protein A-agarose-antigen-antibody complexes were collected by centrifugation at 4°C . The pellets were washed four times with 500 µL IPH buffer for 20 minutes each time at 4°C. Bound proteins were resolved by SDS-PAGE, followed by Western blot as described above. All blots are representative of 3 similar experiments.

**Real-time RT-PCR (rtRT-PCR)**

Total RNA from VSMCs was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. The cDNA was synthesized using the reverse transcriptase (Invitrogen). Real-time PCR was performed using SYBR Green RT-PCR Kit (Invitrogen).

**GST pull-down assay**

For the interaction SM22α or its phosphorylation site mutants and PKCδ in vitro, SM22α or the mutants with GST tag fusion proteins purified from the bacterial lysates of Rosetta competent cells transformed with pGEX-4T1-SM22α, S181D or S181A. Protein expression was induced by reaction with 0.5 mmol/L isopropyl thio-β-D-galactoside at 25°C for 6 hours. Bacterial lysates were purified over glutathione Sepharose beads (Amersham Biosciences). For the pull-down assay, VSMCs lysate was precleared with GST Sepharose beads, then assay mixtures were incubated with GST, GST-SM22α or site-directed mutants Sepharose beads. After centrifugation, the pellets were washed, and the interacting proteins were separated by
SDS-PAGE and identified by Western blot using appropriate antibodies.

**Protein assay**

Subconfluent VSMCs on 12-well culture plates were infected with adenovirus for 24 hours, then incubated with serum-free DMEM for 24 hours following incubation with Ang II (10^{-7} mol/L) for 3 days. After aspiration of the medium, cells were washed twice with ice-cold Hanks’ balanced salt solution. The cell hypertrophy was represented as relative protein amount per cell.

**Actin fractionation**

Cells were scraped, washed with PBS, and lysed in buffer A (20 mmol/L Tris/HCl, pH 7.5, 1% Triton X-100, 5 mmol/L EGTA and 1 mmol/L phenylmethylsulfonyl fluoride) on ice for 20 minutes, and then centrifuged at highest speed and 4 °C for 10 minutes. The supernatants (Sol) were harvested. The pellets were lysed in buffer B (10 mmol/L Tris/HCl, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 1 mmol/L sodium deoxycholate, 2 mmol/L EGTA and 1 mmol/L phenylmethylsulfonyl fluoride) on ice for 30 minutes, and then centrifuged at highest speed and 4 °C for 30 minutes. The supernatants from the lysed pellets (Csk) were harvested. Equal amounts of the supernatant (Sol) and pellet (Csk) were separated by SDS-PAGE and identified by Western blot.

**Immunofluorescence assay**

VSMCs prepared as described. Cells were fixed 4% paraformaldehyde solution for 5 minutes at room temperature and then washed with PBS, followed by incubation in 10% normal goat serum blocking solution for 20 minutes in a humidified chamber at room temperature. Cells were incubated in rabbit anti-SM22α (1:100) and mouse anti-PKCδ antibodies (1:20) mixture, or mouse anti-PKCδ (1:20) and rabbit anti-p47^{phox} antibodies (1:20) mixture for 2 hours at room temperature. Cells were washed 3 times with PBS and incubated in fluorescein-conjugated secondary antibodies (anti-rabbit TRITC, red; anti-mouse FITC, green. 1:100) for 60 minutes at room temperature. The cells were then washed with PBS, mounted with DAPI, and visualized using laser scanning confocal microscope.

**Measurement of ROS levels**

There were two independent approaches for ROS measurement. Superoxide in VSMCs on chamber slides was detected using 2 μmol/L dihydroethidium (DHE) for 30 minutes at 37°C, then visualized using laser scanning confocal microscope or fluorescence microscope. Semi-quantitative analysis of DHE relative fluorescence intensity was performed on 3 independent images (100-300 cells/image) using integrative optical density function in the Image-Pro Plus 6.0 software. The other method was TBA assay for malondialdehyde (MDA), a marker of lipid peroxidation, with a TBA assay kit according to the manufacturer’s protocol.

**Gene transfer to the vascular, drug infusion and assessment of**
hypertrophy

Thirteen- to 16-week-old male Sprague-Dawley rats were anesthetized with pentobarbital (50 mg/kg per day, intraperitoneally) and the neck was dissected to expose the common carotid arteries. 28% pluronic F-127 gel (Sigma) containing adenovirus at a concentration of 10⁹ pfu/mL was spread evenly around the outside of the common carotid artery. After 2 days, the rats were re-anesthetized and osmotic minipumps (Alzet 2ML4) were implanted subcutaneously in the midscapular for either vehicle (saline) or AngII (750 µg/kg/d; in saline) infusion. On day 14 and 28, the middle portions of carotid arteries were harvested and fixed. Each artery was processed, embedded in paraffin, and serially sectioned (5-µm sections). Three 5-µm cross sections were stained with hematoxylin and eosin (HE). To quantify wall thickness, radial lines were drawn to determine the distance from internal elastic lamina to the external lamina at a minimum of 10 locations per aortic section, and a mean value was calculated. To determine medial cross-sectional wall area, the perimeters of the internal and external elastic laminas were traced. All measurements were completed with the use of Image-Pro Plus 6.0 software.

Blood pressure evaluation

Systolic blood pressure was measured on days 3 and 0 (basal) before pump implantation and every 3-6 days after implantation using a standard tail cuff (BP-2010A System, Softron).

Balloon injury and morphometric analysis

Balloon denudation of the left common carotid artery of male Sprague-Dawley rats was performed as described.⁸ ⁹ After balloon injury, 100 µL of Ad-GFP, Ad-GFP-SM22α, Ad-GFP-S181D or Ad-GFP-S181A (5×10⁹ pfu/mL) was infused into the ligated segment of the common carotid artery for 15 minutes. The ligatures and catheter were then removed, the external carotid artery was ligated, and the incision closed. The arteries were collected at day 3, 6 or 14 after balloon injury. Neointima thickening was assessed using the intima to media (I/M) area ratio measured from HE-stained arterial cross sections with a computer-based Image-Pro Morphometric System in a double-blind manner. Cell proliferation in vivo was measured with a BrdU in situ detection kit (BD Pharmingen). BrdU was injected intraperitoneally (50 mg/kg) at 24 and 12 h before euthanasia. Four discontinuous sections from each vessel were measured, and four rats were used in each experimental group.

Immunohistochemistry

For the immunohistochemical analysis, the sections were blocked by 0.3% hydrogen peroxide, followed by preincubation with 5% normal goat serum and then incubated with anti-GFP (1:100) or anti-4-HNE (1:100) antibodies at 4°C overnight. Next, the sections were incubated with the biotinylated secondary antibody streptavidin-horseradish peroxidase followed by diaminobenzidine, and then they
were counterstained with haematoxylin. For the negative controls, the primary antibody was replaced with non-immune rabbit or mouse serum. Staining intensities were determined by measurement of the integrated optical density (IOD) with light microscopy using a computer-based Image-Pro Morphometric System by two independent observers in a double-blind manner.

**Statistical analysis**

Data analysis was performed by using SPSS version 16.0 (SPSS, Inc., Chicago, IL). Data are presented as the means ± SEM. Paired data were compared by Student's t tests. Differences among groups were determined with one-way or two-way analysis of variance (ANOVA) with repeated measures. A probability value of <0.05 was considered significant.
**Supplemental Figures and Legends**

**Online Figure I.** Downregulation of SM22α expression increases oxidative stress in Ang II-induced VSMCs. **A**, Western blot analysis of VSMCs stimulated by Ang II (10^−7 mol/L) for indicated times, respectively. *P*<0.05, **P**<0.01, ***P***<0.001 vs 0 hour control. **B**, VSMCs were preincubated with PEG-SOD (100U/mL) or not for 1 hour, following stimulation with Ang II (10^−7 mol/L) for 10 minutes, then superoxide level was determined by DHE staining. *P*<0.05, **P**<0.01 vs the unstimulation control. **C**, Western blot analysis for Calponin-1 and SM22α expression (below), and DHE staining for superoxide (upper) in VSMCs transfected with siCon or siCalponin-1. Bar graphs show the means ± SEM from 3 independent experiments (n=3). The representative images in the Figure are from 3 independent experiments.
Online Figure II. Disruption of SM22α is required for the phosphorylation of p47phox and ROS production induced by Ang II in VSMCs. A, VSMCs were treated with Ang II (10^{-7} \text{ mol/L}) for 10 minutes (+) or not (-), then membrane translocation of p47phox was determined after subcellular fragmentation by Western blot. B, Immunofluorescent for the membrane translocation of p47phox following Ang II stimulation for 10 minutes or not. Bars: 75 \text{ µm}. C, ROS levels were measured using DHE staining and TBA assay in siRNA-transfected VSMCs treated with Ang II or not. *P<0.05 vs siCon. D, DHE (2 \text{ µmol/L}) staining for superoxide in adenovirus-infected VSMCs stimulated by Ang II for 10 minutes (+) or not (-). *P<0.05 vs Ad-GFP-SM22α. The representative images in the Figure are from 3 independent experiments. Bar graphs show the means ± SEM from 3 independent experiments (n=3).
Online Figure III. Phosphorylation of SM22α at Ser181 mediates p47phox activation and ROS production via promoting interaction of PKCδ with p47phox in VSMCs of rats and human. A and B. Coimmunoprecipitation (IP) for the interaction between PKCδ and SM22α (A), or PKCδ and p47phox (B) in GFP-SM22α, GFP-S181D or GFP-S181A plasmids-transfected 293A cells. C, GST pull down of recombinant GST-SM22α, GST-S181D or GST-S181A with PKCδ from VSMC lysates. D, Western blot analysis for the expression of SM22α in human VSMCs. E, DHE staining for the superoxide in human VSMCs following Ang II (10⁻⁷ mol/L) stimulation for indicated times. *P<0.05, **P<0.01 vs 0 hour control. F, Coimmunoprecipitation (IP) for the interaction among SM22α, p47phox and PKCδ, and the phosphorylation of SM22α and p47phox stimulated by Ang II (10⁻⁷ mol/L) for 10 minutes or not. The representative images in the Figure are from 3 independent experiments. Bar graphs show the means ± SEM from 3 independent experiments (n=3).
Online Figure IV. Downregulation of SM22α may be responsible for the long-term effects of Ang II on oxidative stress via increasing actin dynamics in VSMCs. A, Immunoprecipitation analysis (IP) for SM22α ubiquitination in 293A cells stimulated as indicated. B, The distribution of actin in Sol and Csk fractions of VSMCs stimulated by Ang II (10^{-7} mol/L) for indicated times. Upper, the ratio of Csk/Sol actin. Below, Western blot. *P<0.05 vs 0 hour control. C and D, VSMCs were pretreated with CB (2 µM) or jasplakinolide (JPK, 50 pmol/L) for 2 hours, then stimulated by Ang II (10^{-7} mol/L) for 10 minutes. Coimmunoprecipitation (IP) and Western blot (WB) showed the interaction between p47^{phox} and PKCδ, and the distribution of actin in Sol and Csk in VSMCs. E, The phosphorylation of SM22α, p47^{phox} or PKCδ was detected by immunoprecipitation and Western blot. VSMCs were pretreated with JPK (50 pmol/L) or not for 2 hours, and then stimulated by Ang II (10^{-7} mol/L) for 10 minutes or not. F, The interaction of p47^{phox} with PKCδ was investigated by immunoprecipitation (IP), and the distribution of actin in Sol and Csk was detected by Western blot in siCon- or siSM22α-infected VSMCs. The representative images in the Figure are from 3 independent
experiments. Bar graphs show the means ± SEM from 3 independent experiments (n=3).
Online Figure V. Phosphorylation of SM22α is involved in both hypertrophy and hyperplasia of VSMCs via increased ROS generation in vitro and in vivo. A and B, Vascular hypertrophy in rats. Representative HE-stained cross-sections treated with vehicle or Ang II (n=3). Bars: 20 µm (A) and 100 µm (B). C, Representative immunohistochemical staining for 4-HNE of carotid artery sections (n=3). Bars: 20 µm. D, GFP or 4-HNE immunohistochemical staining, and HE staining of aortic carotid arteries after balloon injury for 3 days (n=4). Bars: 50 µm. E, Immunohistochemical staining of BrdU incorporation in carotid sections 6 days after injury (n=4). Bars: 20 µm. F, HE staining of aortic carotid arteries after balloon injury for 14 days (n=4). Bars: 50 µm.
### Supplemental Tables

**Online Table 1.** Systolic blood pressure during vehicle or Ang II treatments (*n=3*).

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Supplemental References