Phospholipase Cγ1 Mediates Intima Formation Through Akt-Notch1 Signaling Independent of the Phospholipase Activity

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Background—Vascular smooth muscle cell proliferation, migration, and dedifferentiation are critical for vascular diseases. Recently, it was demonstrated that Notch receptors have opposing effects on intima formation after vessel injury. Therefore, it is important to investigate the specific regulatory pathways that activate the different Notch receptors.

Methods and Results—There was a time- and dose-dependent activation of Notch1 by angiotensin II and platelet-derived growth factor in vascular smooth muscle cells. When phospholipase Cγ1 (PLCγ1) expression was reduced by small interfering RNA, Notch1 activation and Hey2 expression (Notch target gene) induced by angiotensin II or platelet-derived growth factor were remarkably inhibited, while Notch2 degradation was not affected. Mechanistically, we observed an association of PLCγ1 and Akt, which increased after angiotensin II or platelet-derived growth factor stimulation. PLCγ1 knockdown significantly inhibited Akt activation. Importantly, PLCγ1 phospholipase site mutation (no phospholipase activity) did not affect Akt activation. Furthermore, PLCγ1 depletion inhibited platelet-derived growth factor–induced vascular smooth muscle cell proliferation, migration, and dedifferentiation, while it increased apoptosis. In vivo, PLCγ1 and control small interfering RNA were delivered periadventitially in pluronic gel and complete carotid artery ligation was performed. Morphometric analysis 21 days after ligation demonstrated that PLCγ1 small interfering RNA robustly attenuated intima area and intima/media ratio compared with the control group.

Conclusions—PLCγ1-Akt–mediated Notch1 signaling is crucial for intima formation. This effect is attributable to PLCγ1-Akt interaction but not PLCγ1 phospholipase activity. Specific inhibition of the PLCγ1 and Akt interaction will be a promising therapeutic strategy for preventing vascular remodeling. (*J Am Heart Assoc*. 2017;6:e005537. DOI: 10.1161/JAHA.117.005537.)

Key Words: Akt • intima formation • Notch1 • phospholipase Cγ1 • vascular smooth muscle cell

L ong-term outcomes of bypass surgery and percutaneous coronary intervention are limited by restenosis accounting for accelerated neointima formation.[1] The excessive proliferation and migration of vascular smooth muscle cells (VSMCs) from the media are critical events for intima thickening at sites of vascular injury.[2] During the progression of intima thickening, platelet-derived growth factor (PDGF) and angiotensin II (Ang II) are excessively released, subsequently stimulating intracellular transduction cascades and promoting proliferation and migration of VSMCs.[3,4] Furthermore, VSMCs are not terminally differentiated and possess the ability to modulate their phenotypes in response to remodeling, sheer stress, and other environmental cues. The phenotypic switch in VSMCs is accompanied by accelerated migration and proliferation, eventually resulting in the formation of vascular neointima.[5]

Clinical Perspective

What Is New?

- Phospholipase Cγ1 is crucial for angiotensin II and platelet-derived growth factor–induced Notch1 activation but not Notch2 degradation in vascular smooth muscle cells.
- Phospholipase Cγ1 recruits 3-phosphoinositide-dependent protein kinase-1 and mammalian target of rapamycin to bind Akt and subsequently facilitate the activation of Akt and Notch1 signaling, independently of the phospholipase enzyme activity.
- Phospholipase Cγ1 mediates intima formation by accelerating vascular smooth muscle cell proliferation, migration, dedifferentiation, and survival.

What Are the Clinical Implications?

- Designing specific small molecules or blocking peptides to interrupt phospholipase Cγ1 and Akt interaction could be promising therapeutic strategies for vascular remodeling–related diseases including restenosis after bypass surgery and percutaneous coronary intervention.

Notch receptors, including Notch1–4, belong to an evolutionarily conserved family of membrane receptors that transduce intercellular signals and play a central role in cardiovascular development and diseases.[6,7] Upon binding to ligands, Notch receptors are catalyzed and cleaved by γ-secretase to allow the Notch intracellular domain to translocate into the nucleus and activate target genes.[8] Various Notch receptors play different roles in vascular remodeling.[9] Notch1 has been shown to increase intima formation induced by carotid artery ligation through promoting VSMC proliferation and migration.[10] Whereas Notch2 has been shown to suppress VSMC proliferation and vascular remodeling by activating p27kip1 in vitro.[11] Notch3 plays an essential role in pulmonary hypertension progression.[12] Notch3 mutations in humans lead to cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy but have no effect on intima formation.[10,13] Notch4 is highly expressed in endothelial cells but not in VSMCs.[14,15] Given the divergent effects of Notch receptors on vascular remodeling, there is no preferred specific Notch inhibitor (such as the γ-secretase inhibitor, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) that may serve as a pharmacological inhibitor for vascular remodeling-related diseases. Therefore, it is critical to investigate the regulatory mechanisms of Notch1 signaling during intima formation.

Phospholipase Cγ1 (PLCγ1), a member of the phosphoinositide-specific phospholipase C (PLC) family, is a key regulator of several growth factor–induced signaling pathways, such as Ang II, PDGF, epidermal growth factor, and fibroblast growth factor.[16] Numerous studies revealed the function of PLC using the inhibitor U73122. Activated PLC hydrolyzes phosphatidylinositol-4,5-bisphosphate to generate the second messengers including diacylglycerol and inositol-1,4,5-trisphosphate. Consequently, protein kinase C (PKC) is activated and intracellular calcium is increased. These processes are critical for cell proliferation, migration, and survival.[17]

However, the physiological function of PLCγ1 has not been well established, especially in vivo.[18] U73122 can inhibit the catalytic activity of PLC, and then inhibit ERK1/2 activation and cell proliferation in VSMCs.[14,18,19] However, U73122 is not specific to PLCγ1.[20–22] In the current study, using PLCγ1 small interfering RNA (siRNA), we revealed that PLCγ1 specifically activates Notch1 through Akt, which is essential for intima formation after vessel injury.

Materials and Methods

Reagents

Recombinant rat PDGF was purchased from R&D Systems. Human Ang II, losartan, sunitinib, wortmannin, and DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) were purchased from Sigma-Aldrich. Primary antibodies of phospho-Akt Ser473, phospho-Akt Thr308, Akt, phospho-PLCγ1 Tyr783, PLCγ1, 3-phosphoinositide-dependent protein kinase-1 (PDK1), mammalian target of rapamycin (mTOR), and Notch2 were purchased from Cell Signaling Technology; Hey2 antibody was purchased from Beijing Biosynthesis Biotechnology; antibody of Notch1 used in Western blot was from Santa Cruz Biotechnology; and Notch1 intracellular domain (N1-ICD) antibody used in immunohistochemistry was from Millipore.

Plasmid Constructs

The pcDNA3.1/Zeo (+) vector containing the entire coding sequence of rat PLCγ1 was generously provided by Bradford Berk (University of Rochester). The phospholipase site–mutated PLCγ1 was generated from rat PLCγ1 plasmid by polymerase chain reaction using primers 5′-ttggatctcttcctcgtttaatacgtatctgact (H335F) and 5′-gatgccagtcatttactttgggcacaccctcacc (H380F) resulting in substitution of phenylalanine for histidine-335 and histidine-380, and then inserted into the pcDNA3.1/Zeo (+) vector (MUT-PLCγ1).

Cell Culture and Transfection

Rat aortic smooth muscle cells (RASMCs) were isolated by enzymatic digestion and maintained in DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37°C in 95% CO_2.[23] Passages 4 to 8 were used for

experiments. siRNA specific for rat PLCγ1 (CAGTCATCCTGTC-CATCGA) and control siRNA (siCtr) (ATCTCCGAACGTGTCAC-GATT) were transfected into RASMCs at 100 nmol/L using HiPerFect Reagent (Qiagen). RASMCs were serum-deprived for 24 hours before experimental treatments.

Human embryonic kidney 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37°C in 5% CO_2. Human embryonic kidney 293T cells were transfected with siRNA or plasmids by Lipofectamine 2000 (Thermo Fisher Scientific).

Western Blot and Immunoprecipitation

RASMCs and carotid arteries were lysed in ice-cold cell lysis buffer (Cell Signaling Technologies) containing a protease inhibitor cocktail (Sigma). Protein concentrations were determined by bicinchoninic acid protein assay. Lysates containing 400 μg of soluble proteins were incubated with anti-Akt, anti-PDK1, or anti-mTOR (diluted 1:100) overnight at 4°C. Antibody complexes were collected by incubation with Protein A/G agarose (Santa Cruz Biotechnology) for 2 hours. Precipitates were washed 5 times with lysis buffer and then resuspended in SDS-PAGE loading buffer. Then Western blot analysis was performed as previously described in our study. After blocking, the membrane was incubated with the following antibodies at 4°C overnight: phospho-PLCγ1 (Y783) (diluted 1:800), PLCγ1 (diluted 1:1000), phospho-Akt (S473) (diluted 1:1000), phospho-Akt (T308) (diluted 1:1000), Akt (diluted 1:1000), PDK1 (diluted 1:1000), mTOR (diluted 1:1000), Notch1 (diluted 1:300), Notch2 (diluted 1:800), and Hey2 (diluted 1:200). After washing and incubation with anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase or IRDye 700/800 for 1 hour, membranes were visualized with the Amersham Imager 600 ECL system (GE Healthcare) or the LI-COR Odyssey infrared scanner (LI-COR Bioscience).

Real-Time Polymerase Chain Reaction Assay

Total RNA was extracted from RASMCs using Trizol Reagent (Invitrogen). Reverse transcription was performed using 1 μg of RNA with a PrimeScript RT Reagent Kit (Takara) and the relative mRNA expression levels were determined by real-time polymerase chain reaction using the SYBR Premix Ex Taq assay (Takara). Primer sequences used in real-time polymerase chain reaction are documented in Table S1.

Cell Proliferation Assay and Scratch Wound Assay

For proliferation assays, RASMCs were seeded in 96-well plates and transfected with 100 nmol/L siRNA using

Hiperfect Reagent for 48 hours. RASMCs were serum-deprived for 24 hours and then treated with or without PDGF (20 ng/mL) for up to 48 hours. Cells were incubated with 20 μL of MTS (Promega) for 2 hours and measured at 490 nm absorbance by an automatic microplate reader (SpectraMaxi3, Molecular Devices). For the scratch wound assay, RASMCs were grown on 35-mm dishes and transfected as described above. The monolayer was scratched with a standard 100-μL pipette tip and initial scrape wound was photographed to calculate the initial wound area. After scratching, the cells were gently washed with normal medium without serum. Then RASMCs were treated with PDGF (20 ng/mL) or dimethyl sulfoxide. The wound healing was calculated as the percentage of remaining cell-free area compared with the original wound area.

Cell Apoptosis Assay

Apoptosis of RASMCs was determined by annexin V-FITC-PI staining (BD Biosciences) and analyzed by flow cytometry (FACSCalibur, BD Biosciences) as described in our previous study.[21] Annexin V–positive cells were considered as early apoptosis, while cells stained by annexin V and propidium iodide were considered as late apoptosis. Moreover, CellEvent Caspase-3/7 Green Detection Reagent (Thermo Fisher Scientific) was also used to analyze RASMC apoptosis according to the manufacturer's instructions. RASMCs were incubated with serum-free medium for 48 hours and then the reagent was added for 30 minutes. The fluorescence images were acquired by Olympus IX83 microscope (Olympus).

Complete Common Carotid Artery Ligation

The experiments outlined in this article conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication, 8th Edition, 2011). All animal procedures were approved by the Animal Care and Use Committees of Shanghai Tenth People's Hospital. Ten-week-old male C57BL/6J mice were subject to complete carotid artery ligation as previously described.[21] Mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The left common carotid artery was completely ligated with a 6-0 silk suture just proximal to the carotid bifurcation. In the right common carotid artery (sham), a similar procedure was performed but without ligation.

Pluronic Gel Delivery of siRNA In Vivo

Pluronic gel (F-127, KeyGen) was made as a 40% solution (1 g dissolved in 2.5 mL of ice-cold sterile PBS) and kept
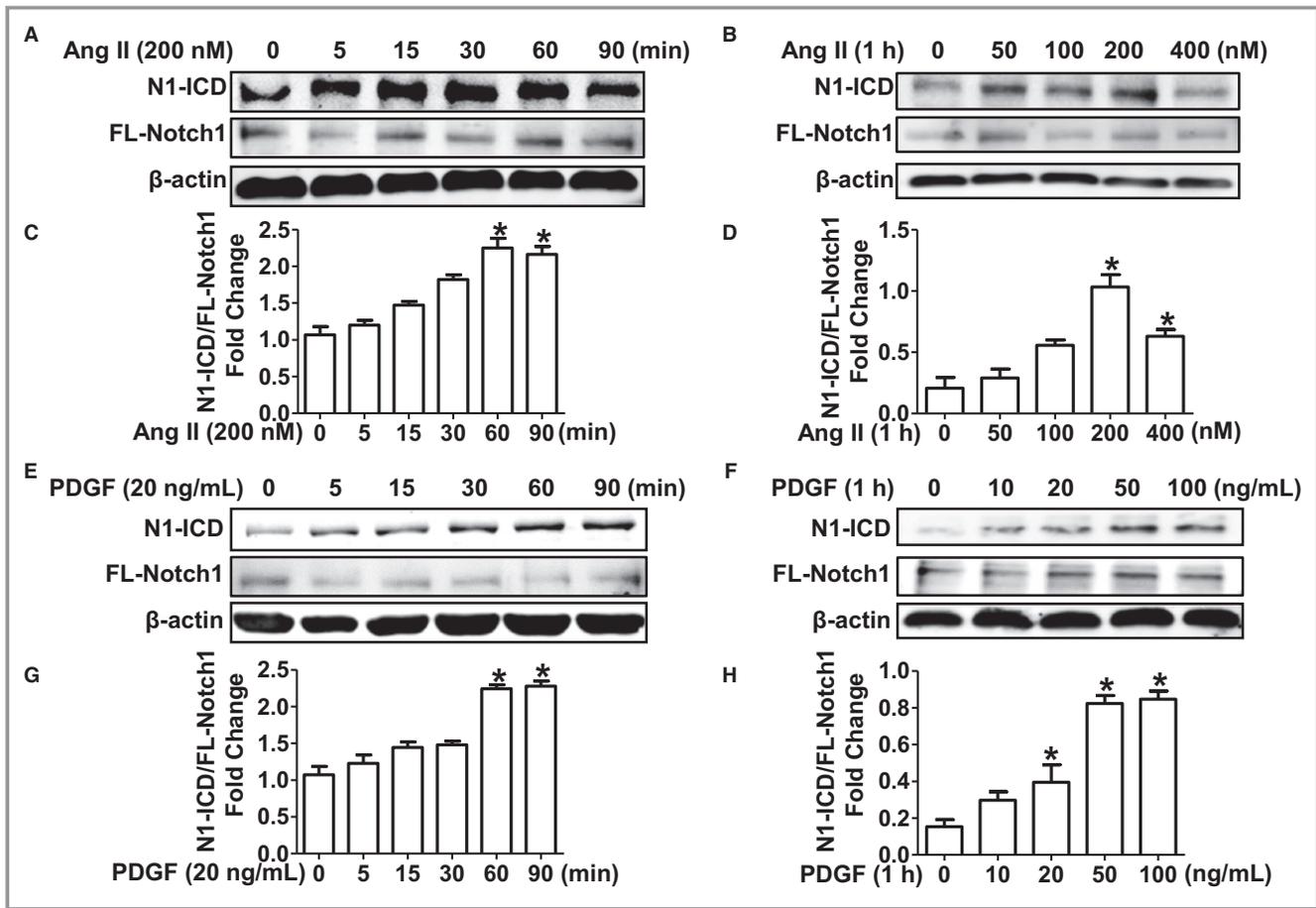
Figure 1. Phospholipase Cγ1 (PLCγ1) is required for Notch1 cleavage stimulated by angiotensin II (Ang II) and platelet-derived growth factor (PDGF). A through D, Rat aortic smooth muscle cells (RASMCs) were treated with 200 nmol/L Ang II for different time (A and C) or different dose of Ang II for 1 hour (B and D). Notch1 intracellular domain (N1-ICD), FL-Notch1, and β-actin expression were measured by Western blot. Notch1 cleavage (measured by N1-ICD/Notch1) after Ang II stimulation was quantified (mean±SEM, n=3) (C and D). *P<0.05 vs the cells not treated with Ang II. E through H, RASMCs were treated with 20 ng/mL of PDGF for different time (E and G) or different dose of PDGF for 1 hour (F and H). N1-ICD, FL-Notch1, and β-actin expression were measured by Western blot. Notch1 cleavage (measured by N1-ICD/Notch1) after PDGF stimulation was quantified (mean±SEM) (G and H). *P<0.05 vs the cells not treated with PDGF. I and J, RASMCs were transfected with control small interfering RNA (siCtr) or PLCγ1 small interfering RNA (siPLCγ1) for 48 hours followed by stimulation of Ang II (200 nmol/L, E) or PDGF (20 ng/mL, F) for 1 hour. N1-ICD, FL-Notch1, N2-ICD, FL-Notch2, t-PLCγ1, and β-actin expression were measured by Western blot. K, PLCγ1 protein levels were quantified (normalized to β-actin after small interfering RNA knockdown) (mean±SEM, n=3). #P<0.05 vs the siCtr group. L and M, Notch1 cleavage was quantified by N1-ICD normalized to FL-Notch1 (mean±SEM, n=3). *P<0.05 vs the siCtr group. #P<0.05 vs the siCtr+PDGF group. Experiments were performed in triplicate.

on ice. The left common carotid artery was dissected free of the surrounding connective tissue. A 6.0 silk suture was placed beneath the common carotid and gently used to lift the carotid out of the neck cavity. Thirty-five microliters of ice-cold pluronic gel containing either siCtr (100 nmol/L) or PLCγ1 siRNA (siPLCγ1) (100 nmol/L) (Sigma) was applied to the neck cavity, the suture was removed to release the carotid back into the neck, and another 35 μL of pluronic gel containing siRNA was applied to the top of the carotid.[24] The incision was sutured, and mice were individually housed and allowed to recover.

Morphometric Analysis

For morphological analysis, mice were perfused and fixed with 10% phosphate-buffered formalin for 10 minutes. The carotid arteries were harvested, fixed overnight, and embedded in paraffin. To evaluate the entire vessel, cross sections of the entire carotid arteries were prepared with 5 sections located at 200-μm intervals from the carotid bifurcation. Vessel areas were measured with ImagePro Plus software (Media Cybernetics Inc) as previously discribed.[21] In brief, the intima area was calculated as the internal elastic lamina area minus the luminal area, the medial area was the external elastic lamina
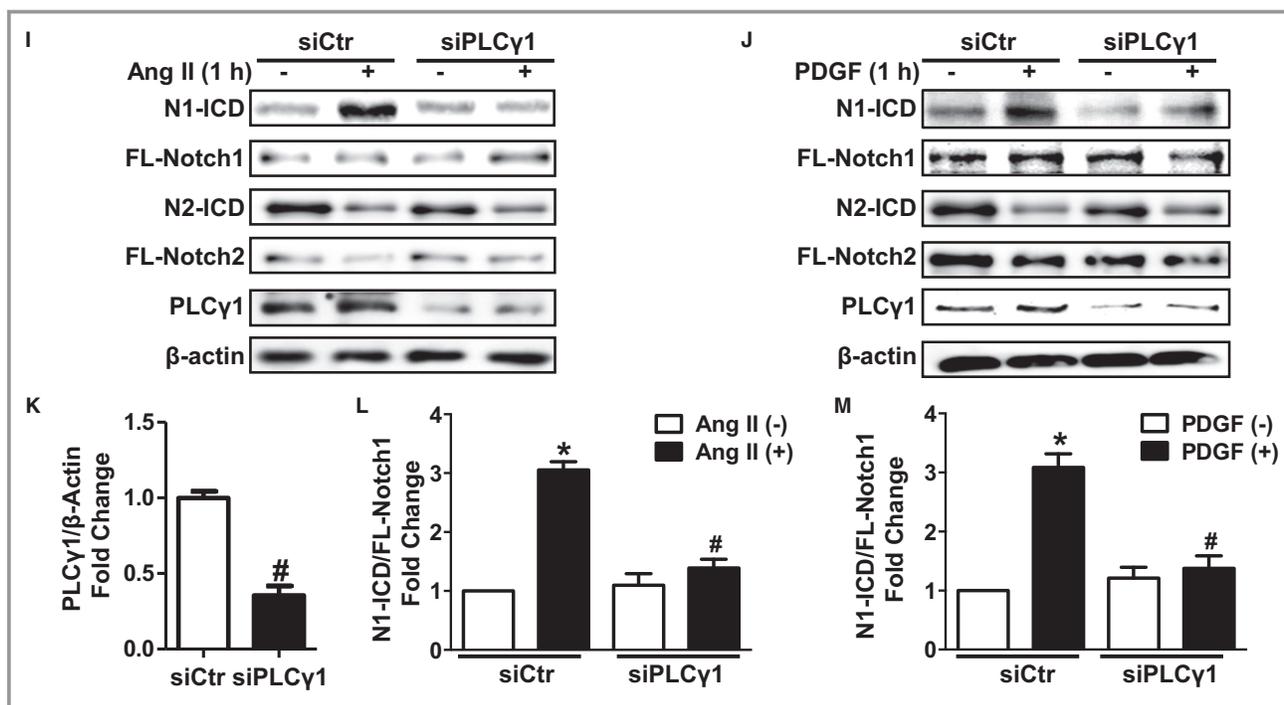
Figure 1. Continued

area minus the internal elastic lamina area, and the adventitial area was the vascular area minus the external elastic lamina area.

Immunohistochemistry and Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Staining

For immunohistochemical staining, cross sections were incubated with N1-ICD antibody (diluted 1:50, Millipore, 07-1232) and Hey2 antibody (diluted 1:50, Biosynthesis Biotechnology, bs-9461R) at 4°C overnight after microwave antigen retrieval in citrate buffer. Immunostaining was visualized using species-specific horseradish peroxidase–conjugated secondary antibodies for 1 hour and DAB for 30 to 90 seconds. Cell apoptosis was determined by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay on paraffin sections of carotid arteries (21 days after complete ligation) according to the manufacturer's instructions (Keygene Biotech). Specimens were counterstained with hematoxylin and images were acquired with Leica DMI6000 microscopy (Leica).

Statistical Analysis

All analyses were performed with SPSS version 14.0 (SPSS Inc). Data are given as mean±SEM unless otherwise indicated. Differences between 2 groups were examined using Student t test. One-way ANOVA was used to compare multiple groups, if appropriate, with Bonferroni correction for post hoc analysis. A P value <0.05 was considered statistically significant. All experiments were performed at least 3 times. The statistics were computed taking all the independent experiments into account.

Results

### PLCγ1 is Required for Notch1 Activation and its Target Gene Expression Induced by Ang II and PDGF in VSMCs

The Notch receptor family comprises highly conserved membrane proteins. Notch receptors are activated (cleaved) by γ-secretase to generate the Notch intracellular domain.[25] Different Notch receptors have different roles in intima formation.[10] Several growth factors, such as Ang II and PDGF, are important for VSMC function and intima formation.[3,4] Therefore, it is critical to study the effects of these growth factors on the activation of various Notch receptors. We determined Notch1 activation (Notch1 cleavage) stimulated by Ang II and PDGF in RASMCs. Ang II–dependent Notch1 activation gradually increased and reached its peak at 60 minutes (Figure 1A and 1C). This response was dose-dependent and began to increase from 50 nmol/L with maximal activation at 200 nmol/L (Figure 1B and 1D). Similarly, cleaved Notch1 was detected in a time-dependent manner with a peak at 60 and 90 minutes following PDGF
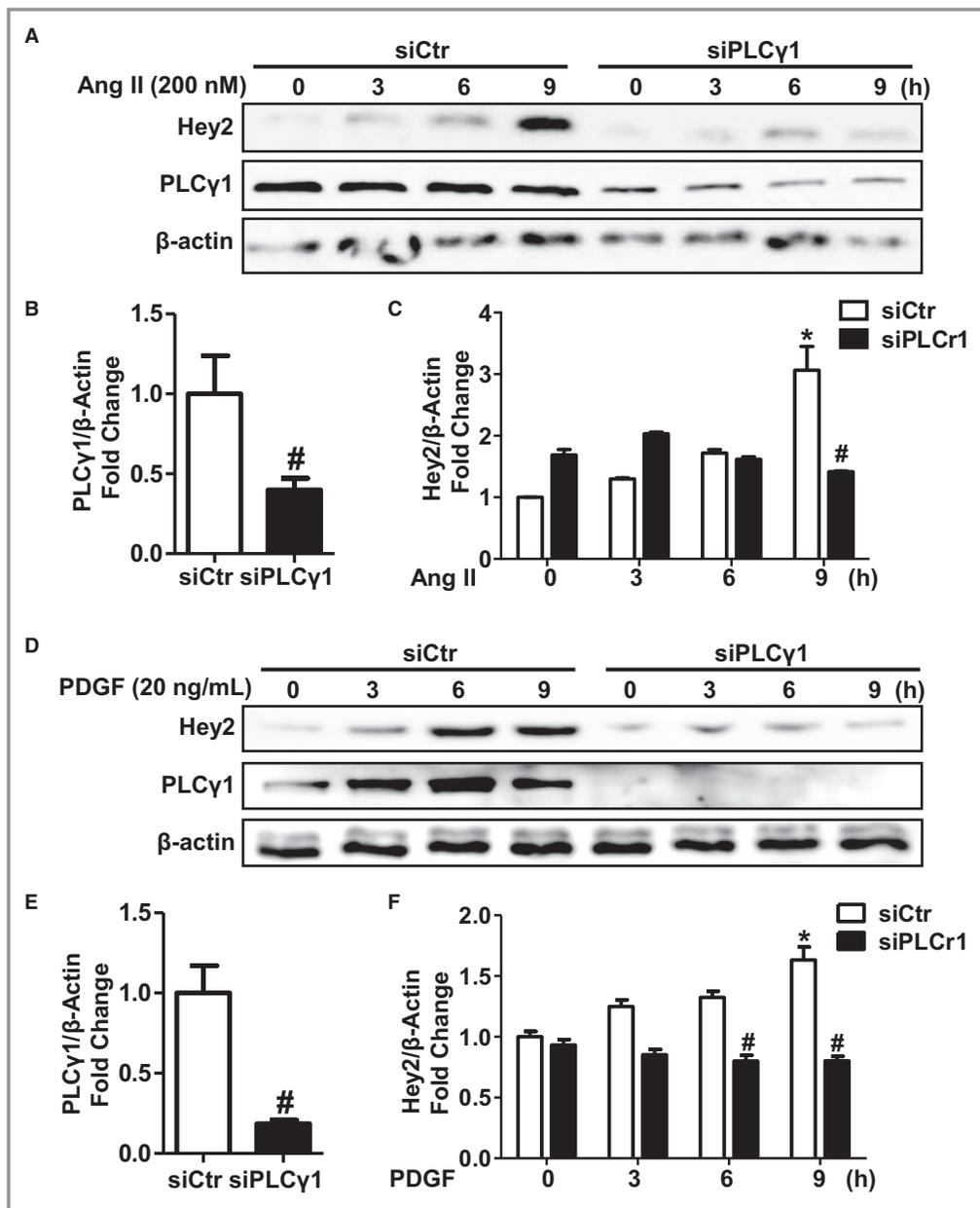
Figure 2. Phospholipase Cγ1 (PLCγ1) depletion suppresses Hey2 expression stimulated by angiotensin II (Ang II) and platelet-derived growth factor (PDGF). A through C, Representative immunoblot (A) and quantitative results (C) of Hey2 expression after PLCγ1 depletion. Rat aortic smooth muscle cells (RASMCs) were transfected with control small interfering RNA (siCtr) or PLCγ1 small interfering RNA (siPLCγ1) for 48 hours and then stimulated by Ang II (200 nmol/L). PLCγ1 levels were quantified after small interfering RNA (siRNA) knockdown (normalized to β-actin) (B). D through F, Representative immunoblot (D) and quantitative results (F) of Hey2 expression induced by PDGF after PLCγ1 depletion. RASMCs were transfected with siCtr or siPLCγ1 for 48 hours and then stimulated by PDGF (20 ng/mL). PLCγ1 levels were quantified after siRNA knockdown (normalized to β-actin) (E). All experiments were performed in triplicate. Quantification of Hey2 was normalized to β-actin (mean±SEM, n=3). *$P<0.05$ vs the siCtr group at baseline. #$P<0.05$ vs the siCtr group with Ang II or PDGF treatment at the same time point.

stimulation (Figure 1E and 1G). This response was also dose-dependent from a minimum at 10 ng/mL to a maximum at 50 ng/mL (Figure 1F and 1H). The total Notch1 level was not affected.

PLCγ1 plays an important role in Ang II and PDGF signaling. However, the effect of PLCγ1 on Notch signaling in VSMCs has not been reported. Thus, N1-ICD levels were assessed after PLCγ1 depletion by siRNA in RASMCs. PLCγ1
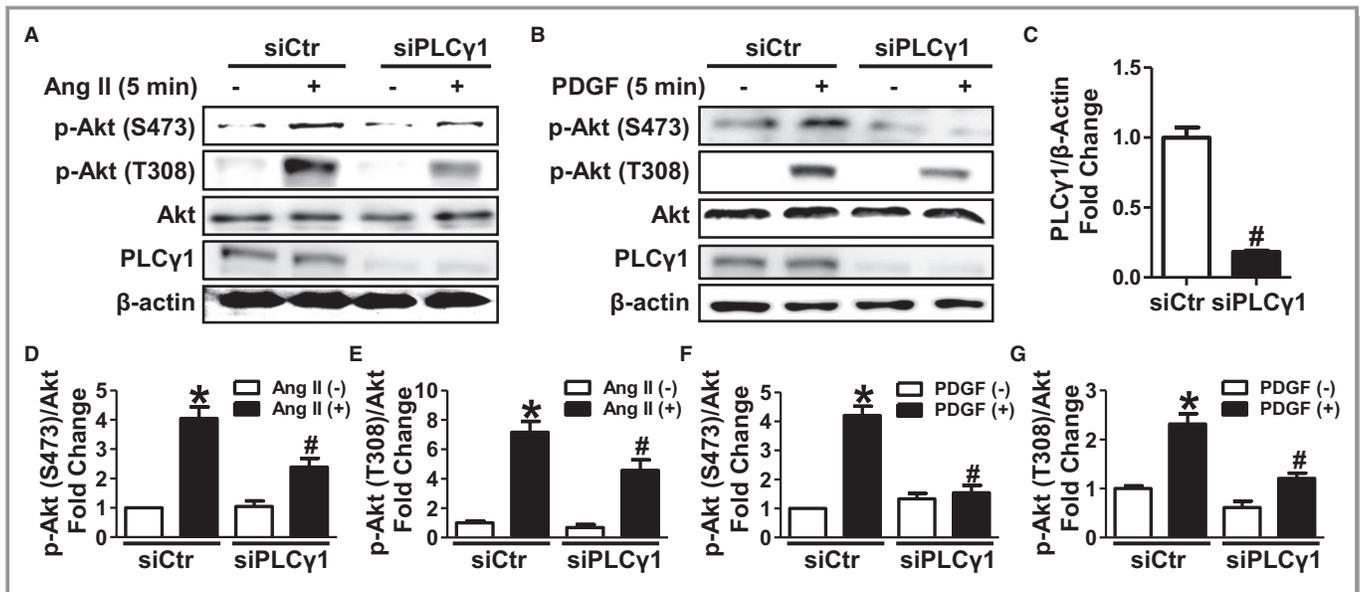
Figure 3. Phospholipase Cγ1 (PLCγ1)–mediated Akt activation is essential for Notch1 cleavage. A through G, Representative immunoblot and quantification of Akt phosphorylation (p-Akt) on S473 and T308 after PLCγ1 depletion. Rat aortic smooth muscle cells (RASMCs) were transfected with control small interfering RNA (siCtr) or PLCγ1 small interfering RNA (siPLCγ1) for 48 hours and then stimulated by angiotensin II (Ang II; 200 nmol/L, A, D, and E) or platelet-derived growth factor (PDGF; 20 ng/mL, B, F, and G) for 5 minutes. PLCγ1 levels were quantified after small interfering RNA (siRNA) knockdown (normalized to β-actin) (C). P-Akt phosphorylation on S473 and T308 were quantified after small interfering RNA knockdown (normalized to total Akt) (D through G). Bars indicate mean±SEM. *$P<0.05$ vs the siCtr group. #$P<0.05$ vs the siCtr with Ang II or PDGF treatment. H, The binding of PLCγ1 and Akt was assayed by immunoprecipitation (IP) with t-Akt antibody and probing for t-PLCγ1. Serum-starved RASMCs were treated with vehicle, 200 nmol/L Ang II, or 20 ng/mL PDGF for 5 minutes. Then cell lysate was collected for IP assay. I and J, The binding of PLCγ1 and mammalian target of rapamycin (mTOR) (I) or 3-phosphoinositide–dependent protein kinase–1 (PDK1) (J) was assayed by IP with mTOR antibody or PDK1 antibody and probing for PLCγ1 in RASMCs. K through M, Akt activation after overexpression of phospholipase site–mutated PLCγ1 (H335F and H380F) was assayed in PLCγ1 knockdown human embryonic kidney 293T cells. Effects of Akt or Notch inhibitors on PLCγ1 and p-Akt and Notch1 cleavage induced by Ang II (N) or PDGF (O). RASMCs were pretreated with 1 μmol/L wortmannin (Wort) and 10 μmol/L N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) for 12 hours and then incubated with Ang II (200 nmol/L) or PDGF (20 ng/mL). All experiments were performed in triplicate. DMSO indicates dimethyl sulfoxide; WT, wild-type; MUT, mutant.

depletion significantly decreased N1-ICD by 57% (Figure 1I and 1L) and 60% (Figure 1J and 1M) induced by Ang II and PDGF, respectively, compared with siCtr. Interestingly, knockdown of PLCγ1 did not affect total Notch2 levels at baseline or following stimulation by Ang II or PDGF (Figure 1I and 1J). As Notch3 has no effects on intima formation in vivo and Notch4 is highly expressed in endothelial cells, we did not detect Notch3/4 protein expression and activation after PLCγ1 knockdown.

Upon activation, N1-ICD translocates to the nucleus and can associate with Notch corepressor RBP-J to enhance the Notch target gene expression.[25] Notch1 target gene Hey2 has been shown to be essential for VSMC function, and Hey2 knockout mice display decreased intima formation after femoral arterial injury.[26,27] Therefore, we measured Hey2 expression in RASMCs. After Ang II and PDGF stimulation, there was a time-dependent increase of Hey2 expression, which peaked at 9 hours. Compared with siCtr, PLCγ1 siRNA markedly repressed Ang II and PDGF-induced Hey2

expression (a 63% reduction and a 50% reduction, respectively) (Figure 2). All these data indicate the specific role of PLCγ1 in Notch1 activation induced by Ang II and PDGF.

PLCγ1-Mediated Akt Activation is Critical for Notch1 Activation

It is reported that in endothelial cells, Akt increases γ-secretase activity and subsequently enhances Notch1 activation (Notch1 cleavage) to promote angiogenesis.[7,28] PLCγ1 binds to Akt and Akt phosphorylates PLCγ1 in response to growth factors in tumor cells.[29] Therefore, we hypothesized that PLCγ1 binds to Akt to facilitate Akt phosphorylation and subsequently increases activation of the Notch1 pathway in VSMC. To test this hypothesis, we first measured the activation of PLCγ1 and Akt induced by Ang II and PDGF.[17,30] Ang II (100–400 nmol/L) and PDGF (20–100 ng/mL) significantly increased phoshorylation of PLCγ1 and Akt (Ser473) in RASMCs (Figure S1A and S1B). In response to 200 nmol/L
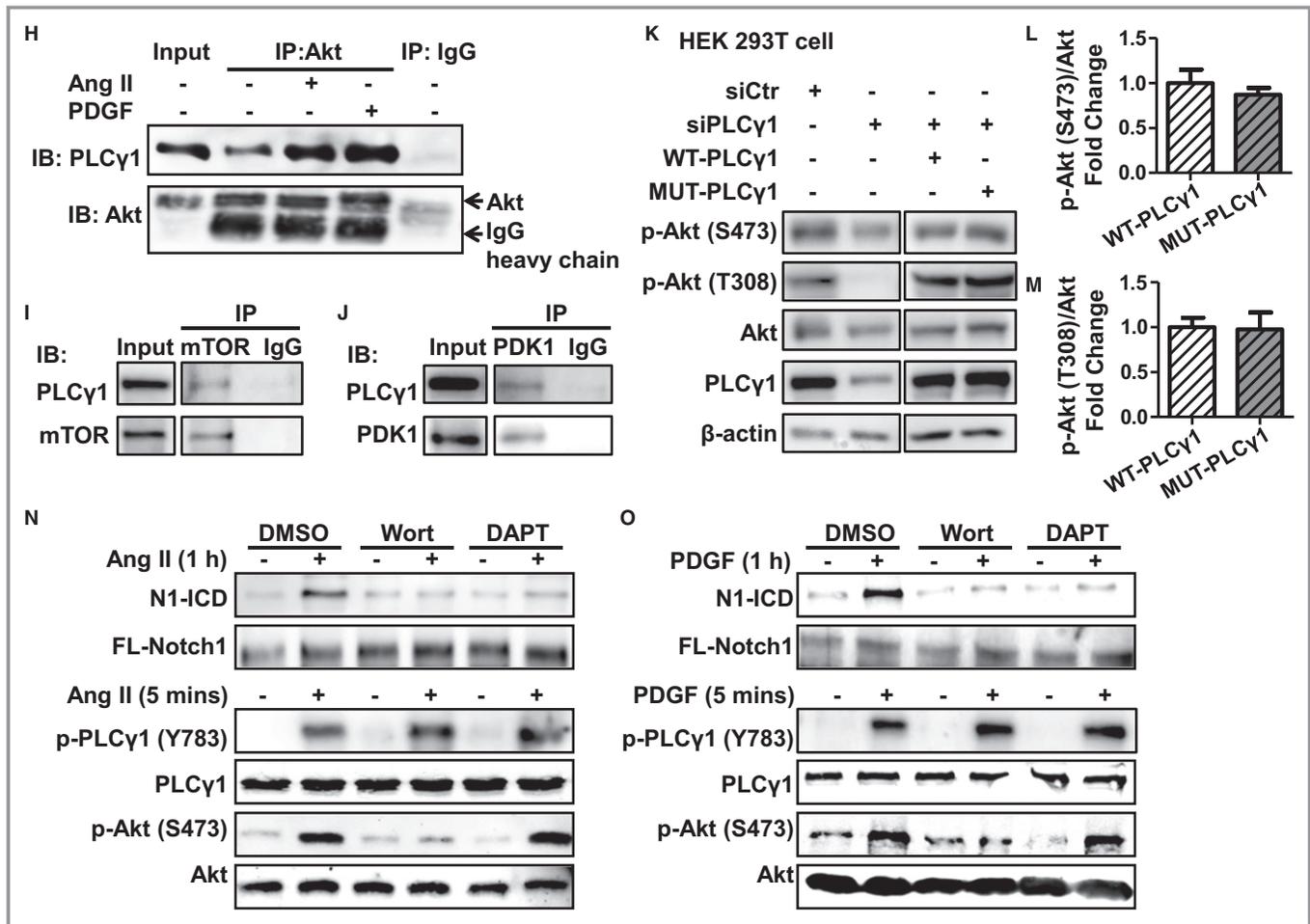
Figure 3. Continued

Ang II and 20 ng/mL PDGF, phospho-PLCγ1 and phospho–Akt rapidly peaked at 5 and 15 minutes, respectively, and then decreased (Figure S1C and S1D).

To determine the effect of PLCγ1 on Akt activation, RASMCs were treated with siCtr or PLCγ1 siRNA. Akt phosphorylation on Ser473 and Thr308 induced by Ang II or PDGF was measured by Western blot. Compared with siCtr, PLCγ1 depletion inhibited Ang II and PDGF mediated Akt phosphorylation on Ser473 by 44% and 61% and Akt phosphorylation on Thr308 by 36% and 48% (Figure 3A through 3G). To further investigate the interaction of PLCγ1 and Akt, we performed a coimmunoprecipitation assay in RASMCs. Without any stimulation, we observed the association of PLCγ1 and Akt (Figure 3H). The level of this interaction was significantly increased after stimulation of Ang II and PDGF for 5 minutes (Figure 3H). In addition, Akt can be phosphorylated on Ser473 by mTOR complex 2 and phosphorylated on Thr308 by PDK1.[31] The association of PLCγ1 with mTOR (the key protein of mTOR complex 2) or PDK1 was also measured. As we expected, PLCγ1 bound to PDK1 and mTOR. These results suggest that PLCγ1 is

required for Akt activation by recruiting PLCγ1-PDK1-Akt or PLCγ1-mTOR-Akt complexes.

It is well established that PLCγ1 activation increases inositol-1,4,5-trisphosphate and diacylglycerol through its catalytic activity, which enhances calcium levels and activation of ERK1/2 and PKC. It is reported that ERK1/2 decreases γ-secretase inhibitor activation in neuron cells.[32] We determined the effects of the ERK1/2 inhibitor (PD98059) and PKC inhibitor (Rottlerin) on Notch1 activation induced by Ang II and PDGF in RASMCs. Interestingly, both the ERK1/2 inhibitor and PKC inhibitor did not affect Notch1 activation (Figure S2), whereas ERK1/2 phosphorylation was decreased. Smith et al[20] demonstrated that His 335 and His 380 are important for PLCγ1 phospholipase activity (<10% of PLCγ1 catalytic activity after site mutation). To determine the role of PLCγ1 phospholipase activity in Akt activation, we overexpressed phospholipase site–mutated PLCγ1 (H335F and H380F) in PLCγ1-depleted human embryonic kidney 293T cells (RASMC is difficult to transfect plasmids). PLCγ1 mutation (phospholipase enzyme catalytically inactive) did not affect Akt activation (Figure 3K). These findings imply the
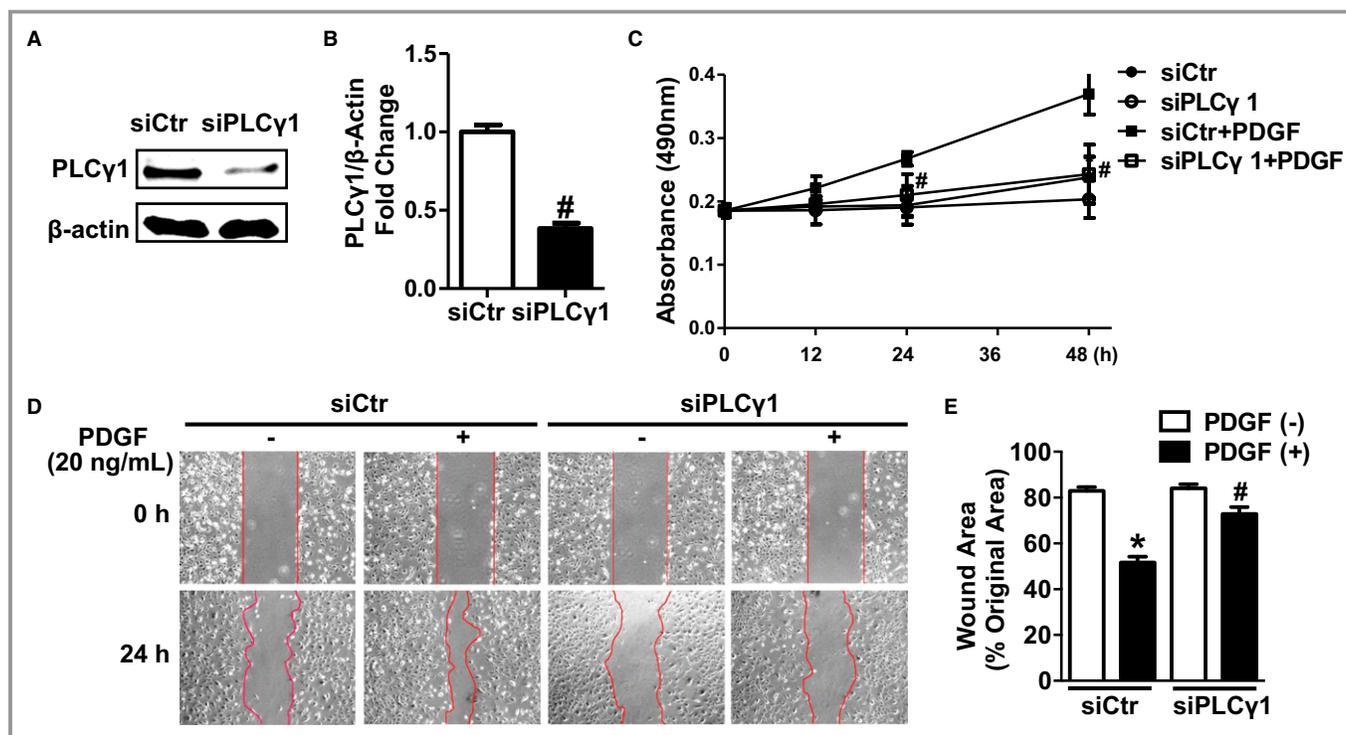
Figure 4. Effects of phospholipase Cγ1 (PLCγ1) knockdown on proliferation, migration, differentiation, and apoptosis in rat aortic smooth muscle cells (RASMCs). A and B, Representative immunoblot and quantification of PLCγ1 in RASMCs after transfection with control small interfering RNA (siCtr) or PLCγ1 small interfering RNA (siPLCγ1) for 48 hours. C, RASMCs are transfected with siCtr and siPLCγ1 for 48 hours. Then proliferation was assayed by MTS in the presence or absence of platelet-derived growth factor (PDGF; 20 ng/mL) at the indicated time points. D and E, After transfection with siCtr or siPLCγ1 for 48 hours, cell migration was assessed by scratch wound assay in RASMCs with or without PDGF (20 ng/mL) stimulation for 24 hours. Wound area was analyzed by ImagePro Plus software. F and G, Representative flow cytometry images and statistical results show apoptosis ratio of serum-starved RASMCs pretreated with siCtr or siPLCγ1. H and I, Representative images and statistical results show ratio of serum-starved RASMCs with activated caspase-3/7 pretreated with siCtr or siPLCγ1. White arrows indicate green nuclear signals of RASMCs in which caspase-3/7 was activated and cleaved DEVD peptide so that DNA-binding dye conjugated to DEVD was released and stained the nucleus. J, Quantification of the mRNA levels of VSMC differentiation marker genes by real-time polymerase chain reaction. Each experiment was repeated at least 3 times. Bars indicate mean±SEM. *$P<0.05$ vs the siCtr group. #$P<0.05$ vs the siCtr+PDGF group.

specific role of PLCγ1-Akt pathway in Notch1 activation in VSMC, which depends on the scaffold effect of PLCγ1 and is independent of PLCγ1 phospholipase activity.

To confirm the roles of the Ang II and PDGF receptors in PLCγ1-Akt-Notch1 signaling, we measured the effects of sunitinib (a PDGF inhibitor) and losartan (an Ang II type 1 receptor inhibitor) on Notch1 activation. As expected, sunitinib and losartan significantly inhibited Notch1 cleavage, PLCγ1 phosphorylation, and Akt phosphorylation induced by Ang II and PDGF (Figures S3 and S4). To prove the role of Akt in Notch1 activation, RASMCs were pretreated with the PI3K/Akt inhibitor wortmannin and then stimulated with Ang II or PDGF. Notch1 and Akt activation induced by Ang II and PDGF was markedly decreased, yet PLCγ1 phosphorylation was not affected (Figure 3N and 3O). As a positive control, Notch1 cleavage was blocked by pretreatment of the γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, whereas PLCγ1 and Akt phosphorylation

was comparable to vehicle control (Figure 3N and 3O). These results indicate that Akt activation is required for Notch1 cleavage.

PLCγ1 is Important for VSMC Proliferation, Migration, Dedifferentiation, and Apoptosis

The function of PLCγ in VSMC proliferation and migration and intima formation was determined using U73122.[17] As previously mentioned, U73122 is not specific to PLCγ1 but to the phospholipase activity of PLC. Moreover, PLCγ1 knockdown did not affect ERK1/2 activation but U73122 did.[33] Therefore, we used PLCγ1 siRNA to study the biological function of PLCγ1 in VSMCs. RASMCs proliferation, migration, dedifferentiation, and apoptosis were assessed after PLCγ1 siRNA treatment. PDGF increased RASMCs proliferation, whereas knockdown of PLCγ1 significantly inhibited proliferation (Figure 4C). Next, we performed scratch wound assays. In
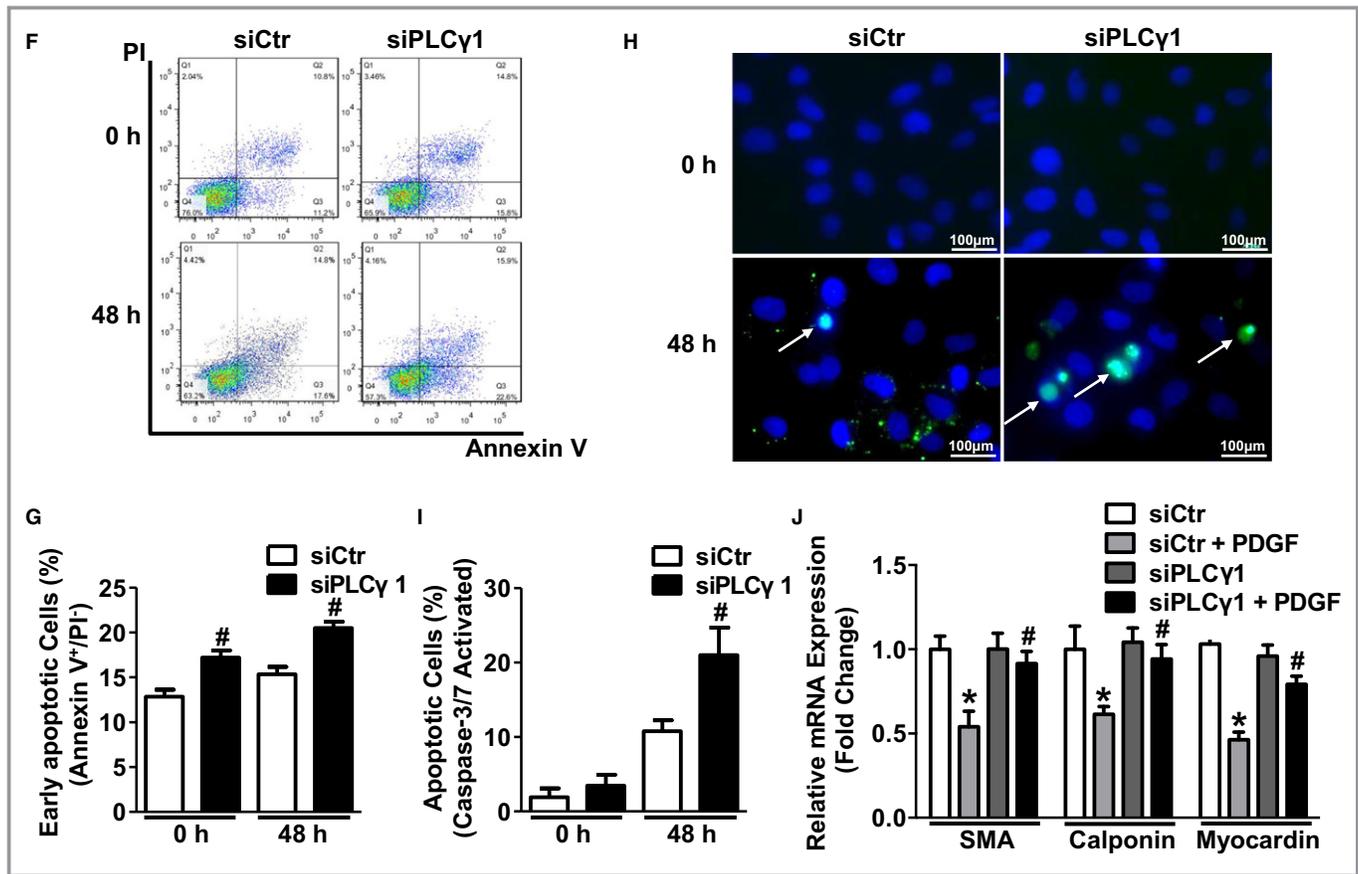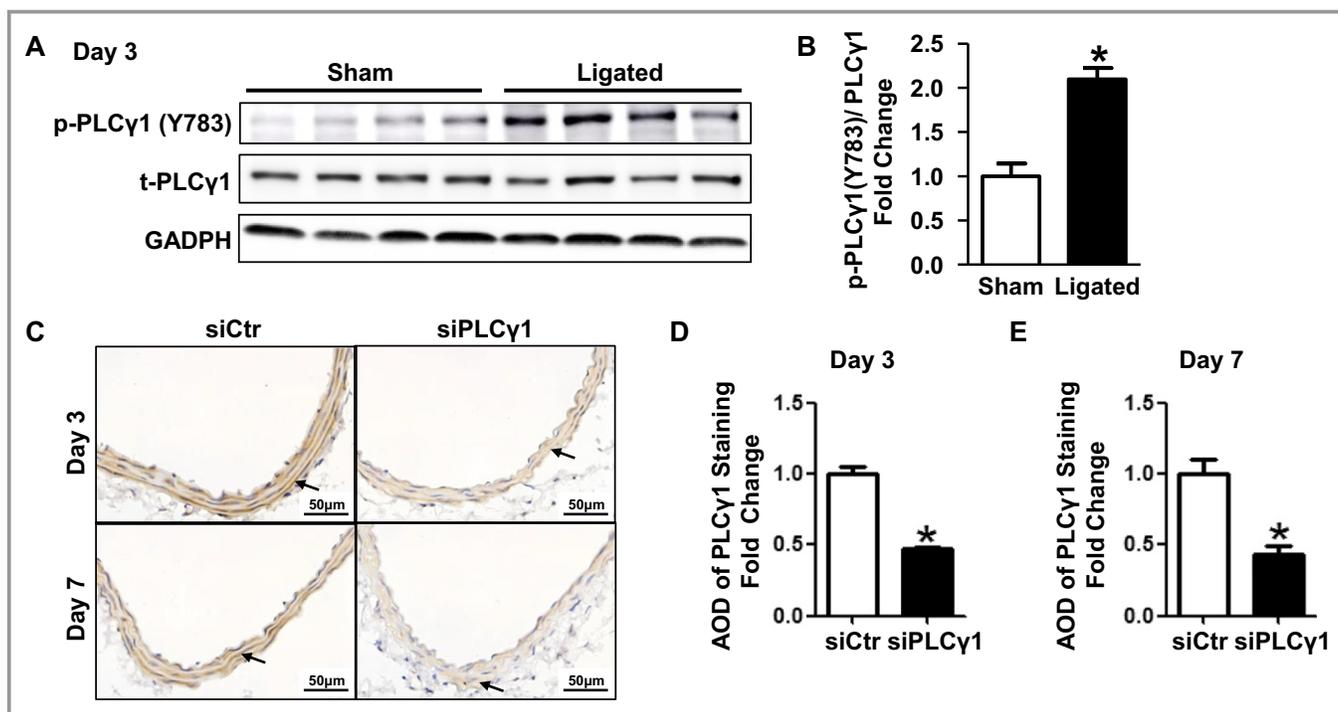
Figure 4. Continued

VSMC apoptosis is another critical process during vascular remodeling.[34] Early-stage apoptosis was detected by annexin V staining. In baseline conditions, depletion of PLCγ1 increased cell early apoptosis by 1.3-fold. After 48 hours of serum starvation, early apoptotic RASMCs were greatly increased in the PLCγ1 knockdown group compared with the siCtr group (20.5±0.8% versus 14.3±0.7%, $P<0.01$) (Figure 4F and 4G). Activation of caspase-3/7 is another essential event during apoptosis. We measured caspase-3/7 activation in RASMCs transfected with control or PLCγ1 siRNA after 48 hours of serum starvation. Compared with the siCtr group, PLCγ1 knockdown increased caspase-3/7–activated cells by 1.95-fold (Figure 4H and 4I).

Differentiated VSMCs express a variety of contractile proteins, including smooth muscle α-actin, calponin, and myocardin. When VSMCs are switched from a contractile to a synthetic phenotype during vascular remodeling, these differentiation markers are downregulated. Thus, we used quantitative polymerase chain reaction to measure the expression levels of VSMC differentiation markers. Compared with quiescent RASMCs, the expression levels of smooth muscle α-actin, calponin, and myocardin were respectively decreased by 47%, 44%, and 58% after PDGF treatment, whereas their expression levels were significantly reversed by knockdown of PLCγ1 (siPLCγ1 versus siCtr, smooth muscle α-actin: 0.93±0.19 versus 0.56±0.24, $P=0.01$; calponin: 0.89±0.22 versus 0.62±0.91, $P=0.02$; myocardin: 0.72±0.12 versus 0.41±0.13, $P=0.04$) (Figure 4J).

PLCγ1 is Required for Intima Formation After Carotid Artery Ligation

To determine the role of PLCγ1 in intima formation, we measured PLCγ1 and its signaling activation in carotid arteries after complete ligation in C57BL/6J mice. PLCγ1 phosphorylation rapidly increased and peaked at day 3, while Akt phosphorylation peaked at day 7 after carotid ligation (Figures 5A and 6A). These data suggest the important role of PLCγ1-Akt signaling during vascular remodeling. Targeted deletion of PLCγ1 leads to embryonic lethality caused by defects in vessel development. Thus, we knocked down

the absence of PDGF, depletion of PLCγ1 had no effects on wound area. Following PDGF treatment, knockdown of PLCγ1 significantly retarded RASMC migration (wound area relative to origin, siPLCγ1 versus siCtr: 73.7±4.8% versus 46.5±2.4%; $P=0.03$) (Figure 4D and 4E).

Figure 5. Phospholipase Cγ1 (PLCγ1) depletion attenuates intima formation after vessel injury. A, Representative immunoblot of PLCγ1 in sham and ligated carotid arteries 3 days after ligation. B, Quantification of PLCγ1 phosphorylation in carotid arteries on day 3 after ligation. Bars indicate mean±SEM. *P<0.05 vs the sham group. C through E, Representative immunohistochemical staining and quantification of PLCγ1 on day 3 or day 7 after control small interfering RNA (siCtr) or PLCγ1 small interfering RNA (siPLCγ1) delivery periadventitially in pluronic gel. Black arrows indicate smooth muscle cells in the media layer. Images were analyzed and PLCγ1 expression was quantified by fold changes of the average optical density (AOD) of staining. F, Representative cross sections of carotid arteries on day 21 (hematoxylin and eosin staining). siCtr or siPLCγ1 were delivered periadventitially in pluronic gel. Black line indicates intimal width. G through I, Quantification of intimal area (G), medial area (H), and intima/media (I/M) ratio (I) of carotid arteries on day 21 (mean±SEM, siCtr: n=10, siPLCγ1: n=9). J, Immunostaining of proliferating cell nuclear antigen (PCNA) in ligated carotid arteries transfected with siCtr or siPLCγ1 on day 21. Red arrows indicate PCNA-positive cells. K, PCNA-positive cells were quantified by examining every section of 5 sections in each artery (mean±SEM, n=5). *P<0.05 vs the siCtr group. L, Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining of ligated carotid arteries transfected with siCtr or siPLCγ1 for day 21. Red arrows indicate TUNEL-positive cells. M, TUNEL-positive cells were quantified by examining every section of 5 sections in each artery (mean±SEM, n=5). *P<0.05 vs the siCtr group.

PLCγ1 in the carotid artery with periadventitial delivery of PLCγ1 siRNA using pluronic gel. We first determined the deletion efficiency of PLCγ1 siRNA in the carotid artery after treatment for 3 or 7 days without ligation. Results from immunostaining showed that in the siCtr group, PLCγ1 was highly expressed in both endothelial cells and smooth muscle cells but much less in cells in adventitia. In contrast, PLCγ1 expression decreased by ≈50% in both endothelial cells and smooth muscle cells after PLCγ1 siRNA delivery for 3 days and the knockdown effects lasted until day 7 after siRNA delivery (Figure 5C through 5E). To prove the crucial role of PLCγ1 in intima formation, we then performed complete carotid ligation in C57BL/6J mice with periadventitial delivery of control or PLCγ1 siRNA using pluronic gel. Consistent with the results observed in vitro, the ligated arteries in the PLCγ1 siRNA group exhibited a profound decrease in intima area (siPLCγ1 versus siCtr: 2.53±0.64 versus 4.30±0.89, P=0.04) and intima/media ratio (siPLCγ1 versus siCtr: 0.43±0.10

versus 0.89±0.19, P=0.03), indicative of lessened intima formation (Figure 5F through 5I). VSMC proliferation, shown by proliferating cell nuclear antigen–positive staining cells, was reduced in the PLCγ1 siRNA group compared with the siCtr group (11.2±2.2% versus 20.7±2.2%, P=0.01) (Figure 5J and 5K). To determine the role of PLCγ1 in VSMC apoptosis in vivo, we performed terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays on carotid arteries 21 days after ligation. The number of apoptotic cells in the PLCγ1 siRNA group was significantly increased compared with the siCtr group (22.7±3.32% versus 35.7±2.76%, P=0.01) (Figure 5L and 5M).

PLCγ1 is Important for Akt Phosphorylation and Notch1 Signaling Activation In Vivo

To confirm the role of PLCγ1 in the Akt and Notch1 pathway in vivo, we examined the expression levels of phospho-Akt,
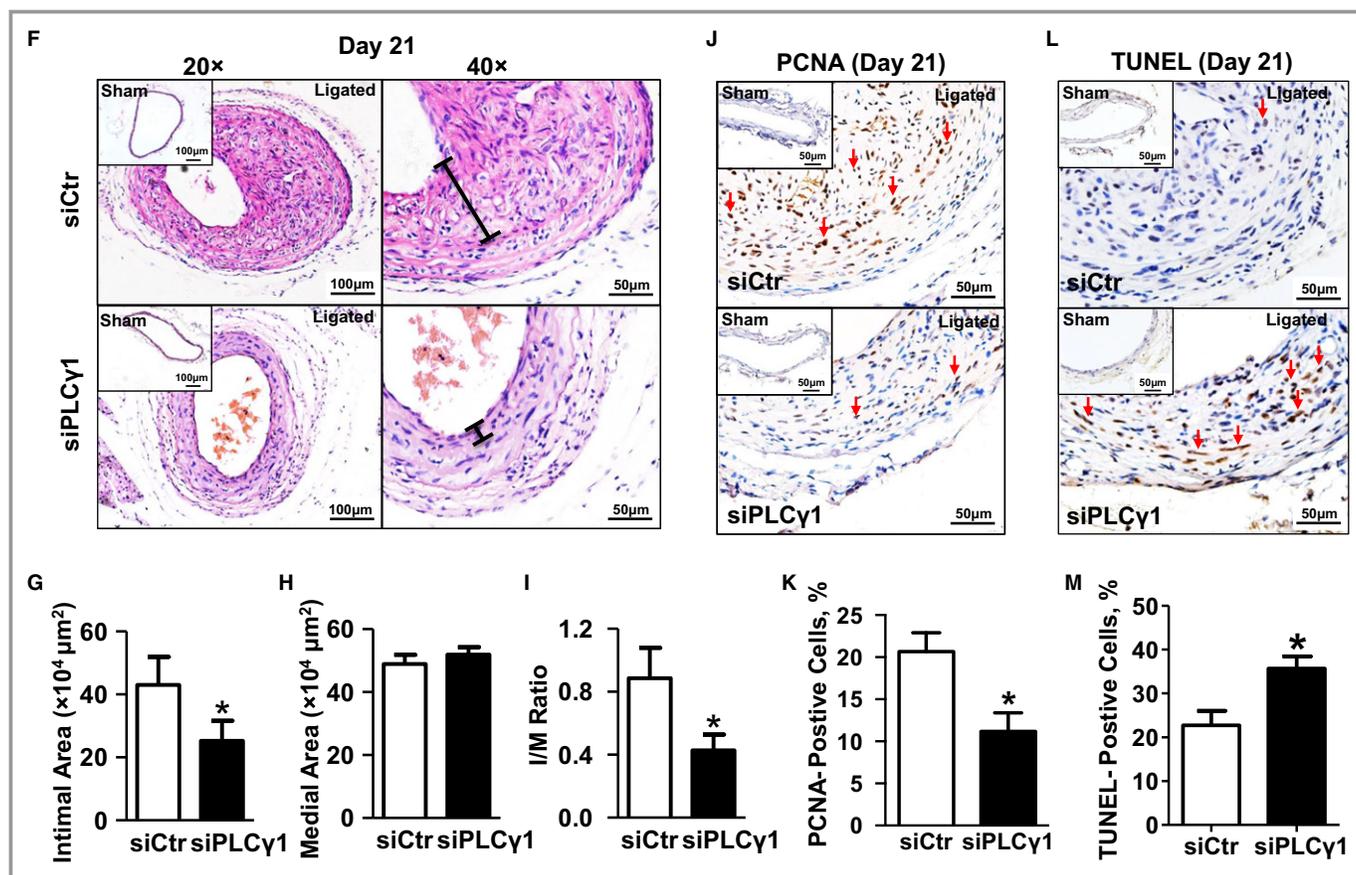
Figure 5. Continued

N1-ICD, and Hey2 in carotid arteries from siPLCγ1- and siCtr-treated mice. On day 7 after ligation, PLCγ1 expression decreased 87.6% compared with siCtr. PLCγ1 silencing significantly suppressed Akt phosphorylation by 62% in ligated carotid arteries compared with siCtr-treated mice (Figure 6A and 6C). Consistent with decreased Akt activation, results from immunohistochemistry staining showed that knockdown of PLCγ1 decreased N1-ICD as well as its target gene, Hey2, expression in ligated carotids (Figure 6D through 6G).

Discussion

The major finding of this paper is that PLCγ1-mediated Akt-Notch1 signaling is essential for intima formation in response to injury (Figure 7). In vitro, we demonstrated that PLCγ1 depletion significantly inhibited Ang II and PDGF-induced Notch1 cleavage and Notch1 target gene Hey2 expression through inhibiting Akt activation, and that the effects of PLCγ1 are phospholipase-independent. VSMC proliferation, migration, and dedifferentiation were decreased, whereas apoptosis was increased by PLCγ1 knockdown. In vivo, PLCγ1 siRNA–treated mice showed a remarkable decrease of intima formation. Akt activation and Notch1 signaling were also decreased in ligated arteries after PLCγ1 siRNA administration.

The critical role of the Notch family of receptors in cardiovascular development is well established.[35,36] However, how Notch signaling is regulated in various physiological and pathological conditions in adults is seldom investigated. Our results demonstrate that Ang II and PDGF are strong activators of Notch1 signaling. Although some γ-secretase inhibitors have been used in clinical trials for cancer therapy,[37,38] targeting γ-secretase to inhibit all Notch signaling is not an ideal therapeutic strategy for vascular remodeling–related diseases since different notch receptors show opposite effects on VSMC function. Defining the specific regulating mechanisms of notch signaling is important.

We demonstrated that PLCγ1 is crucial for Notch1 activation and increased Hey2 expression in VSMCs, whereas no effects on Notch2 expression were observed. Ang II and PDGF stimulate PLCγ1 enzyme activity to generate inositol-1,4,5-trisphosphate and diacylglycerol, which subsequently enhance calcium levels, Ras-Raf-ERK1/2, and PKC signaling.[39–43] It also has been shown that ERK1/2 can increase γ-secretase activity to cleave Notch1 in cortical cells.[32] We expected that PLCγ1 would affect Notch1 activation through ERK1/2. Surprisingly, our results showed that inhibition of ERK1/2 or PKC signaling did not affect Notch1 activation induced by Ang II and PDGF, whereas Akt did. This observation is
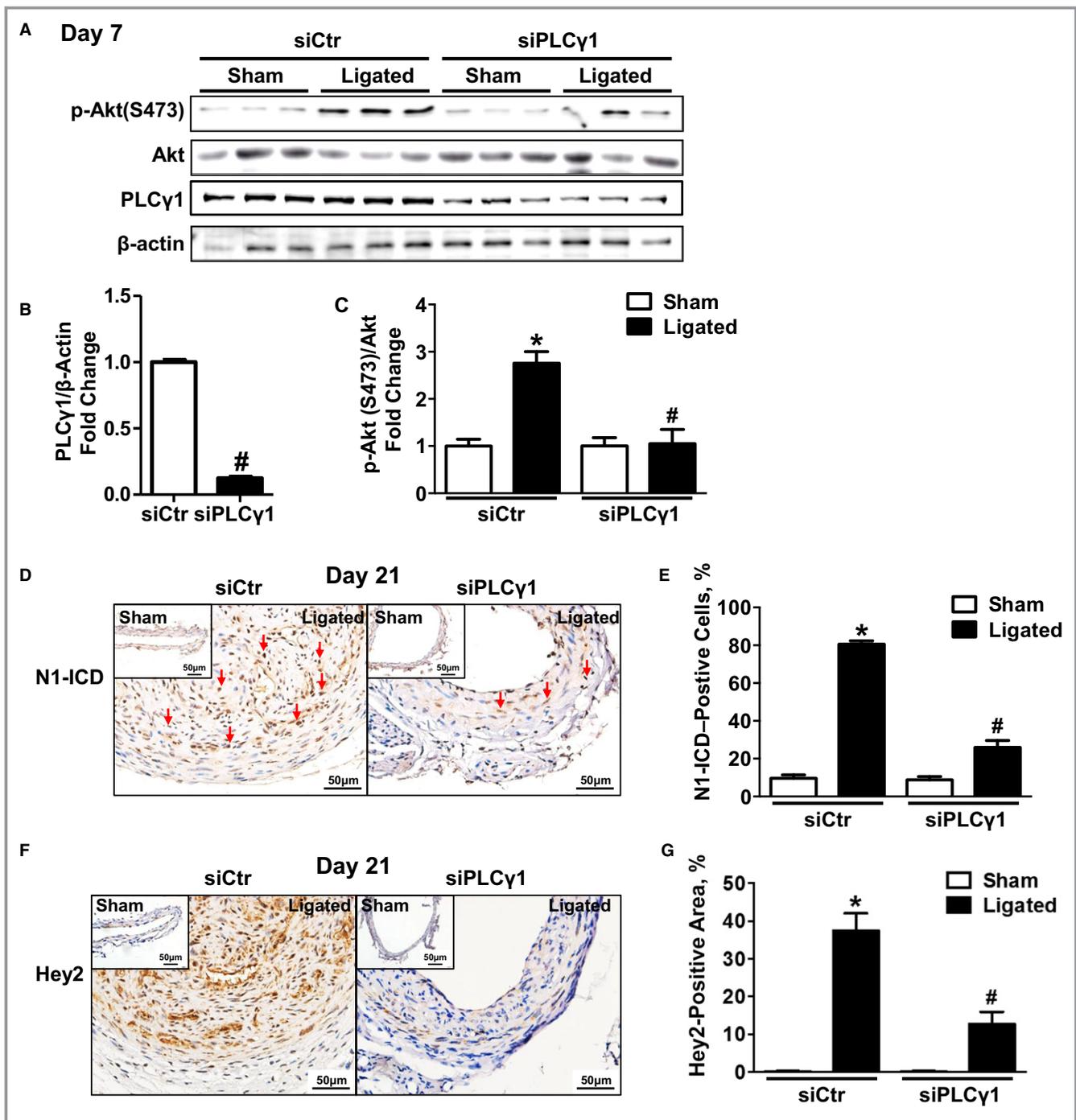
Figure 6. Phospholipase Cγ1 (PLCγ1) is critical for Akt and Notch1 activation in the carotid artery after ligation. A, Representative immunoblot of Akt phosphorylation (p-Akt) in carotid arteries transfected with control small interfering RNA (siCtr) or PLCγ1 small interfering RNA (siPLCγ1) 7 days after ligation. B, Quantification of PLCγ1 protein levels after small interfering RNA knockdown on day 7 (normalized to β-actin) (mean±SEM). C, Quantification of p-Akt in carotid arteries on day 7 (mean±SEM, n=5). D, Immunostaining of Notch1 intracellular domain (N1-ICD) in ligated carotid arteries on day 21. Red arrows indicate N1-ICD–positive cells. E, N1-ICD–positive cells were quantified by examining every section of 5 sections in each artery (mean±SEM, n=5). F, Immunohistochemistry of Hey2 in carotid arteries on day 21. G, Hey2 expression was quantified by examining every section of 5 sections in each artery (mean±SEM, n=5). *$P<0.05$ compared with the siCtr group. #$P<0.05$ compared with the siCtr+PDGF group in (B, D, and F).

consistent with results from Dr Liao's group,[7] which showed that Akt activated γ-secretase to promote angiogenesis through Notch1 in endothelial cells.

Akt is essential for VSMC proliferation, survival, and migration.[44,45] Activated growth factor receptors can activate PI3K to generate phosphatidylinositol 3,4,5 trisphosphate,
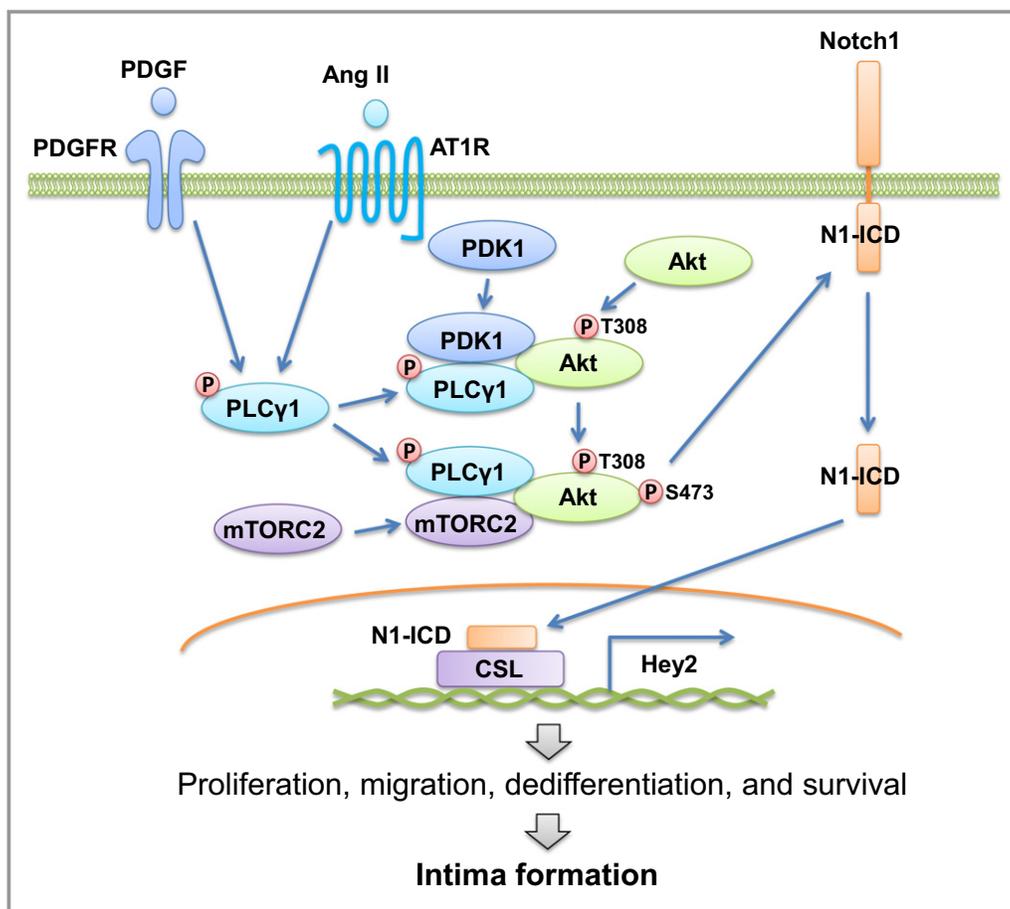
Figure 7. Proposed model of phospholipase Cγ1 (PLCγ1)–mediated Akt-Notch1 signaling in vascular smooth muscle cell (VSMC) function. After vessel injury, angiotensin II (Ang II), and platelet-derived growth factor (PDGF) are excessively released and activate their receptors on VSMC, and then induce PLCγ1 phosphorylation. Phosphorylated PLCγ1 recruits 3-phosphoinositide-dependent protein kinase-1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2) to form PLCγ1-PDK1-Akt or PLCγ1-mTOR-Akt complexes and promotes Akt phosphorylation. Activated Akt facilitates Notch1 cleavage and Notch1 intracellular domain (N1-ICD) translocates to the nucleus, inducing the expression of Hey2 and other target genes. These transduction cascades promote VSMC proliferation, migration, survival, and dedifferentiation, leading to intima formation. AT1R indicates Ang II receptor 1; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor.

which recruits Akt and PDK1 to the cell membrane, and PDK1 phosphorylates Akt on T308. Subsequently, mTOR complex 2 catalyzes Akt activation to the maximum by phosphorylating Akt on S473.[31] Our findings demonstrated that PLCγ1 knockdown inhibits Akt phosphorylation on both T308 and S473. Mechanistically, PLCγ1 may be acting as a scaffold protein instead of an enzyme to recruit PDK1 and mTOR to bind Akt and subsequently facilitate the activation of Akt and Notch1 signaling (Figure 7). The concept of scaffold function of PLCγ1 was supported by our results showing that catalytically inactive PLCγ1 has a comparable effect on Akt phosphorylation on T308 and S473 compared with wild-type PLCγ1. Smith et al[20] showed that PLCγ1 induces DNA synthesis independently of its lipase activity. Xie et al[46] also demonstrated that mutated PLCγ1 has similar

mitogenesis effects compared with wild-type PLCγ1. Interestingly, PLCγ1 had no effects on Ang II and PDGF-induced Notch2 degradation. These results imply that Notch signaling is context-dependent.

Functionally, PLCγ1 is important for cell migration and proliferation in tumor cells and human VSMCs.[47–50] Consistent with these reports, we observed that knockdown of PLCγ1 inhibited RASMC proliferation and migration. We further observed the unappreciated roles of PLCγ1 in cell dedifferentiation, respectively mediated by Akt and Hey2 (Hey2 is considered a key mediator in VSMC differentiation via interfering with myocardin transcription).[26,51,52] Homozygous null mice of PLCγ1 are embryonic lethal caused by cardiovascular defects, which precludes investigation of the role of PLCγ1 in intima formation.[53] By delivering PLCγ1

siRNA in pluronic gel around the injured vessel, we successfully depleted PLCγ1. PLCγ1 depletion significantly retarded intima formation after vessel injury compared with the siCtr treatment group. Although it is known that PLCγ1 is universally expressed, we found that PLCγ1 is highly expressed in endothelial cells and smooth muscle cells, but much less in cells of adventitia. These data imply that the critical role of PLCγ1 in smooth muscle cells and PLCγ1 expression is cell-specific. Combining in vivo results and in vitro data, we believe that PLCγ1 in VSMCs plays an important role in vascular remodeling, but we cannot exclude the effects of PLCγ1 in other cell types, such as endothelial cells and inflammatory cells. Considering localized administration of PLCγ1 siRNA, the role of PLCγ1 in other cell types during remodeling should be minor compared with PLCγ1 in smooth muscle cells. These results fill the knowledge gap of PLCγ1 function in VSMCs in vivo.

Our findings are important for developing new PLCγ1 and specific Notch1 inhibitors. PLCγ1 is a universally expressed protein and it is important for various cell functions such as cell migration and cell proliferation in different cell types.[42,47,48,54,55] Currently, U73122, a commonly used phosphoinositol-PLC inhibitor, blocks the PLCγ1 phosphatase activity to inhibit different cell functions.[43,55] This compound shows significant toxic effects. Moreover, Cetin et al[19] found that U73122 was capable of attenuating mitogen-activated protein kinase activity in VSMCs. In contrast, we failed to confirm that silencing of PLCγ1 abolished Ang II–induced ERK1/2 phosphorylation (data not shown). Hunter et al[33] also revealed that PLCγ1 is not required for ERK1/2 activation but ERK1/2 nuclear translocation in vitro. However, our in vivo data showed that ERK1/2 translocation of VSMCs in ligated carotid arteries was not affected by PLCγ1 siRNA transfection (data not shown), implying that ERK1/2 may not be involved in the function of PLCγ1 in VSMCs. This discrepancy could be explained by the fact that, besides PLCγ1, U73122 could moderately inhibit other members of the PLC family.[20–22] For example, PLCδ1, another PI-PLC, is also highly expressed in VSMCs.[18,20–22] Ideally, cell-specific PLCγ1 inhibitors are needed. Wang et al[29] showed that the SH2 domain is required for PLCγ1 and Akt interaction in tumor cells. Furthermore, we found that PLCγ1 has no effect on Akt and Notch1 activation in HUVEC (data not shown), implying the specific role of PLCγ1 in Akt-Notch1 signaling in VSMCs.

Conclusions

PLCγ1-mediated Akt-Notch1 signaling plays a crucial role in intima formation. Designing specific small molecules or blocking peptides to interrupt PLCγ1 and Akt interaction could be promising therapeutic strategies for vascular remodeling–related diseases in the future.

Disclosures

None.

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SUPPLEMENTAL MATERIAL

Table S1. Summary of primer sequences used for RT-PCR

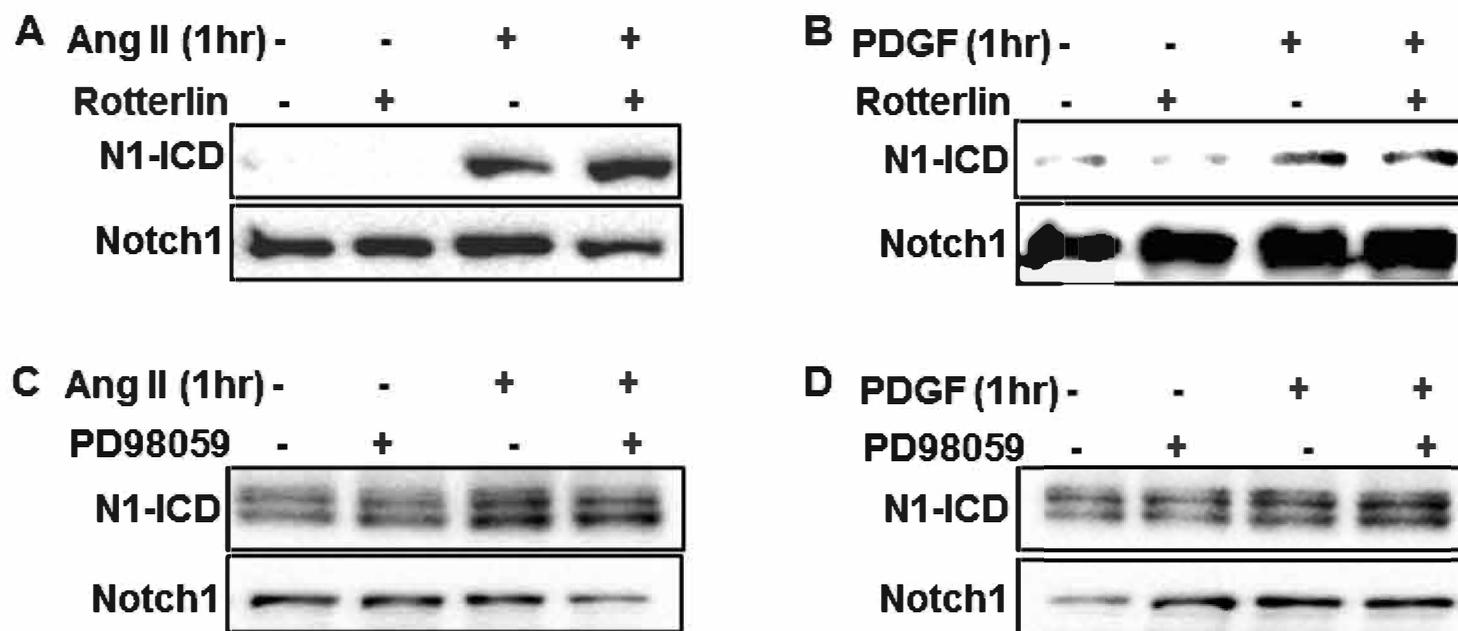| Gene	Sequence
α-SMA (Rat)	Forward: 5' TAA CGG AGG CGC CGC TGA AC 3'
	Reverse: 5' GGG ACA GCA CAG CCT GAA TAG CC 3'
Calponin (Rat)	Forward: 5' ACC AAG CGG CAG ATC TTT GA 3'
	Reverse: 5' CAT CTG CAA GCT GAC GTT GA 3'
Myocardin (Rat)	Forward: 5' ACG AGA AGA TTG CTC TCC GCC CA 3'
	Reverse: 5' TGC TGC TTT GGA GAG CTC ACC T 3'
GADPH (Rat)	Forward: 5' AAG CTC ACT GGC ATG GCC TT 3'
	Reverse: 5' CGG CAT GTC AGA TCC ACA AC 3'

Figure S1
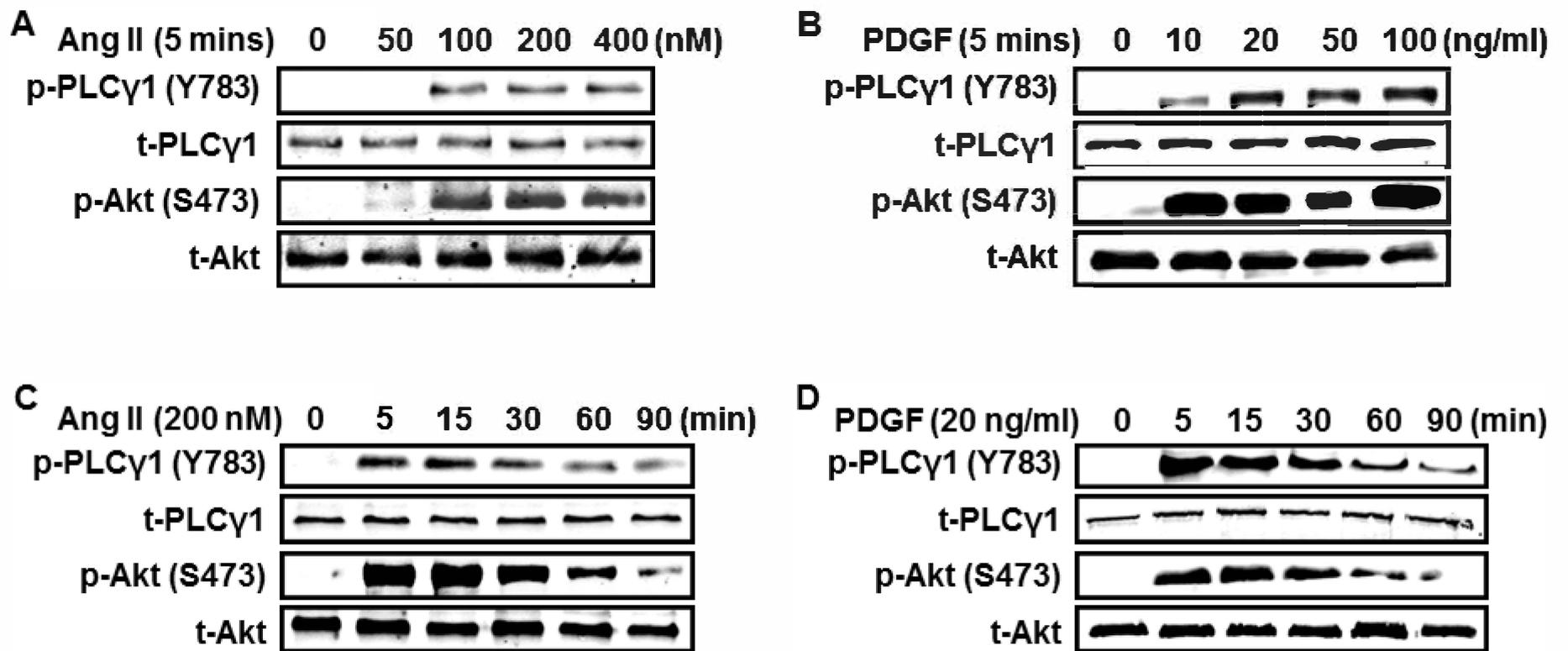
Angiotensin II (Ang II) and platelet-derived growth factor (PDGF) induce phospholipase C gamma-1 (PLCγ1) and Akt phosphorylation in time and dose-dependent manners **A-B.** Serum-starved rat aortic smooth muscle cells (RASMCs) were stimulated with different dose of Ang II (A) and PDGF (B) for 5 mins. p-PLCγ1 (Y783), p-Akt (S473), t-PLCγ1, and t-Akt were measured by western blot. **C-D.** Serum-starved RASMCs were stimulated with Ang II (200 nM, C) and PDGF (20 ng/ml, D). Cells were collected at indicated time points and p-PLCγ1 (Y783), p-Akt (S4 73), t-PLCγ1, and t-Akt were measured by western blot.

Figure S2

A Ang II (1hr) -   -   +   +
Rotterlin   -   +   -   +

N1-ICD

Notch1

B PDGF (1hr) -   -   +   +
Rotterlin   -   +   -   +

N1-ICD

Notch1

C Ang II (1hr) -   -   +   +
PD98059   -   +   -   +

N1-ICD

Notch1

D PDGF (1hr) -   -   +   +
PD98059   -   +   -   +

N1-ICD

Notch1

ERK1/2 and PKC-mediated signaling have no effects on Notch1 activation induced by angiotensin II (Ang II) and platelet-derived growth factor (PDGF). Rat aortic smooth muscle cells (RASMCs) were pretreated with 20 µM Roterllin (PKC inhibitor, A-B) or 30 µM PD98058 (ME$_K$1 inhibitor, C-D) for 1 hour and then stimulated with AngII (200 nM) or PDGF (20 ng/ml). Notch1 activation was detected by N1-ICD (Notch1 intracellular domain).
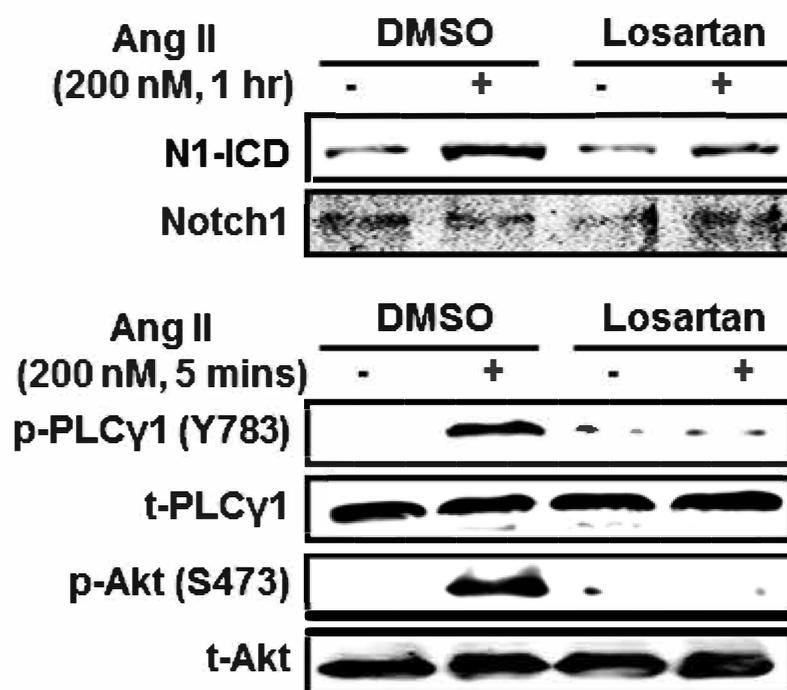
Figure S3
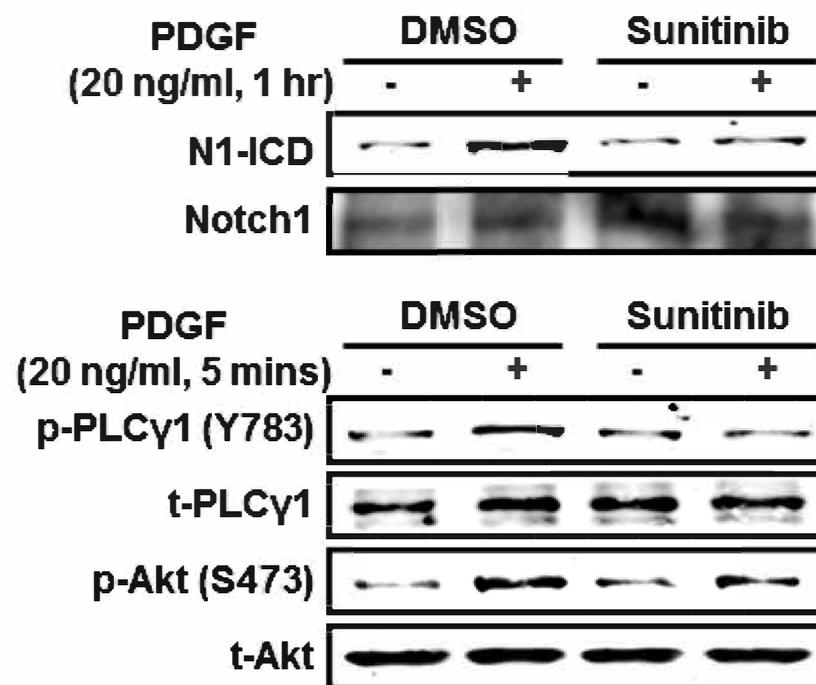
Inhibition of platelet-derived growth factor (PDGF) receptor by Sunitinib suppresses phospholipase C gamma-1(PLCγ1) and Akt phosphorylation and Notch1 cleavage in rat aortic smooth muscle cells (RASMCs).

RASMCs were pretreated with 10 μM Sunitinib for 1 hour and then stimulated with PDGF (20 ng/ml). Notch1 and N1-ICD (Notch1 intracellular domain) were measured by western blot 1 hr after PDGF stimulation, and p-PLCγ1 (Y783), p-Akt (S473), t-PLCγ1, and t-Akt were measured by western blot 5 mins after PDGF stimulation.

Figure S4

Inhibition of angiotensin II (Ang II) receptor by Losartan suppresses phospholipase C gamma-1 (PLCγ1) and Akt phosphorylation and Notch1 cleavage in rat aortic smooth muscle cells (RASMCs).

RASMCs were pretreated with 100 nM Losartan for 1 hour and then stimulated with Ang II (200 nM). Notch1 and N1-ICD (Notch1 intracellular domain) were measured by western blot 1 hr after Ang II stimulation, and p-PLCγ1 (Y783), p-Akt (S473), t-PLCγ1, and t-Akt were measured by western blot 5 mins after Ang II stimulation.