Novel Role of Protein Kinase C-δ Tyr^{311} Phosphorylation in Vascular Smooth Muscle Cell Hypertrophy by Angiotensin II

Hidekatsu Nakashima, Gerald D. Frank, Heigoro Shirai, Akinari Hinoki, Sadaharu Higuchi, Haruhiko Ohtsu, Kunie Eguchi, Archana Sanjay, Mary E. Reyland, Peter J. Dempsey, Tadashi Inagami, Satoru Eguchi

Abstract—We have shown previously that activation of protein kinase C-δ (PKCδ) is required for angiotensin II (Ang II)–induced migration of vascular smooth muscle cells (VSMCs). Here, we have hypothesized that PKCδ phosphorylation at Tyr^{311} plays a critical role in VSMC hypertrophy induced by Ang II. Immunoblotting was used to monitor PKCδ phosphorylation at Tyr^{311}, and cell size and protein measurements were used to detect hypertrophy in VSMCs. PKCδ was rapidly (0.5 to 10.0 minutes) phosphorylated at Tyr^{311} by Ang II. This phosphorylation was markedly blocked by an Src family kinase inhibitor and dominant-negative Src but not by an epidermal growth factor receptor kinase inhibitor. Ang II-induced Akt phosphorylation and hypertrophic responses were significantly enhanced in VSMCs expressing PKCδ wild-type compared with VSMCs expressing control vector, whereas the enhancements were markedly diminished in VSMCs expressing a PKCδ Y311F mutant. Also, these responses were significantly inhibited in VSMCs expressing kinase-inactive PKCδ K376A compared with VSMCs expressing control vector. From these data, we conclude that not only PKCδ kinase activation but also the Src-dependent Tyr^{311} phosphorylation contributes to Akt activation and subsequent VSMC hypertrophy induced by Ang II, thus signifying a novel molecular mechanism for enhancement of cardiovascular diseases induced by Ang II. (Hypertension. 2008;51:232-238.)

Key Words: angiotensin II • AT_{1} receptor • signal transduction • protein kinase C δ • Src • hypertrophy • vascular smooth muscle cells

Angiotensin II (Ang II) plays a major role in vascular remodeling outside of its hemodynamic effects. In cultured vascular smooth muscle cells (VSMCs), cardiac myocytes, and cardiac fibroblasts, Ang II has been shown to promote hypertrophy and/or hyperplasia. There are 2 subtypes of Ang II receptors, AT_{1} and AT_{2}, although the major physiological and pathophysiologically actions of Ang II are facilitated through the AT_{1} receptor. In VSMCs, activation of the AT_{1} receptor coupled to G_{q} increases intracellular Ca^{2+} and activates protein kinase C (PKC).^{1-5} In this regard, several PKC isoforms, including PKCδ, are believed to be activated by Ang II in VSMCs.^{3-5} In addition, various tyrosine kinases and serine/threonine kinases are rapidly activated by Ang II and likely play important roles in mediating vascular remodeling induced by Ang II.^{6,7} However, the detailed role of each PKC isoform in mediating Ang II–induced vascular remodeling, as well as the possible signal cross-talk with other kinases, has been insufficiently characterized.

Increasing evidence suggest that PKCδ is involved in many mechanisms promoting VSMC remodeling and dysfunction.^{8-11} It was reported that PKCδ is activated by mechanical stress, and VSMCs from PKCδ-null mice migrate slower than control VSMCs.^{12} Previously, we have shown that PKCδ kinase activity is required for activation of several tyrosine kinases by Ang II or reactive oxygen species in VSMCs.^{4,13,14} Moreover, we have reported recently that PKCδ is required for activation of Rho, Rho-kinase and c-Jun NH_{2}-terminal kinase and subsequent migration of VSMCs by using kinase-inactive PKCδ overexpression.^{15} These data suggest an important role of PKCδ in mediating vascular remodeling induced by Ang II.

PKCδ is also phosphorylated on tyrosine residues in many cells, including VSMCs and cardiac myocytes.^{15,16-18} Although there are multiple tyrosine phosphorylation sites on PKCδ, Tyr^{311} located between the regulatory and catalytic

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From the Cardiovascular Research Center and Department of Physiology (H.N., H.S., A.H., S.H., H.O., K.E., S.E.) and Department of Anatomy and Cell Biology (A.S.), Temple University School of Medicine, Philadelphia, Pa; the Department of Biochemistry (G.D.F., T.I.), Vanderbilt University School of Medicine, Nashville, Tenn; the Department of Craniofacial Biology, School of Dentistry, and Department Cell and Developmental Biology, School of Medicine (M.E.R.), University of Colorado Health Science Center, Aurora; and the Departments of Pediatrics and Molecular and Integrative Physiology (P.J.D.), University of Michigan, Ann Arbor.
The first 2 authors contributed equally to this work.
Correspondence to Satoru Eguchi, Cardiovascular Research Center and Department of Physiology, Temple University School of Medicine, 3420 N Broad St, Philadelphia, PA 19140. E-mail seguchi@temple.edu
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domains is of particular interest. This is because the Tyr\(^{311}\) phosphorylation has been linked to increased kinase activity in cells treated with H\(_2\)O\(_2\).\(^{19}\) PKC\(\delta\) phosphorylation at Tyr\(^{311}\) may also affect the selectivity of substrates.\(^{17}\) Taken together with the above information, we have tested the hypothesis that PKC\(\delta\) Tyr\(^{311}\) phosphorylation plays a major role in Ang II-induced vascular hypertrophy. We found that PKC\(\delta\) phosphorylation at Tyr\(^{311}\) was induced by Ang II through a Src family kinase and that this phosphorylation was involved in Akt activation and subsequent VSMC hypertrophy.

**Materials and Methods**

An expanded Methods section describing reagents, primary antibodies, cell culture, and statistical analysis is available at http://hyper.ahajournals.org.

**Retrovirus Infection**

Wild-type or Y311F PKC\(\delta\) containing enhanced green fluorescent protein (GFP) at the C terminus\(^{20}\) was cloned into the pBM-IRES-PURO vector, and high titer retroviral supernatants were generated.\(^{21}\) VSMCs were infected with retrovirus, and the infected VSMCs were selected as described previously.\(^{22,23}\) To assess complete viral transformation after an antibiotic selection, in addition to the detection of the overexpression by immunoblotting, we routinely confirmed >99% infection efficiency of our retrovirus vectors by the GFP tagged to the mutants and detected under a fluorescence microscope.

**Adenovirus Infection**

The generation of adenovirus encoding wild-type and a kinase-inactive K376A PKC\(\delta\) mutant construct and dominant-negative K295M + Y527F Src was described previously.\(^{24,25}\) The titer (plaque-forming units per milliliter) of adenovirus was determined by Adeno-XTM Rapid Titer kit (BD Biosciences). VSMCs were infected with adenovirus for 2 days, as described previously.\(^{14}\) To assess complete viral transformation, we routinely confirmed >99% infection efficiency of our adenovirus vectors by GFP encoded by these vectors separately and detected under a fluorescence microscope.

**Immunoblotting**

Cell lysates were subjected to SDS-PAGE and transferred to a nitrocellulose membrane, as described previously.\(^ {26}\) The membranes were then exposed to primary antibodies overnight at 4°C. After incubation with the peroxidase-linked secondary antibody (Amer sham Biosciences) with dilution between 1:1000 and 1:10 000 (depending on the primary antibody) for 1 hour at room temperature, the immunoreactive proteins were visualized by a chemiluminescence reaction kit (Pierce).

**Protein Assay**

VSMCs on 12-well culture plates were incubated with serum-free DMEM for 3 days in retrovirus-infected VSMCs. For adenovirus infection, VSMCs were incubated with serum-free DMEM for 1 day and infected with adenovirus at 100 multiplicity of infection in serum-free DMEM for 2 days. The cells were further incubated with or without 100 nmol/L of Ang II for 3 days. After aspiration of the medium, cells were washed twice with ice-cold Hanks’ balanced salt solution, and the total amount of cellular protein was measured as described previously.\(^ {27}\)

**Cell Volume Assay**

After the pretreatments described in the protein assay, VSMCs were washed with Hanks’ balanced salt solution and trypsinized. The cells were then suspended in PBS, and the cell volume was measured by Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter).\(^ {27}\)

**Results**

**Phosphorylation of PKC\(\delta\) at Tyr\(^{311}\) by Ang II Through the G\(_{\text{q}}\)-Coupled AT1 Receptor**

In 3-day serum-starved rat aortic VSMCs, Ang II (100 nmol/L) stimulated phosphorylation of PKC\(\delta\) at Tyr\(^{311}\) in a rapid (within 30 seconds) and transient manner with a peak of 2 to 5 minutes (Figure 1). The phosphorylation returned to the baseline level at 40 minutes (Figure S1A). Thus, in subsequent experiments, unless otherwise stated, VSMCs were stimulated with Ang II for 2 minutes for evaluation of the PKC\(\delta\) phosphorylation. Pretreatment with an AT\(_1\) receptor antagonist, R116270, totally blocked PKC\(\delta\) phosphorylation by Ang II (Figure S1B). The AT\(_1\) receptor is mainly coupled to the heterotrimeric G protein G\(_{\text{q}}\), whereas G protein–independent signal transduction by the AT\(_1\) has been reported.\(^ {2}\) Thus, we determined whether G\(_{\text{q}}\) contributed to the Ang II-induced PKC\(\delta\) phosphorylation. Pretreatment with a selective G\(_{\text{q}}\) inhibitor, YM-254890,\(^ {22,28}\) completely blocked PKC\(\delta\) phosphorylation at Tyr\(^{311}\) by Ang II (Figure S1C), indicating that Ang II-induced phosphorylation of PKC\(\delta\) at Tyr\(^{311}\) was mediated through G\(_{\text{q}}\) activation.

**Involvement of Src in PKC\(\delta\) Tyr\(^{311}\) Phosphorylation by Ang II**

Activation of the AT\(_1\) receptor by Ang II leads to rapid transactivation of the epidermal growth factor (EGF) recep-
tor, which seems to mediate many key components of downstream signal transduction in VSMCs, whereas an Src family kinase has been implicated as a PKC/H9254/Tyr311 kinase.

To clarify the involvement of Src family kinase and/or EGF receptor transactivation in PKC/H9254 phosphorylation, we pre-treated VSMCs with PP2, an Src family kinase inhibitor, or AG1478, an EGF receptor kinase inhibitor. Interestingly, Ang II-induced PKC/H9254/Tyr311 phosphorylation was markedly blocked by PP2 (5 μmol/L), whereas AG1478 (1 μmol/L) had no inhibitory effect. As expected, AG1478, but not PP2, inhibited Ang II-induced EGF receptor transactivation as detected by its autophosphorylation at Tyr1068 (Figure 2A). Also, PP3 (5 μmol/L), the inactive control chemical for PP2, had no inhibitory effect on Ang II-induced PKC/H9254/Tyr311 phosphorylation (Figure S1D). To support these pharmacological experiments, the effect of dominant-negative Src was examined. Infection of adenovirus encoding dominant-negative Src, but not the control vector, markedly inhibited PKC/H9254/Tyr311 phosphorylation induced by Ang II, whereas neither virus affected the EGF receptor transactivation (Figure 2B). Sufficient overexpression of the dominant-negative Src mutant, as compared with endogenous Src, was confirmed (Figure 2B). These data suggest that Src, but not the EGF receptor, mediates Ang II-induced PKC/H9254/Tyr311 phosphorylation.

Involvement of PKCδ Tyr311 Phosphorylation and PKCδ Kinase Activity in Ang II–Induced VSMC Hypertrophy

To verify the functional significance of the Tyr311 phosphorylation, we established VSMCs that overexpress wild-type PKCδ or a PKCδ Y311F mutant containing GFP at the C terminus using retrovirus infection (Figure 3A). In wild-type PKCδ-expressing VSMCs, the Ang II-induced increase in cellular protein was significantly enhanced compared with the control VSMCs. However, the enhancement was much less in Y311F-expressing cells (Figure 3B). Moreover, in wild-type PKCδ VSMCs, Ang II significantly increased the cell volume, whereas no enhancement was observed in Y311F mutant-expressing cells (Figure S2A). There was no significant change in cell number among these VSMCs stimulated by Ang II (Figure S2B). The confluence state of these VSMCs at the time of the measurements was <90%, because without a mitogen, the VSMC did not significantly proliferate after serum starvation. Also, 5-bromodeoxyuridine incorporation was not significantly changed by Ang II, regardless of wild-type PKCδ overexpression in VSMCs.
In addition, there was no enhancement of an apoptotic marker, cleaved caspase-3, detected in either control or PKC\(\beta\)/H9254 overexpressing VSMCs with 4 hours of Ang II stimulation (Figure S3).

To investigate whether the kinase activity of PKC\(\beta\)/H9254 is also required for Ang II-induced protein synthesis in VSMCs, we infected VSMCs with an adenovirus encoding a kinase-inactive PKC\(\beta\)/H9254 mutant (K376A). In VSMCs expressing K376A, both Ang II–induced protein synthesis (Figure 4) and the increase in cell volume (Figure S4A) were significantly inhibited compared with control VSMCs. Again, there was no significant change in cell number among these VSMCs stimulated by Ang II (Figure S4B). These data suggest that PKC\(\beta\) Tyr\(^{311}\) phosphorylation and PKC\(\beta\) kinase activity are both required for Ang II–induced hypertrophy in VSMCs.

It has been demonstrated that both Akt and extracellular signal regulated kinase (ERK) 1/2 are involved in Ang II–induced VSMC hypertrophy.\(^{30-32}\) To assess the functional role of PKC\(\beta\) Tyr\(^{311}\) phosphorylation and kinase activity in Ang II–induced hypertrophic signaling, we examined Akt and ERK 1/2 activation in the above-treated cells. In wild-type PKC\(\beta\) expressing VSMCs, Ang II–induced Akt phosphorylation was markedly enhanced, whereas no enhancement of Akt phosphorylation by Ang II was seen in Y311F-expressing VSMCs (Figure 5A). In contrast, neither wild-type nor Y311F expression altered Ang II–induced ERK phosphorylation in VSMCs. Also, PKC\(\beta\) Tyr\(^{311}\) phosphorylation by Ang II precedes the Akt phosphorylation. We have demonstrated previously that K376A PKC\(\beta\) had no inhibitory effect on ERK 1/2 phosphorylation induced by Ang II in VSMCs.\(^{15}\) In contrast, Akt phosphorylation induced by Ang II was markedly inhibited in K376A-expressing VSMCs (Figure 5B). These data suggest that Ang II–induced VSMC hypertrophy is positively regulated by PKC\(\beta\) kinase activation and Tyr\(^{311}\) phosphorylation through their involvement with Akt activation but not ERK activation.

**Discussion**

The major novel findings of this study revealed that PKC\(\beta\) activation associated with Tyr\(^{311}\) phosphorylation mediates Ang II–induced VSMC hypertrophy through a mechanism involving Akt. Also, Ang II seems to induce PKC\(\beta\) Tyr\(^{311}\) phosphorylation through the \(G\)\(_{q}\)-coupled AT\(_{1}\) receptor via Src. These findings provide a new signaling mechanism by which...
the AT₁ receptor activation leads to PKCδ-mediated vascular remodeling and may serve as a potential therapeutic target toward cardiovascular diseases.

Ang II–induced rapid PKCδ Tyr311 phosphorylation has been reported with a slightly more sustained time course, which may be because of a shorter serum starvation than in the present study. However, the upstream regulators of PKCδ Tyr311 phosphorylation have not yet been identified. The present study using a selective Gq inhibitor indicates that the phosphorylation is mediated through Gq activation. This is in agreement with our recent observation suggesting that Ang II–induced PKCδ Tyr311 phosphorylation in VSMCs requires intracellular Ca²⁺ elevation. Because the AT₁ receptor is the dominant receptor expressed in our cultured VSMCs, we have not evaluated the possible confounding of these signal transductions by the AT₂ receptor. Increasing evidence suggests the counterregulatory functions of the AT₂ receptors toward the AT₁ receptor–dependent functions, including vascular hypertrophy, as well as hyperplasia in vivo. Therefore, it will be interesting to further characterize a possible signal cross-talk of the PKCδ regulation between these subtype receptors in vivo.

Here, we report that PKCδ phosphorylation at Tyr311 by Ang II is at least in part Src dependent in VSMCs. Supporting this finding is the fact that several others have reported that PKCδ Tyr311 phosphorylation in select cell types depended on Src family kinases when stimulated with various non-G protein–coupled receptor agonists. Also, Src family kinases have been shown to be complexed with PKCδ in several cell types, including VSMCs. However, possible contribution of other Src family kinases (Fyn and yes) expressed in VSMCs in Ang II–induced PKCδ Tyr311 phosphorylation remains to be determined. Although we have not studied Ang II–induced Src phosphorylation, such as at the positive regulatory Tyr416 residue, the Src inhibitor PP2 used in this study has been shown to block this phosphorylation effectively in VSMCs. In addition, our data suggest that the involvement of EGF receptor transactivation in the PKCδ phosphorylation by Ang II is unlikely. However, the EGF receptor kinase inhibitor AG1478, if used at 10 times more concentration than in the present study, partially attenuated c-Src phosphorylation at Tyr416 induced by Ang II in VSMCs. Thus, further careful evaluation may be necessary regarding the possible partial but minor involvement of the EGF receptor transactivation in this PKCδ cascade.

We have previously used a PKCδ inhibitor, rottlerin, to elucidate the role of this PKC isofrom in signal transductions of the AT₁ receptor in VSMCs. However, we have not used this inhibitor in the present study because of the reported off-target effects, which would be inappropriate for our long-term hypertrophic experiments. In PKCδ-deficient VSMCs, cytoskeletal signaling, reorganization, and subsequent migration in response to mechanical stress were diminished. Also, an overexpression study using the wild-type PKCδ suggested that PKCδ mediates p38 mitogen-activated protein kinase activation induced by high glucose in VSMCs. However, by using the K376A mutant, as well as rottlerin, our previous studies have shown that PKCδ kinase activity is essential for Ang II–induced activation of a select set of protein kinases, which include JAK2, Rho-kinase, p21-activated kinase, and c-Jun NH₂-terminal kinase but not ERK or p38 mitogen-activated protein kinase. Thus, involvement of PKCδ in p38 mitogen-activated protein kinase activation may be agonist dependent.

It has been reported that PKCδ-deficient mouse VSMCs are resistant to apoptotic responses compared with control VSMCs. Overexpression of PKCδ in VSMC cell lines also results in G1 arrest and apoptosis. These apoptotic or necrotic changes, if they occur, could be associated with enlargement of cell volume. However, this scenario is quite unlikely in our present study, because there was no difference in caspase-3 cleavage or cell viability with PKCδ overexpression regardless of Ang II stimulation, as shown in Figure S3.

Here, we further revealed Akt as a PKCδ-dependent kinase in VSMCs stimulated by Ang II, which plays a significant role in VSMC hypertrophy. To support our notion, a similar link between PKCδ and Akt was observed in thrombin-induced nuclear factor κB activation in endothelial cells. In addition, other mechanisms may coordinately regulate VSMC hypertrophy on PKCδ activation by Ang II, such as expression of Smad3 and transforming growth factor-β, and the Tyr311 phosphorylation of PKCδ.

Interestingly, our data suggest that Ang II–induced PKCδ Tyr311 phosphorylation is also required for enhanced Akt activation and VSMC hypertrophy observed in VSMCs overexpressing wild-type PKCδ. However, the PKCδ Y311F mutant did not show a dominant-negative effect to inhibit Akt activation below the vector-transfected cells, and one of the hypertrophic responses was still slightly greater than the control cells, demonstrating distinct characteristics of the PKCδ mutants. The kinase-inactive mutant, K376A, not only loses its wild-type ability to positively regulate Akt activation and subsequent hypertrophy but also competes with endogenous PKCδ and, thus, acts as a dominant-negative PKCδ inhibitor. Y311F mutant also loses most of its own hypertrophic characteristics, whereas it is unable to interfere with the endogenous wild-type PKCδ. Although PKCδ Tyr311 phosphorylation has been proposed to enhance kinase activity, recent findings suggest additional roles of the Tyr311 phosphorylation in mediating unique functions of this PKC isoform. The Tyr311 phosphorylation may be the additional component required for the complex formation among PKCδ, Akt, and its upstream kinase, 3-phosphoinositide-dependent kinase 1/3-phosphoinositide-dependent kinase 1 and subsequent Akt activation, which seems to require the PKCδ kinase activity. Taken together, it is attractive to speculate that the PKCδ phosphorylation may contribute to Akt activation and subsequent hypertrophy independent from the kinase activity. To support this notion, we observed a comparable autophosphorylation of Y311F PKCδ at Ser307 phosphorylation to that of wild-type in Ang II–stimulated VSMCs (unpublished observation), thus reflecting the kinase activity remains intact in the Y311F mutant.

In the present study, we have not used a standard protein synthesis assay measuring a radiolabeled leucine incorporation. However, we believe that our 2 distinct methods used here measure the hypertrophic effects of Ang II just as sufficiently and perhaps more directly. Our data demonstrat-
ing hypertrophic responses by Ang II stimulation in VSMCs are consistent with highly cited past articles using intact aortic segments and cultured aortic VSMCs. Moreover, no significant enhancement of DNA synthesis was observed in Ang II-stimulated VSMCs regardless of PKCδ overexpression. However, because our data rely on overexpression strategies, a future study should be conducted by using specific RNA silencing to evaluate the overall roles of PKCδ in mediating VSMC hypertrophy induced by Ang II. In addition, slight distinctions in control cell responses between Figures 3B and 4A may be caused by distinct control vectors used, as well as by a selection of the permanently infected cells in the retroviral experiment. It is also unlikely that the PKCδ Y311F mutant affects other PKC isoforms expressed in VSMCs nonspecifically, because this residue is unique to PKCδ. Other than the data shown in Figure 3A, we and others have demonstrated previously the specificities of the PKCδ mutants used in the present study. A recent study using proteomic analysis of PKCδ-deficient VSMCs revealed that >30 proteins are altered, including enzymes related to glucose and lipid metabolism, thus highlighting the critical role of PKCδ in the development of cardiovascular diseases. PKCδ activation increases O2- derived free radical generation from mitochondria and thereby promotes a pro-oxidant state. Therefore, it will be interesting to expand the present findings by evaluating the regulation of proteins, such as pyruvate dehydrogenase and heat shock proteins, which are likely involved in atherosclerosis, as well as metabolic disorders.

Perspectives

We found that, in addition to PKCδ kinase activity, PKCδ phosphorylation at Tyr311 seems to be required for Akt activation and subsequent VSMC hypertrophy induced by Ang II, which is considered a potential mechanism of atherosclerosis and restenosis after vascular injury. However, our findings are limited within cell culture experiments. Therefore, further clarification of the signal transduction in vivo could contribute to a better understanding of the molecular mechanism of cardiovascular diseases, as well as to the development of better strategies for their treatment.

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Disclosures

None.

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Novel Role of Protein Kinase C Delta Tyr311 Phosphorylation in Vascular Smooth Muscle Cell Hypertrophy by Angiotensin II

Hidekatsu Nakashima1*, Gerald D. Frank2*, Heigoro Shirai1, Akinari Hinoki1, Sadaharu Higuchi1, Haruhiko Ohtsu1, Kunie Eguchi1, Archana Sanjay3, Mary E. Reyland4, Peter J. Dempsey5, Tadashi Inagami2, Satoru Eguchi1**

1. Cardiovascular Research Center and Department of Physiology, Temple University School of Medicine, Philadelphia, PA.
2. Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN.
3. Department of Anatomy and Cell Biology, Temple University School of Medicine, Philadelphia, PA.
4. Department of Craniofacial Biology, School of Dentistry, and Cell and Developmental Biology, School of Medicine, University of Colorado Health Science Center, Aurora, CO.
5. Departments of Pediatrics and Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI.

Short title: PKC delta in Angiotensin II-induced Hypertrophy

*These authors contributed equally to this work.

**Correspondence to Satoru Eguchi, MD, PhD, FAHA: Cardiovascular Research Center and Department of Physiology, Temple University School of Medicine, 3420 N. Broad Street, Philadelphia, PA 19140. Tel & FAX 215-707-8378, E-mail seguchi@temple.edu
**Supplemental Methods and Figures**

**Supplemental Methods**

**Reagents and Antibodies**
AngII was purchased from Sigma Chemical Co. (St. Louis, MO). PP2, PP3, AG1478 and staurosporine were purchased from Calbiochem (La Jolla, CA). RNH6270 and YM-254890 were gifts from Sankyo Pharmaceutical Co Ltd. (Tokyo, Japan) and Astellas Pharma Inc. (Tokyo, Japan), respectively.

Sources and dilutions of the primary antibodies used are as follows. Phospho-specific antibodies for Tyr$^{311}$-phosphorylated PKCδ (1:10,000) and Tyr$^{1068}$-phosphorylated EGF receptor (1:6,000) were purchased from Biosource (Camarillo, CA). Phosphospecific antibody for Ser$^{473}$-phosphorylated Akt (1:4,000), total Akt antibody (1:6,000) and cleaved caspase-3 antibody (1:10,000) were purchased from Cell Signaling (Beverly, MA). Phosphospecific antibody for Tyr$^{202}$-phosphorylated ERK1/2 (1:20,000) and antibodies against PKCδ (1:10,000), PKCα (1:10,000) and Src (1:10,000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for GAPDH (1:10,000) was purchased from Chemicon International (Temecula, CA). Antibody for GFP (1:10,000) was purchased from BD Biosciences (Palo Alto, CA).

**Cell culture**
Rat aortic VSMCs were obtained and maintained as described previously. Subcultured cells from passages 3-12 were used in the experiments and showed more than 99% positive immunostaining of smooth muscle α-actin antibody $^1$. For both short-term (2-10 min) and long-term (3 days) experiments, cells were cultured to ~80% confluency and then made quiescent by incubating in serum-free media for 2-3 days. Average confluency before AngII stimulation is a little more than 80% but still less than 90% since VSMCs do not proliferate significantly after the serum starvation without a mitogen.

**Statistical Analysis**
Unless otherwise stated, the data presented in this study are representative of a minimum of three independent experiments yielding similar results. The data were analyzed using one-way ANOVA followed by a post-hoc modified t-test (Figure 1, 2A and 2B) or a two-way ANOVA (Figure 3B, 4 and 5).

**Reference**
Supplemental Figure Legends

Figure S1: A. VSMCs were stimulated with 100 nmol/L AngII for the indicated time periods. B and C. VSMCs were pretreated with (A) AT1 receptor blocker, RNH6270 (10 µmol/L) or (B) a selective Gq inhibitor, YM-254890 (1 µmol/L) for 30 min and stimulated with AngII (100 nmol/L) for 2 min. D. VSMCs were pretreated with a Src family kinase inhibitor, PP2 (5 µmol/L) or its negative control, PP3 (5 µmol/L) for 30 min and stimulated with 100 nmol/L AngII for 2 min. The cell lysates were immunoblotted with phospho-selective antibody which detects PKCδ Tyr311 phosphorylation and anti-PKCδ antibody. The PKCδ phosphorylation at Tyr311 was measured by densitometry and shown as mean ± SEM for three independent experiments. *P < 0.05 compared to the basal control. †P < 0.05 compared to the stimulated control.

Figure S2: A. VSMCs were infected with retrovirus encoding control vector, wild type PKCδ tagged with GFP, or PKCδ Y311F mutant tagged with GFP. VSMCs were then stimulated with 100 nmol/L AngII for 3 days. Cell volume was analyzed with a Coulter counter. B. VSMCs were infected with retrovirus encoding control vector, wild type PKCδ, or PKCδ Y311F mutant. The cells were stimulated with 100 nmol/L AngII for 3 days. Afterwards, cell proliferation was measured by a CellTiter 96 Aqueous cell proliferation/viability assay kit. The data was presented as fold basal mean ± SEM for three independent experiments. C. VSMCs were infected with adenovirus encoding control vector or wild type PKCδ. The cells were stimulated with 100 nmol/L AngII for 24 hours. Afterwards, DNA synthesis was assessed by a BrdU incorporation kit. The data was presented as mean ± SEM (n=5).

Figure S3: A. VSMCs were infected with adenovirus encoding wild type PKCδ or control empty vector, and stimulated with 100 nmol/L AngII for 4 hours. B. VSMCs were stimulated with 100 nmol/L AngII or 1 mmol/L staurosporine (Staur) for 4 hours. The cell lysates were immunoblotted with cleaved caspase-3 selective antibody, anti-GAPDH antibody and anti-PKCδ antibody, as indicated. Note that an apoptosis inducer, staurosporine, markedly stimulated cleavage of caspase 3. Data shown are representative from three independent experiments.

Figure S4: A. VSMCs were infected with adenovirus encoding a kinase-inactive PKCδ mutant (K376A) or control empty vector, and stimulated with 100 nmol/L AngII for 3 days. Cell volume was analyzed with a Coulter counter. B. VSMCs were infected with adenovirus encoding K376A or control vector. The cells were stimulated with 100 nmol/L AngII for 3 days. Afterwards, cell proliferation was measured by a CellTiter 96 Aqueous cell proliferation/viability assay kit. The data was presented as fold basal mean ± SEM for three independent experiments.
Supplement Figure S2

A

Cell Number (%) vs. Cell Volume (µm³)

- basal
- AngII

vector

B

Proliferation (%)

- basal
- AngII

vector PKCδ Y311F

C

BrdU uptake (OD)

PKCδ - + - +
AngII - - + +
Supplement Figure S3

A

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Cleaved caspase-3 (19/17 kDa)

GAPDH

PKCδ

B

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Cleaved caspase-3 (19/17 kDa)

GAPDH