Protein kinase C δ regulates the release of collagen type I from vascular smooth muscle cells via regulation of Cdc42

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ABSTRACT Collagen type I is the most abundant component of extracellular matrix in the arterial wall. Mice knocked out for the protein kinase C δ gene (PKCδ KO) show a marked reduction of collagen I in the arterial wall. The lack of PKCδ diminished the ability of arterial smooth muscle cells (SMCs) to secrete collagen I without significantly altering the intracellular collagen content. Moreover, the unsecreted collagen I molecules accumulate in large perinuclear puncta. These perinuclear structures colocalize with the trans-Golgi network (TGN) marker TGN38 and to a lesser degree with cis-Golgi marker (GM130) but not with early endosomal marker (EEA1). Associated with diminished collagen I secretion, PKCδ KO SMCs exhibit a significant reduction in levels of cell division cycle 42 (Cdc42) protein and mRNA. Restoring PKCδ expression partially rescues Cdc42 expression and collagen I secretion in PKCδ KO SMCs. Inhibition of Cdc42 expression or activity with small interfering RNA or se-cramine A in PKCδ WT SMCs eliminates collagen I secretion. Conversely, restoring Cdc42 expression in PKCδ KO SMCs enables collagen I secretion. Taken together, our data demonstrate that PKCδ mediates collagen I secretion from SMCs, likely through a Cdc42-dependent mechanism.

INTRODUCTION Type I collagen—the most abundant collagen in the blood vessel wall and other tissues in the human body—is a critical structural and functional component of a healthy artery. Collagen in the arterial wall is produced by fibroblasts and smooth muscle cells (SMCs); either too much or too little collagen can contribute to the pathogenesis of vascular disease (Rudijanto, 2007). The presence of excessive collagen in the atherosclerotic plaque is believed to be an important element of atherothrombotic disease (Libby et al., 2010). Furthermore, collagen and other matrix proteins are not inert by-standers; rather, they contribute to arterial homeostasis and pathogenesis by influencing the proliferative behavior of SMCs (Hollenbeck et al., 2004). Too little collagen or thinning and weakening of collagen in the fibrous cap region of atherosclerotic plaques is believed to contribute to plaque rupture (Rekhter, 1999), a complex pathological event that is frequently responsible for heart attacks and strokes.

Because of all the aforementioned factors, understanding the process by which collagen is synthesized, trafficked, and secreted in SMCs provides opportunities for the treatment of arterial diseases, as well as systemic fibrotic states.

Collagen type I, along with types II, III, V, and IX, forms into fibrils. Type I is a heterotrimer composed of two \( \alpha 1 \) chains and one \( \alpha 2 \) chain. Collagen chains are translated and initially processed in the rough endoplasmic reticulum (ER), where the heterotrimer is formed with the aid of protein disulfide isomerase. The newly synthesized collagen molecules are then transported through the cisternae of the Golgi complex through a cisternal maturation mechanism (Bonfanti et al., 1998). Finally, the collagen molecules are packaged in...
post-Golgi vesicular carriers at the trans-Golgi network (TGN) and secreted (Canty et al., 2004). Although much work has been performed on collagen transcription, the signaling mechanisms that control collagen trafficking and secretion have yet to be fully elucidated.

Protein kinase C δ (PKCδ) is a 78-kDa member of the novel PKC family of serine/threonine kinases. PKCδ regulates multiple cellular processes, including proliferation (Fukumoto et al., 1997) and apoptosis (Leitges et al., 2001; Ryer et al., 2005) of vascular SMCs. More recently, PKCδ has been found to play an important role in the synthesis of extracellular matrix proteins. In vascular SMCs, PKCδ activity is necessary for induction of fibronectin production by transforming growth factor β (TGFβ; Ryer et al., 2006). Studies of PKCδ knockout (KO) mouse reveal that mice lacking PKCδ develop normally but exhibit an antiapoptotic phenotype when subjected to models of vascular injury such as vein graft or carotid artery ligation (Leitges et al., 2001; Bai et al., 2010; Yamanouchi et al., 2010). More recently, PKCδ was reported to be involved in the regulation of chemokine expression and consequently proinflammatory signaling (Liu et al., 2010).

In human fetal lung fibroblasts, PKCδ stimulates elastin expression by stabilizing its mRNA (Kucich et al., 2002). In renal mesangial cells, PKCδ was found to be important for TGFβ-dependent transcription of the gene that encodes collagen α2 chain COL1A2 (Runyan et al., 2003). However, the potential roles of PKCδ in other important steps of collagen synthesis besides gene transcription, such as translation, posttranslational modifications, and trafficking, remain undetermined. It has been shown that PKCδ localizes to the Golgi complex in fibroblasts (Goodnight et al., 1995; Kajimoto et al., 2001; Schultz et al., 2004) and that it regulates the retrograde transport of Shiga toxin from early endosomes to the Golgi complex. However, a role of PKCδ in anterograde transport has not been reported.

During trafficking through the Golgi apparatus, secreted proteins are subject to posttranslational processing and are incorporated into post-Golgi transport carriers (PGTCs) for secretion. These processes are regulated by multiple factors, including a recently described Golgi- 

![Figure 1](https://via.placeholder.com/150.png?text=FIGURE%201%3A%20PKCδ%20is%20necessary%20for%20efficient%20collagen%20I%20secretion.%20(A)%20Representative%20mouse%20aortic%20sections%20immunostained%20for%20collagen%20I%20(left)%20or%20van%20Geison%20stains%20(right).%20Scale%20bar%2C%20100%20μm.%20(B)%20Primary%20mouse%20aortic%20SMCs%20isolated%20from%20PKCδ%20KO%20mice%20or%20their%20WT%20littermates%20were%20starved%20for%2048%20h%20and%20then%20treated%20for%2048%20h%20with%20or%20without%205%20ng/ml%20TGFβ).%20Extracellular%20(media)%20and%20intracellular%20(cell%20lysate)%20collagen%20I%20contents%20were%20analyzed%20by%20Western%20blot.%20Representative%20blots%20and%20quantifications%20are%20shown.%20Optical%20density%20of%20secreted%20collagen%20I%20was%20normalized%20to%20the%20total%20collagen%20I%20density%20(secreted%20plus%20intracellular).%20Equal%20loading%20of%20cell%20lysate%20was%20confirmed%20by%20probing%20with%20an%20anti-β-actin%20antibody.%20Values%20are%20expressed%20as%20mean%20±%20SEM%20(n%20= 3). *p%20<%200.05. 

**RESULTS**

**PKCδ is a critical mediator of collagen I secretion in vascular smooth muscle cells**

Aortas were harvested from PKCδ KO mice or their wild-type (WT) littermates and analyzed for collagen I content by immunohis- 

tochemistry. WT mice revealed considerable collagen I staining (Canty et al., 2006). The best molecular candidate to regulate actin dynamics is the Rho GTPase Cdc42, known to associate with the Golgi complex in an ADP-ribosylation factor– and brefeldin A–dependent manner (Erickson et al., 1996) and to regulate the post-Golgi transport of several membrane and secretory proteins (Musch et al., 2001; Pelish et al., 2006). More recently, it has been shown that reduction of Cdc42 activation and its recruitment to the Golgi by the guanine nucleotide exchange factor faciogenital dysplasia protein 1 small interfering RNA (siRNA) blocks the pro–collagen I trafficking at the Golgi complex (Egorov et al., 2009).

In the present study, we investigated the role of PKCδ in collagen secretion from vascular SMCs. Our results suggest that PKCδ regulates trafficking of collagen I by controlling its exit from the trans-Golgi network through a mechanism involving Cdc42.
Similarly, PKCδ gene deficiency selectively diminished collagen I in the extracellular compartment (Figure 1B) in the presence of TGFβ. To test whether a similar collagen I phenotype can be produced by transient inhibition of PKCδ, we acutely inhibited PKCδ activity in A10 cells—a rat aortic smooth muscle cell line—with the chemical inhibitor rottlerin. As seen in Supplemental Figure S2A, rottlerin reduced the amount of secreted collagen in the presence or absence of TGFβ. In contrast, intracellular collagen content was not significantly altered by the rottlerin treatment (Supplemental Figure S2A). In comparison, PKCδ gene deficiency produced different effects on collagen type III and tropoelastin. As shown in Supplemental Figure S3, PKCδ KO SMCs displayed greatly reduced levels of collagen III as compared with WT cells in both extracellular and intracellular compartments. However, PKCδ gene deficiency slightly increased levels of tropoelastin detected in the culture media without altering the level of this matrix protein inside the cell (Supplemental Figure S3). Taken together, these data suggest a role of PKCδ in regulation of the basic collagen secretion mechanism.

PKCδ inhibition causes intracellular accumulation of pro–collagen I in trans-Golgi

We next studied the intracellular localization of the collagen molecules that were retained in cells lacking PKCδ activity. Inhibition of PKCδ with rottlerin in A10 cells caused similar perinuclear clusters of collagen I (Supplemental Figure S2B, arrowhead). To determine the organelle in which collagen I and pro–collagen I were trapped, we double stained SMCs with antibodies specific to pro–collagen I and markers for cis- and trans-Golgi network (GM130, or GM130, and TGN38) or early endosomes (EEA1). Immunofluorescence was performed in aortic SMCs isolated from PKCδ KO mice or their WT littermates. WT SMCs showed the TGN arranged symmetrically around the nuclei and procollagen distributed evenly throughout the cytoplasm (Figure 2A, WT). KO SMCs displayed focal dilations in the TGN that colocalized with perinuclear clumps of procollagen I (Figure 2A, PKCδ KO). Compared to the WT cells, PKCδ KO cells showed a slight accumulation of procollagen I in cis-Golgi (Figure 2B). Comparison of EEA1 staining with that of procollagen I failed to show any significant colocalization of procollagen I clusters and endosomes (Figure 2C). Similar colocalization patterns were observed in rottlerin-treated A10 cells (Supplemental Figure S4). Taken together, these data suggest that in the cell PKCδ deficiency induces the perinuclear localization of procollagen I, but it does not colocalize with cis-Golgi (B, right) or endosomes (C, right).

Because SMCs are often exposed to various profibrotic factors such as TGFβ in a disease state such as occlusive vascular disease, we then repeated the experiment in the presence of 5 ng/ml TGFβ.
absence of PKCδ, collagen I is transported across the Golgi to reach the TGN but does not exit the TGN toward the cell surface.

**High PKCδ protein expression is localized to the trans-Golgi**
To further explore the role of PKCδ in collagen trafficking, we next examined the intracellular localization of PKCδ in relation to the cis and trans portions of the Golgi stack. As seen in Figure 3, PKCδ co-localized with both TGN38 and GM130 markers, with high abundance in the trans-Golgi networks. Such an intracellular distribution pattern supports the involvement of PKCδ in regulation of Golgi exit of post-Golgi transport carriers.

**Cdc42 is impaired in PKCδ KO SMCs**
Because the Rho GTPase Cdc42 has been implicated in post-Golgi protein trafficking (Kroschewski et al., 1999; Musch et al., 2001; Pelish et al., 2006), we evaluated the effect of PKCδ gene deficiency on Cdc42. Primary mouse aortic SMCs isolated from PKCδ KO mice or their WT littermates were analyzed for Cdc42 activity by measuring GTP-bound Cdc42. As shown in Figure 4A, PKCδ gene deficiency led to a ~50% reduction of GTP-bound Cdc42, indicating decreased levels of total and active Cdc42 in PKCδ KO cells. Western blotting and RT-PCR analysis revealed a similar reduction in the level of Cdc42 protein and mRNA in PKCδ KO SMCs when compared with wild-type SMCs (Figure 4, B and C). To test whether the lack of PKCδ affects the Golgi localization of Cdc42, we performed coimmunostaining of Cdc42 and TGN38 in PKCδ WT and KO SMCs. Whereas the intensity of Cdc42 was less in KO cells, cells of both genotypes displayed similar distribution patterns of Cdc42 within the Golgi stack (Figure 4D).

Western blot analysis of PKCδ WT and KO SMCs revealed that other members of the Rho GTPase family—specifically RhoA, RhoB, and RhoC—were also down-regulated in PKCδ gene–deficient SMCs. Rac1/2/3 did not appear to be altered by PKCδ gene deficiency (Supplemental Figure S5).

**Cdc42 is an important effector necessary for PKCδ-mediated collagen I secretion**
If Cdc42 is a critical effector with PKCδ, inhibition of this GTPase should mimic the collagen phenotype we observed with PKCδ gene deficiency. To test this, we treated A10 cells with the Cdc42-specific inhibitor secramine A or solvent (dimethyl sulfoxide) for 48 h. As shown in Figure 5A, secramine A eliminated extracellular collagen I but did not significantly affect the amount of collagen detected in cell lysate. Furthermore, immunocytochemistry performed on A10 cells treated with secramine A displayed perinuclear clumping of pro–collagen I co-localized with TGN38, similar to results seen in PKCδ gene–deficient SMCs (Figure 5B).

To confirm the results obtained with secramine A, we inhibited Cdc42 by silencing
To further support the role of PKCδ in collagen trafficking and Cdc42 expression, we attempted to restore PKCδ expression in the knock-out SMCs with an adenovirus vector that expresses the wild-type PKCδ (AdPKCδ). Primary mouse aortic SMCs from PKCδ KO mice were infected with AdPKCδ or a control vector AdLacZ. Forty-eight hours after viral infection, cells were lysed for Western analysis. AdPKCδ but not AdLacZ significantly restored PKCδ expression in PKCδ KO SMCs (Figure 6A). Furthermore, SMCs infected with AdPKCδ but not AdLacZ produced extracellular collagen I at a level that was comparable to the wild-type SMCs (Figure 6A). As shown in Figure 6B, intracellular Cdc42 levels were also partially restored by AdPKCδ, further implying a relationship between these two signaling proteins.

**DISCUSSION**

Tight regulation of collagen is critical to homeostasis of the arterial wall, as well as to stability of atherosclerotic plaque. Malfunction in any of the regulatory steps, that is, synthesis, secretion, and degradation, can potentially lead to abnormal structure of the blood vessel or plaque rupture. By using a combination of pharmacological, molecular, and genetic approaches, we provided evidence that suggests a novel role of PKCδ in the regulation of collagen secretion. The requirement of PKCδ activity in normal trafficking of type I collagen through the Golgi apparatus is demonstrated by our observation that pro–collagen I molecules were trapped in the TGN in arterial SMCs that lack PKCδ activity or expression. To our knowledge, this is the first report on a regulatory role of PKCδ in collagen trafficking.

We did not observe significant collagen accumulation in cis-Golgi or the EEA1–positive endosomes in PKCδ-null or rottlerin-treated cells, suggesting this PKC isoform may be specifically required for its expression with siRNA. The wild-type mouse aortic SMCs were transfected with either siRNA to Cdc42 or a control siRNA. At 24 and 48 h posttransfection, cell lysate and media were collected for Western blot analysis. At both time points, siRNA to Cdc42 was able to significantly ablate collagen trafficking to the media with no significant effects on the intracellular contents of collagen I or PKCδ, despite the marked reduction in Cdc42 protein levels (Figure 5C). Similar to secramine A, siRNA to Cdc42 caused PKCδ WT cells to display perinuclear clumping of pro–collagen I colocalized with TGN38 (Figure 5D). Collectively, our results suggest that Cdc42 is a critical mediator in collagen I trafficking out of the SMCs.

**Restoration of PKCδ expression rescues collagen I secretion and Cdc42 expression**

To further support the role of PKCδ in collagen trafficking and Cdc42 expression, we attempted to restore PKCδ expression in the knock-out SMCs with an adenovirus vector that expresses the wild-type PKCδ (AdPKCδ). Primary mouse aortic SMCs from PKCδ KO mice were infected with AdPKCδ or a control vector AdLacZ. Forty-eight hours after viral infection, cells were lysed for Western analysis. AdPKCδ but not AdLacZ significantly restored PKCδ expression in PKCδ KO SMCs (Figure 6A). Furthermore, SMCs infected with AdPKCδ but not AdLacZ produced extracellular collagen I at a level that was comparable to the wild-type SMCs (Figure 6A). As shown in Figure 6B, intracellular Cdc42 levels were also partially restored by AdPKCδ, further implying a relationship between these two signaling proteins.

**FIGURE 5:** Cdc42 is necessary for collagen I trafficking. (A, B) A10 SMCs were treated with secramine A or solvent (control) for 48 h. (A) Media conditioned by SMCs or cell lysate were harvested and analyzed for collagen I by Western blotting. Equal loading was confirmed by reprobing for tubulin. (B) Cells were coimmunostained using antibodies specific to pro–collagen I (green) or TGN38 (red). Nuclei were counterstained with DAPI (blue). Scale bar, 20 μm. (C, D) Primary mouse aortic SMCs were transfected in Opti-MEM I medium with 20 nM of siRNA for mouse Cdc42 or scramble siRNA. (C) Cells and media were harvested 24 and 48 h posttransfection. Media were evaluated by Western blotting for collagen I content and quantified. Values are expressed as mean ± SEM (n = 3). *p < 0.05. Cell lysate was evaluated by Western blotting for collagen I, PKCδ, Cdc42, and β-actin. (D) Cells were coimmunostained using antibodies specific to procollagen I throughout the cytoplasm, similar to results seen in WT SMCs (Figure 7B). Western blotting of the extracellular proteins in the media demonstrated that ectopic expression of either Cdc42 construct was able to partially rescue the collagen-trafficking phenotype in the absence of PKCδ (Figure 7A). Furthermore, immunocytochemistry performed on PKCδ KO SMCs transfected with Cdc42 WT or Cdc42 V12 for 48 h displayed even distribution of pro–collagen I throughout the cytoplasm, similar to results seen in WT SMCs (Figure 7B).
whether PKC bound form to the active, GTP-bound form, catalyzed by guanine of GTP-bound Cdc42 in PKC 
elates Golgi-mediated protein trafficking through Cdc42-dependent
secretion and trafficking led us to hypothesize that PKC duplicated with Cdc42 mRNA knockdown. The striking similarity be
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(fig 3), 2009), the involvement of Cdc42 in col
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terograde traffic at the Golgi level.

As an essential player in controlling protein secretion (Kroschewski et al., 1999) and post-Golgi protein trafficking (Pelish et al., 2006; Egorov et al., 2009), the involvement of Cdc42 in collagen I trafficking is not unexpected. Secramine A, a small chemical inhibitor, has been found to inhibit the activation of Cdc42 by inhibiting its binding to membranes, GTP, and effectors in a Rho GDP dissociation inhibitor–dependent manner (Pelish et al., 2006). Consistent with the prior reported effect of secramine A on Golgi-mediated protein trafficking, we found that secramine A eliminated secretion of collagen I by vascular SMCs, an observation that was duplicated with Cdc42 mRNA knockdown. The striking similarity between the effect of PKC8 inhibition and Cdc42 inhibition on collagen secretion and trafficking led us to hypothesize that PKC8 regulates Golgi-mediated protein trafficking through Cdc42-dependent mechanisms. Indeed, we found a substantial reduction in the level of GTP-bound Cdc42 in PKC8-null cells.

Activation of Cdc42 involves conversion from an inactive, GDP-bound form to the active, GTP-bound form, catalyzed by guanine nucleotide exchange factors (Sinha and Yang, 2008). It is unclear whether PKC8 gene deficiency affected this conversion and led to a lower level of GTP-bound active form of Cdc42. Because a similar

collagen I secretion vesicles to bud off the TGN. A study by Tisdale (2003) showed that inhibition of PKCδ, in HeLa cells resulted in newly synthesized reporter protein being trapped in the ER (it is possible that different PKC isoforms differentially regulate distinct steps of protein secretion). It has been shown that PKCδ localizes at the Golgi complex in fibroblasts (Goodnicht et al., 1995; Kajimoto et al., 2001; Schultz et al., 2004). Our immunohistochemistry analysis confirmed a similar localization of PKCδ in vascular SMCs, particularly rich in trans-Golgi stacks. Furthermore, PKCδ is involved in the endosomes of Golgi trafficking of Shiga toxin in HeLa cells (Torgerson et al., 2007), raising the possibility that PKCδ might also affect anterograde traffic at the Golgi level.

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FIGURE 6: Restoration of PKCδ expression rescues collagen I secretion and Cdc42 expression in PKCδ-knockout SMCs. Primary mouse aortic SMCs from PKCδ KO mice were infected with AdPKCδ or a control vector AdLacZ. (A) Representative Western blot of media conditioned by SMCs analyzed for collagen I. Quantification of secreted collagen is expressed as relative band density. Values are expressed as mean ± SEM (n = 3). *p < 0.05. Cell lysates were blotted with the antibodies specific to PKCδ, collagen I, or β-actin. (B) Representative Western blot of cell lysate analyzed for Cdc42.

MATERIALS AND METHODS
General materials and reagents
Rottlerin was obtained from Sigma-Aldrich (St. Louis, MO). DMEM and other tissue culture reagents were from Invitrogen (San Diego,
FIGURE 7: Restoration of Cdc42 expression enhances collagen I secretion by PKCδ-knockout SMCs. Primary mouse aortic SMCs from PKCδ KO were transfected with WT Cdc42 DNA plasmid, V12 Cdc42 DNA plasmid, or a control GFP plasmid. (A) At 48 h posttransfection, media or cell lysate were harvested and analyzed by Western blotting. Representative Western blot of media conditioned by SMCs was analyzed for collagen I. Quantification of secreted collagen is expressed as relative band density. Values are expressed as mean ± SEM (n = 3). *p < 0.05. Cell lysates were blotted with the antibodies specific to PKCδ, collagen I, Cdc42, or β-actin. (B) At 48 h posttransfection, cells were immunostained using antibodies specific to pro–collagen I (green) and TGN38 (red). Nuclei were counterstained with DAPI (blue). Colocalization of pro–collagen I and TGN38 was quantified by Pearson coefficient analysis (bottom graph). Pearson coefficient values range from −1 to 1, where 1 means complete colocalization. Each point in the graph represents a Pearson colocalization coefficient for each field of a slide. Each line represents an independent experiment. Confocal fluorescence images were acquired with a BD Biosciences pathway confocal microscope using a 60 × 1.42 objective. Scale bar, 20 μm.

CA). Antibodies used include collagen I, collagen III (Fitzgerald Industries, Acton, MA), Cdc42, Rho GTPase antibody sampler kit (Cell Signaling Technologies, Danvers, MA), PKCδ (Santa Cruz Biotechnology, Santa Cruz, CA), TGN38 (rat antibody), EEA1, β-actin (Sigma-Aldrich, St. Louis), TGN38 (mouse antibody), GM130, tropoelastin, α-tubulin (Abcam, Cambridge, MA), and pro–collagen I (Developmental Studies Hybridoma Bank, Iowa City, IA). Other chemicals and reagents if not specified were purchased from Sigma-Aldrich.

Cell culture
The mouse aortic SMCs were isolated from the thoracic and abdominal aorta based on a protocol described by Clowes et al. (1994). Primary SMCs were grown at 37°C in 5% CO2 in DMEM modified to contain 1 g/l d-glucose, 4.5 g/l glucose, 1 mM sodium pyruvate supplemented with 10% fetal bovine serum (FBS; Gemini, Woodland, CA), and antibiotics. Cells between three and seven passages were used for all experiments. The generation of mice with targeted deletion of PKCδ was described elsewhere (Miyamoto et al., 2002). Rat aortic A10 SMCs were obtained from American Type Culture Collection (Manassas, VA) and grown as recommended in DMEM modified to contain 4 mM l-glutamine, 4.5 g/l glucose, 1 mM sodium pyruvate, and 1.5 g/l sodium bicarbonate supplemented with 10% FBS and antibiotics.

Transfection
Plasmid DNAs encoding Cdc42 wild-type or constitutive mutant V12 have been described previously (Musch et al., 2001). siRNA to Cdc42, UUUGGUCCCAA-CAAGCAAGAAAGG, or its scrambled control was obtained from Invitrogen (Grand Island, NY). Transfection of plasmid DNA was carried out using Nucleofector technology (Amaxa Biosystems, Lonza, Cologne, Germany). For each treatment group, one million cells were suspended in 100 μl of Nucleofector Solution R, mixed with 5 μg of plasmid DNA, and electroporated using the proprietary program D-033. Cells were then plated onto six-well plates or seeded on chamber slides (BD Biosciences, San Jose, CA) containing 10% FBS DMEM. For siRNA transfection, primary mouse SMCs were plated onto six-well plates or seeded on chamber slides at 50–60% confluence and incubated for 24 h. Cells were then transfected in Opti-MEM I medium with 20 nM of siRNA for mouse Cdc42 or control siRNA using Lipofectamine RNAiMAX transfection reagent as described by the manufacturer’s protocol (Invitrogen). At 12 h posttransfection Opti-MEM I medium was replaced with DMEM containing 10% FBS.

Adenoviral infection
The construction of the PKCδ-expressing adenoviral vector has been previously described (Ryer et al., 2006). Infection was carried out by incubating SMCs with adenovirus (30,000 particles/cell) in DMEM/2% FBS for 4 h. After the removal of adenovirus, cells were cultured containing 10% FBS and then in DMEM/0.5% FBS for 48 h.

Immunoblotting
The same number of PKCδ WT and KO SMCs was seeded to each plate and cultured simultaneously. Cells were made quiescent at the same time by incubation in medium containing 0.5% FBS for 48 h and then collected. SMCs were lysed in radioimmunoprecipitation assay buffer consisting of 50 mM Tris HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS (all reagents from Sigma-Aldrich). Then equal amounts of protein extract were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes as described previously. To analyze secreted proteins, media were collected from treated cell culture and concentrated using Ultracel 10K Centrifugal Filters (Millipore, Billerica, MA). Each media sample was centrifuged at 2000 RCF for 10 min, ultimately yielding a 40× concentration.
For Western blotting, the membranes were incubated with rabbit polyclonal antibodies to collagen I, collagen III, tropoelastin, Cdc42, RhoA, RhoB, RhoC, Rac1/2/3, and mouse monoclonal antibodies to β-actin and pro–collagen I, followed by horseradish peroxidase–labeled goat anti–rabbit or anti–mouse immunoglobulin G (Bio-Rad, Hercules, CA). Labeled proteins were visualized with an enhanced chemiluminescence system (PerkinElmer-Cetus, Boston, MA). For quantification of secreted proteins, optical density of secreted proteins, determined by ImageJ (National Institutes of Health, Bethesda, MD), was normalized to the total protein density (secreted plus intracellular).

**Immunohistochemistry and immunocytochemistry**

Mouse aortas were embedded in OTC compound, frozen, and then cut into 7-μm sections. For immunohistochemical analysis, protocols were supplied by the diaminobenzidine (DAB) kit (DakoCyto- mation, Carpenteria, CA), and collagen I and pro–collagen I antibodies were selected based on institutional experience. Antigen retrieval was performed in a citrate buffer water bath for 10 min. Endogenous peroxidase was quenched by submerging slides in 3% hydrogen peroxide for 5 min, followed by two washes in phosphate-buffered saline (PBS). Sections were then incubated for 30 min in 2% bovine serum albumin to minimize nonspecific binding and then for 1 h at room temperature with either anti–collagen I at 1:50 or anti–pro–collagen I (for human tissue) at 1:1000. Sections were washed three times in PBS and incubated with horseradish peroxidase–labeled anti–primary antibody (1:1000) for 30 min, followed by incubation in freshly prepared EnVision+ solution and developed with DAB. All sections were then counterstained with hematoxylin and mounted.

For immunofluorescence, SMCs were seeded on chamber slides (BD Biosciences) and fixed in 2% paraformaldehyde at room temperature for 10 min. After permeabilization in 0.5% bovine serum albumin, 1% goat serum, and 0.1% saponin, the cells were decorated with primary antibodies against pro–collagen I, PKCδ, Cdc42, TGN38, GM130, and EEA-1, followed by secondary antibodies tagged with Alexa 488 and 568. Images were generated with a Zeiss Axio-Observer spinning disk confocal microscope (Carl Zeiss, Jena, Germany) or a BD Biosciences pathway confocal microscope. Colocalization between TGN38, GM130, or EEA-1 and pro–collagen I or Cdc42 was quantified by Pearson coefficient analysis with ImageJ. For Pearson coefficient analysis, measurements were performed on ≥10 cells in multiple fields and repeated in two or three independent experiments.

**RNA isolation and quantitative real-time PCR**

Total RNA was isolated from WT or PKCδ KO mice aortic SMCs using RNeasy Plus Mini Kit (Qiagen, Valencia, CA). Two micrograms of total RNA was used for the first-strand cDNA synthesis (Applied Biosystems). Each cDNA template was amplified in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) with gene-specific primers for Cdc42, RhoA, RhoB, RhoC, Rac1/2/3, and mouse monoclonal antibodies to β-actin and pro–collagen I, followed by horseradish peroxidase–labeled goat anti–rabbit or anti–mouse immunoglobulin G (Bio-Rad, Hercules, CA). Labeled proteins were visualized with an enhanced chemiluminescence system (PerkinElmer-Cetus, Boston, MA). For quantification of secreted proteins, optical density of secreted proteins, determined by ImageJ (National Institutes of Health, Bethesda, MD), was normalized to the total protein density (secreted plus intracellular).

**Cdc42 activation assay**

Primary SMCs were grown to 70% confluence and serum starved for 48 h and then lysed according to the protocol supplied by the Cdc42 Activation Assay Biochem Kit (Cytoskeleton, Denver, CO). Briefly, cell lysates were incubated in a Cdc42-GTP affinity plate for 15 min. The wells were probed with anti-Cdc42 monoclonal antibody and a secondary antibody. Finally, the plate was developed with a colorimetric substrate, and the absorbance was read at 490 nm.

**Statistical analysis**

Unpaired Student's t test was used to evaluate the statistical differences between control and treated groups. Differences with p < 0.05 were considered significant. All experiments were repeated at least three times. Error bars on the graphs represent SEM. Values were expressed as mean ± SEM.

**ACKNOWLEDGMENTS**

We acknowledge the technical support of the Hospital of Special Surgery Musculoskeletal Core Center, which is funded by National Institutes of Health Grant AR46121, and thank Henry Pelish at Harvard Medical School (Boston, MA) for the generous gift of se-cranine A and Drew Roenneburg for technical assistance in histology. This work was supported by National Institutes of Health Grants R01HL081424 (B.L.), R01GM34107 (E.B.), and T32HL083824 (A.Z.). The hybridoma antibody developed by Heinz Furthmayr was ob-tained from the Developmental Studies Hybridioma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biology, University of Iowa (Iowa City, IA).

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