Elevated Protein Kinase C-δ Contributes to Aneurysm Pathogenesis Through Stimulation of Apoptosis and Inflammatory Signaling

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Objective—Apoptosis of smooth muscle cells (SMCs) is a prominent pathological characteristic of abdominal aortic aneurysm (AAA). We have previously shown that SMC apoptosis stimulates proinflammatory signaling in a mouse model of AAA. Here, we test whether protein kinase C-δ (PKCδ), an apoptotic mediator, participates in the pathogenesis of AAA by regulating apoptosis and proinflammatory signals.

Methods and Results—Mouse experimental AAA is induced by perivascular administration of CaCl₂. Mice deficient in PKCδ exhibit a profound reduction in aneurysmal expansion, SMC apoptosis, and transmural inflammation as compared with wild-type littermates. Delivery of PKCδ to the aortic wall of PKCδ⁻/⁻ mice restores aneurysm, whereas overexpression of a dominant negative PKCδ mutant in the aorta of wild-type mice attenuates aneurysm. In vitro, PKCδ⁻/⁻ aortic SMCs exhibit significantly impaired monocyte chemoattractant protein-1 production. Ectopic administration of recombinant monocyte chemoattractant protein-1 to the arterial wall of PKCδ⁻/⁻ mice restores inflammatory response and aneurysm development.

Conclusion—PKCδ is an important signaling mediator for SMC apoptosis and inflammation in a mouse model of AAA. By stimulating monocyte chemoattractant protein-1 expression in aortic SMCs, upregulated PKCδ exacerbates the inflammatory process, in turn perpetuating elastin degradation and aneurysmal dilatation. Inhibition of PKCδ may serve as a potential therapeutic strategy for AAA. (Arterioscler Thromb Vasc Biol. 2012;32:2493-2502.)

Key Words: aneurysms ■ apoptosis ■ vascular biology ■ inflammation ■ protein kinase C-δ

Abdominal aortic aneurysm (AAA), a progressive aortic dilation, is a common vascular disease associated with high mortality. Aneurysm results from the culmination of a series of events that lead to disruption of structural integrity and segmental weakening of the abdominal aortic wall. An incomplete understanding of the biological mechanisms underlying the disease has limited the development of therapeutic treatment and diagnostic strategies, thus leaving surgical and endovascular procedures as the only treatment options for patients with AAA.

Histologically, aneurysmal tissues are characterized by disruption of the elastic fibers in the aortic wall and extensive transmural infiltration of macrophages and lymphocytes.1-3 These features have been consistently duplicated in animal models of AAA.4 The prevailing view is that inflammatory cells, mainly macrophages, are the major source of matrix-degrading enzymes, such as matrix metalloproteinases5-9 and proinflammatory cytokines.10-12 Anti-inflammatory strategies, such as those that deplete neutrophils, lymphocytes, mast cells, or proinflammatory cytokines, have been shown to prevent the upregulation of matrix metalloproteinases and attenuate aneurysm formation in mouse models of AAA.13-16

Although the depletion of vascular smooth muscle cells (SMCs) is well documented in human aneurysmal tissues,17 potential interactions between SMCs and infiltrating inflammatory cells remain unclear. We have recently demonstrated that blocking apoptosis with a pan caspase inhibitor protected mice from angiotensin II–induced aneurysm expansion.18 The caspase inhibitor not only prevented SMC depletion but also diminished infiltration of macrophages and lymphocytes, suggesting a potential link between the apoptotic process and inflammatory signaling in the pathogenesis of aneurysm.

Protein kinase C-δ (PKCδ), a member of the PKC family of serine and threonine kinases, is a crucial mediator of SMC apoptosis.10-21 Studies of PKCδ knockout (KO) mice reveal that mice lacking PKCδ develop normally but exhibit an apoptosis-resistant phenotype when subjected to models of vascular injury, such as vein graft or carotid artery ligation.22,23 Conversely, gene transfer of PKCδ via an adenoviral vector led to excessive apoptosis of vascular SMCs in a rat carotid balloon injury model.24 More recently, we showed that PKCδ may...
also be involved in the regulation of chemokine expression. Inhibition of PKCδ with rottlerin profoundly decreases the production of monocyte chemotactic protein-1 (MCP-1) by aortic vascular SMCs and, subsequently, inhibits chemotaxis of inflammatory cells toward SMC-conditioned media.\(^{21}\)

We have previously shown that the expression of PKCδ is significantly higher in human aneurysmal aortic tissues as compared with normal arteries.\(^{21}\) The collection of these tissues at the time of surgical repair precluded analysis of a potential causal relationship between PKCδ and aneurysm, specifically, whether PKCδ upregulation contributes to the pathophysiology of aneurysm or is merely a resultant phenomenon. To determine whether PKCδ is an integral mediator of SMC apoptosis and vascular inflammation during aneurysm pathogenesis, the current study tests the effects of PKCδ gene deficiency on aneurysm formation using the CaCl\(_2\) mouse model. In addition, we explored the potential molecular mechanisms by which PKCδ regulates the proinflammatory signals produced by apoptotic SMCs.

**Materials and Methods**

A detailed description of the Materials and Methods are shown in online-only Data Supplement.

**Mouse Models of AAA**

The generation of PKCδ target deletion in mice was described elsewhere.\(^{24}\) PKCδ KO mice and their wild-type (WT) littermates were generated by mating heterozygous pairs. C57BL/6 mice and apolipoprotein E–deficient mice were purchased from Harlan Laboratories (Madison, WI) and Jackson Laboratory (Bar Harbor, ME), respectively. Green fluorescent protein transgenic mice were gifted by Dr William Burlingham of the University of Wisconsin–Madison.

Male mice, aged 12 weeks, underwent a CaCl\(_2\)-induced AAA model as described previously.\(^{26–28}\) Briefly, the infrarenal region of the aorta was isolated and treated with 0.5 mol/L CaCl\(_2\) perivascularly via gauze for 20 minutes. Control mice were similarly treated with 0.5 mol/L of NaCl. Tissues were fixed in 4% formaldehyde in PBS, embedded and cut to 6- or 8-µm sections. Green fluorescent protein expression was confirmed with rottlerin. Additionally, levels of the apoptosis-associated catalytic fragment of PKCδ became readily detectable in CaCl\(_2\)-treated group (Figure 1D).

**Immunohistochemistry**

Antibodies were purchased from Abcam (Cambridge, MA; interferon-γ, interleukin [IL]-6, monocyte+macrophage antibody, myosin heavy chain, and CD45), Santa Cruz (Santa Cruz, CA; CD3, MCP-1, Mac3, PKCδ, Ly6G, and CD68), Sigma-Aldrich (smooth muscle actin), and Cell Signaling (Danvers, MA; Cleaved caspase-3). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining kit was purchased from Roche (Madison, WI). Van Geison stains were carried out using Chromaview Van Geison kit (Richard Allan Scientific, Kalamazoo, MI).

**Cell Culture**

The murine macrophage cell line RAW264.7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Primary mouse aortic SMCs from the aorta of both PKCδ KO and WT mice were isolated based on a protocol described by Clowes et al.\(^{29}\)

**Migration Assay**

The murine macrophage cell line RAW264.7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Primary mouse aortic SMCs from the aorta of both PKCδ KO and WT mice were isolated based on a protocol described by Clowes et al.\(^{29}\)

In vitro migration assay was carried out, as previously described.\(^{16}\) Briefly, RAW264.7 macrophages, or CD11b+ cells isolated from bone marrow, were placed in a 5-µm pore Transwell insert. Conditioned and treated media were used as chemottractants. After 6 hours of incubation, inserts were removed and stained with hematoxylin to facilitate nuclei visualization. The mean value of migrated cells was counted in 8 high-power fields per membrane.

**Bone Marrow Isolation and Sorting**

Bone marrow was isolated from long bones, washed with PBS, and counted. Monocytes were collected from bone marrow by magnetic sorting using CD11b microbeads (Miltenyi Biotec, Boston, MA). Purity of the resulting CD11b+ cells was assessed by flow cytometry using antibodies to CD3, CD11b, and CD45/B220 (Miltenyi Biotec).

**Statistical Analysis**

Values were expressed as mean±SE. Experiments were repeated at least 3 times unless stated otherwise. Differences between the 2 groups were analyzed by Student \(t\) test. For time course comparison, 1-way ANOVA analysis was followed by Bonferroni correction to adjust for multiple comparisons. Values of \(P<0.05\) were considered significant.

**Results**

**PKCδ Expression in Experimental Aneurysms**

We subjected C57BL/6 male mice to perivascular treatment of 0.5 mol/L CaCl\(_2\), or equal concentration of NaCl, to the infrarenal region of the aorta and euthanized the animals at selected time points. Administration of CaCl\(_2\) led to gradual aortic dilatation associated with elastin fragmentation (Figure 1 in the online-only Data Supplement). Immunohistochemical analysis showed a profound upregulation of PKCδ protein in the aortic media 3 and 7 days after the CaCl\(_2\) treatment (Figure 1A), a time frame at which aortic expansion was barely visible. The temporal and spatial pattern of PKCδ expression mirrored that of the TUNEL positivity (Figure 1B). Confocal images confirmed the colocalization between PKCδ upregulation and apoptosis (TUNEL). Furthermore, PKCδ-positive cells were primarily SMCs, as identified by myosin heavy chain (Figure 1C). A similar expression pattern of PKCδ and its association with apoptosis was also observed in angiotensin II–induced aneurysm in apolipoprotein E–deficient mice (Figure II in the online-only Data Supplement). Western blot analysis confirmed the elevated level of PKCδ protein in CaCl\(_2\)-treated aortas as compared with the NaCl-treated controls (Figure 1D and 1E). Additionally, levels of the apoptosis-associated catalytic fragment of PKCδ became readily detectable in CaCl\(_2\)-treated group (Figure 1D).

**Mice Lacking PKCδ Are Resistant to AAA Induction**

To prove a potential role of PKCδ in AAA formation, we subjected PKCδ KO mice and their WT littermates to aneurysm induction by CaCl\(_2\). Forty-two days after the CaCl\(_2\) treatment, abdominal aorta of WT mice were visibly inflamed and dilated whereas the arteries of KO mouse appeared minimally affected (Figure 2A). The maximal external diameter of the abdominal aorta was measured immediately before CaCl\(_2\), and at the time of tissue harvest. As seen in Figure 2B, the baseline aortic diameters are comparable in PKCδ WT and KO mice. Six weeks after the CaCl\(_2\) treatment, arteries of WT mice expanded to 1.04±0.08 mm (96.6±31%), whereas arteries of KO mice expanded only to 0.74±0.06 mm (39.7±9%) (Figure 2B). Similarly, PKCδ was shown to play a role in the elastase perfusion model of murine AAA. Inactive
elastase produced minimal dilation of the artery in both WT and KO animals (0.77±0.06 mm and 0.76±0.02 mm, respectively; n=2), whereas active elastase treatment produced a more severe dilation in WT animals (1.47±0.16 mm) compared with KO (0.97±0.29 mm) (Figure III in the online-only Data Supplement).

Histological analysis performed at 7 days after CaCl2 treatment revealed similar elastin degradation in KO and WT arteries (Figure IV in the online-only Data Supplement). However, the same histological analysis 42 days after the CaCl2 treatment showed elastin fibers in arteries harvested from KO mice appeared continuous and organized, similar to NaCl-treated controls, whereas elastin fibers in CaCl2–treated WT arteries appeared fragmented and disoriented (Figure 2C and 2D). Furthermore, PKCδ KO tissue harvested at 7 days displayed significantly reduced SMC apoptosis, as evidenced by confocal staining showing colocalization of myosin heavy chain and apoptosis (TUNEL), as compared with WT samples (Figure 2E). Accordingly, cleaved Caspase-3 was nearly undetectable in PKCδ KO arteries, whereas it was abundant in WT controls.

Figure 1. Protein kinase C-δ (PKCδ) expression correlates with apoptosis in an experimental aneurysm model. Aortas of C57B/6 mice were treated with CaCl2 or NaCl and harvested 3, 7, and 14 days (CaCl2 group) or 7 days (NaCl group) after surgery. A, Cross sections stained for PKCδ (green) or apoptosis (terminal deoxynucleotidyl transferase dUTP nick end labeling [TUNEL], red), and nuclei (DAPI, blue). Scale bar, 200 μm; magnification, 10×. B, Positive cell ratio calculated as number of apoptotic (TUNEL+) or PKCδ-positive cells divided by respective number of DAPI-positive cells. *P<0.05 compared with NaCl control; n=6. C, Representative confocal images for colocalization analysis in cross sections harvested 7 days after CaCl2 treatment. Top panel: Costain TUNEL (red) and PKCδ (green). Bottom panel: Costain for smooth muscle cells (myosin heavy chain [MHC], red) and PKCδ (green). Scale bar, 50 μm; magnification, 40× Overlay with DAPI (blue). D, Representative Western blot analysis of PKCδ expression in tissues harvested from 2 different aortas of C57B/6 mice 7 days after NaCl or CaCl2 treatment. E, Quantification of PKCδ expression from Western blot, normalized to β-actin. PKCδ expression shown as a total of both cleaved and full-length portions. *P<0.05; n=4.

Figure 2. Protein kinase C-δ (PKCδ) knockout (KO) mice are resistant to aneurysm induction. A, Representative photos of abdominal aortas of PKCδ wild-type (WT) and knockout (KO) mice, taken 42 days after the CaCl2 treatment. Scale bar, 5 mm. B, Aortic diameter measured before (white bars) and 42 days after (black bars) CaCl2 treatment. *P<0.05 compared with the CaCl2–treated KO arteries; n=6. C, Representative photos of 42-day aortic sections stained for elastin (Van Gieson); scale bar, 100 μm; magnification, 40×. D, Grading of elastin degradation in Van Gieson–stained arteries harvested 42 days after surgery. *P<0.05 WT compared with KO; n=4. E, Representative confocal images of arterial sections costained for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, red) and myosin heavy chain (MHC, green) overlaid with DAPI (blue); arteries harvested 7 days after surgery; scale bar, 50 μm; magnification 40×.
PKCδ-Deficient Aortic SMCs Are Impaired in MCP-1 Expression

The diminished inflammatory infiltrate in PKCδ KO mice could be caused by a lack of PKCδ-mediated chemokine production in the aortic wall or diminished migratory property of monocytes. A complete blood count performed on WT and KO animals showed no significant difference in white blood cell or red blood cell populations between the 2 genotypes (Table I in the online-only Data Supplement). Furthermore, the percentage of monocytes (CD11b+) in the bone marrow was comparable between the genotypes (Figure VIIA and VIIIB in the online-only Data Supplement). In a chemotaxis assay, CD11b+ monocytes isolated from KO and WT animals migrated with equal efficiency toward recombinant MCP-1 protein (Figure VIIIC in the online-only Data Supplement). Together, these data suggest that neither number nor migratory capability of bone marrow monocytes are affected by PKCδ gene deficiency.

Real-time polymerase chain reaction analysis of aortic SMCs showed KO cells to have a nearly absolute impairment of MCP-1 production. Expression of interferon-γ and IL-6 also appeared to be modulated by PKCδ, albeit to a lesser degree (Figure 4A). The dependence of MCP-1 expression on PKCδ was further demonstrated by ELISA measurement of MCP-1 production by cultured SMCs. After treatment with tumor necrosis factor-α, WT SMCs are shown to produce significantly more MCP-1 as compared with KO SMCs (Figure 4B). Furthermore, overexpression of PKCδ using adenoviral-mediated gene delivery (AdPKCδ) further enhanced the production of MCP-1 in WT SMCs (Figure 4C).

To further test the notion that PKCδ gene deficiency reduces the presence of proinflammatory aneurysm signals produced by the aortic wall, we examined the ability of aortic SMCs to attract RAW264.7 monocyte/macrophages. As shown in Figure 4D, the number of RAW264.7 cells that migrated toward media conditioned by KO SMCs was ≈50% less than in the WT arteries (Figure VA and VB in the online-only Data Supplement). Furthermore, the percentage of nuclei staining positive for TUNEL was decreased from 24.1±3.4 in WT arteries to 12.5±2.9 in KO arteries (data not shown). In vitro analysis of cultured SMCs confirmed the apoptosis-resistant phenotype. The lack of PKCδ diminished the ability of SMCs to undergo apoptosis in response to tumor necrosis factor-δ, which was rescued by restoration of PKCδ expression with an adenoviral vector (Figure VC and VD in the online-only Data Supplement).

**PKCδ Is Critical for the Inflammatory Response**

Next, we analyzed macrophage infiltration, another important characteristic of aneurysm, in the aortas of both WT and KO animals. Immunohistochemical analysis revealed a profound reduction in the number of macrophages (Mac-3+, CD68+) detected in the aorta of PKCδ KO mice as compared with their WT littermates (Figure 3A and 3B). Additionally, neutrophils (Ly6G+), leukocytes (CD45+), and T cells (CD3+) were shown to be present in the aortic samples of the PKCδ WT mice, mostly prevalent in the adventitia, and almost entirely absent in KO aortas (Figure VI in the online-only Data Supplement). Similarly, levels of AAA-associated inflammatory cytokines IL-6 and MCP-1 were markedly decreased by PKCδ gene deficiency (Figure 3C). To better quantify the altered cytokine expression, we analyzed aortic tissues using real-time polymerase chain reaction analysis. As shown in Figure 3D, PKCδ gene deficiency caused a 50.7% and 48.1% reduction in mRNA levels of IL-6 and MCP-1 in tumor necrosis factor-δ–treated SMCs, respectively. Additionally, aneurysm-associated induction of IL-1β and IFN-δ was also significantly blunted in PKCδ KO mice (Figure VII in the online-only Data Supplement). There was also a small but statistically insignificant trend of reduction in the tumor necrosis factor-δ mRNA abundance.
that toward media conditioned by WT SMCs. Furthermore, administration of an MCP-1–neutralizing antibody completely eliminated the ability of WT SMCs to stimulate migration, suggesting MCP-1 to be a critical proinflammatory signal released by aortic SMCs (Figure 4D).

Exogenous PKCδ Reverses the Aneurysm-Resistant Phenotype of KO Mice

Data derived from the above in vitro analyses suggest that PKCδ gene deletion attenuates aneurysm development primarily through preventing aortic SMCs from undergoing apoptosis and producing proinflammatory chemokines, specifically MCP-1. To test this hypothesis, we developed an aortic tissue–specific gene transfer method to restore PKCδ expression in the arterial wall of KO mice. As described in the Materials and Methods section, adenovirus was administered to the aortic wall immediately after the removal of CaCl2. This gene transfer method produced a localized transgene expression as illustrated by using an adenovirus encoding enhanced green fluorescent protein (AdGFP). While producing abundant green fluorescent protein expression in the infrarenal region of the aortic wall, aortic administration of adenovirus encoding green fluorescent protein did not produce transgene expression in circulating white blood cells (Figure IXA and IXB in the online-only Data Supplement).

To restore PKCδ expression in the aortas of PKCδ KO mice, adenovirus expressing either PKCδ or β-galactosidase (AdPKCδ or AdLacZ, respectively) was administered to the infrarenal aorta of PKCδ KO mice. Mice were euthanized after 7 or 42 days for histological and morphological analyses, respectively. Delivery of AdPKCδ successfully induced localized aortic expression of PKCδ in KO mice, mostly in the perivascular region and to a lesser degree in the smooth muscle actin positive media (Figure IXC and IxD in the online-only Data Supplement). Forty-two days after the CaCl2 treatment, AdLacZ-treated PKCδ KO mice displayed minimum aortic expansion, with a final diameter measurement of 0.67±0.07 mm (29.2±15.9%), indicating that viral infection alone did not alter the aneurysm-resistant phenotype of KO mice. In comparison, delivery of AdPKCδ produced significant aortic expansion in KO mice (final diameter 1.11±0.21 mm; 114.8±28.3%), an induction comparable with that seen in WT mice (Figure 5A and 5B). The apparent restoration of aneurysm formation shown to accompany aortic gene transfer of PKCδ is further evidenced by fragmented elastin fibers, as well as TUNEL-positive apoptotic cells and infiltrating monocytes/macrophages, at 7 days after surgery (Figure 5C).
Aortic Inhibition of PKCδ Attenuates Aneurysm Formation in C57B/6 Mice

To further demonstrate the importance of aortic PKCδ expression in aneurysm development, we tested the effect of aortic inhibition of PKCδ. After the routine CaCl₂ treatment, C57BL/6 mice were subjected to local infection with either a dominant negative PKCδ mutant adenovirus or AdLacZ as control. Mice were euthanized after 7 or 42 days for histological and morphological analyses, respectively. As shown in Figure 5D and 5E, treatment with dominant negative PKCδ mutant produced a moderate but significant attenuation in aneurysm formation in C57B/6 mice as reflected by a reduction in aortic expansion as compared with the AdLacZ-treated mice (final aortic diameter measurement 0.74±0.11 mm or 54.7±28.3%, and 1.05±0.06 mm or 126.5±15.7%, respectively). Accordingly, local inhibition of PKCδ activity diminished elastin degradation, apoptotic activity, and infiltration of monocytes/macrophages in the arterial wall (Figure 5F).

Exogenous MCP-1 Protein Restores Aneurysm to PKCδ KO Animals

Various studies have implicated an important role for both MCP-1/C-C chemokine receptor 2 signaling in vascular diseases, including atherosclerosis and AAA. 30–33 Both in vivo and in vitro analyses within the current study indicate a reduction of MCP-1 expression caused by PKCδ gene deficiency; this prompted us to test whether delivery of exogenous MCP-1 to the arterial wall of PKCδ KO mice could restore aneurysm formation. Immediately after the CaCl₂ treatment, recombinant MCP-1 protein suspended in pluronic gel was delivered to the infrarenal aortic region of KO mice. As vehicle controls, parallel groups of KO and WT mice were treated with pluronic gel + solvent. As shown in Figure 6A and 6B, solvent-treated WT aortas developed aneurysmal expansion comparable with those previously observed in these mice at 42 days after the CaCl₂ treatment (0.84±0.04 mm; 73.7±8.2%). At this same time point, pluronic gel + solvent-treated PKCδ KO aortas maintained their aneurysm-resistant phenotype despite the
administration of pluronic gel, a stark contrast to the KO aortas treated with recombinant MCP-1 (aortic diameter 0.62±0.06 mm or 27.1±12.5%, and 0.91±0.04 mm or 89.6±9.4%, respectively). Administration of recombinant MCP-1 in KO aorta created elastin degradation similar to that seen in the solvent-treated WT aorta, whereas solvent-treated KO aortas remained largely unaffected. Further histological analysis of these samples revealed a marked increase of macrophage infiltration in the MCP-1–treated mice as compared with solvent-treated PKCδ KO mice. Of note, the level of aortic SMC apoptosis in PKCδ KO mice was not significantly altered by the MCP-1 administration (Figure 6C).

Discussion

Our data for the first time provide direct evidence that PKCδ is an integral signaling molecule in the pathogenesis of AAA. We showed that inhibition of PKCδ, either through targeted gene deletion or overexpression of a dominant negative mutant, protected mice from developing characteristic features of aneurysm, including inflammation, disruption of elastin fibers, and loss of vascular SMCs. Additionally, the aneurysm-resistant phenotype was accompanied by diminished inflammatory infiltration, cytokine production, and medial apoptosis. These results not only confirm the importance of PKCδ in the regulation of SMC apoptosis but also indicate a novel role for this kinase in the proinflammatory signaling cascade, at least in the aneurysm setting.

Although PKCδ is ubiquitously expressed in many tissues and cell types, results reported here suggest that the role of this signaling protein in aneurysm pathophysiology may be primarily localized in vascular SMCs. Furthermore, our evidence suggests PKCδ may act largely through regulating expression of proinflammatory chemokines and cytokines, notably MCP-1. This notion is supported by several in vivo and in vitro findings: (1) PKCδ gene deficiency reduced the production of MCP-1 and other cytokines by aortic SMCs, but did not significantly alter the ability of monocytes to migrate; (2) an adenovirus-mediated delivery of PKCδ locally to the arterial wall was sufficient to rescue aneurysm development in PKCδ KO mice; (3) aorta-specific inhibition of PKCδ delivered a moderate but significant level of protection in C57B/6 mice; and (4) ectopic administration of MCP-1 to the aortic wall of PKCδ KO mice sufficiently rescued aneurysm development.

It has been postulated that vascular SMCs are the soil of AAA development.14 Being a major source of extracellular matrix proteins, SMCs would be critical in counter balancing the upregulated proteolytic activities present in aneurysmal tissue. As such, the depletion of medial SMCs eliminates a cell population capable of directing connective tissue repair and may, thus, potentiate the degradation of the arterial wall and facilitate eventual rupture.15 This study contains data supporting the idea that the dearth of connective tissue in AAA can be reversed in the presence of healthy SMCs, thus either preventing or even reversing aneurysm growth. Specifically, we showed that the arteries of CaCl2-treated KO mice sustained a similar degree of initial damage to aortic elastin fibers as WT aorta but by 42 days elastin integrity is restored in KO arteries.

Results from the current study further illustrate another important function of SMCs in vascular disease, (ie, providing proinflammatory signals). The potential link between SMC apoptosis and the production of proinflammatory chemokines has been previously indicated in atherosclerosis. Using a mouse atherosclerosis model, Clarke et al18 convincingly demonstrated that SMC apoptosis induces MCP-1 expression, inflammatory infiltrate, and other features of plaque rupture. Recently, our own group demonstrated that blocking apoptosis with a pan-caspase inhibitor protected mice from angiotensin II–induced vascular inflammation and aneurysm expansion.18 These data suggest that, although apoptosis and inflammation are most commonly considered unrelated events, apoptosis in an aneurysm setting may promote the inflammatory response. Such interaction between apoptosis and inflammation has been explored in atherosclerosis. Clarke et al18 suggest that the proinflammatory property of apoptotic SMCs may be attributed to inhibited phagocytosis generated in the hyperlipidemic environment in atherosclerotic arteries. Although AAA is commonly associated with atherosclerosis, these 2 diseases...
are believed to be caused by distinct pathological processes. However, deficient phagocytosis is also being investigated as an underlying pathophysiological event in other types of inflammatory disorders, including autoimmune diseases, thus warranting the exploration of this process in the pathogenesis of AAA.

Another important finding described in this work is the apparent critical role of PKCδ in MCP-1 function during formation and progression of aneurysm. In vitro and in vivo evidence suggests that impaired production of MCP-1 expression by aortic SMCs was the primary mechanism underlying the aneurysm-resistant phenotype of PKCδ KO mice. Importantly, this notion is further supported by the evidence that localized aortic administration of recombinant MCP-1 to the aorta of PKCδ KO mice restored vascular inflammation, elastin degradation, and aneurysm expansion. Although several groups have explored the role of the CCR2/MCP-1 signaling axis in the aneurysm progression, this work provides what we believe to be the first evidence suggesting MCP-1 to be a critical downstream effector of PKCδ signaling in the pathogenesis of aneurysm.

Although our study has implicated a critical role for MCP-1 in AAA, it is important to consider the large number of cytokines that likely play a role in AAA development and progression. Our real-time polymerase chain reaction analysis identified additional cytokines that may be regulated by PKCδ and require further investigation. Interestingly, localized aortic delivery of exogenous MCP-1 failed to reverse the apoptosis-resistant phenotype of PKCδ KO mice. A similar number of TUNEL+ cells were found in MCP-1 and solvent–treated PKCδ KO mice. In contrast, a similar rescue experiment delivering exogenous PKCδ to the arterial wall restored all aneurysm-related cellular events (ie, inflammation, apoptosis, and elastin degradation in CaCl₂, PKCδ KO arteries). These results not only underscore the critical role of PKCδ in the apoptotic response of SMCs but also provide support for a novel relationship between PKCδ, MCP-1, and AAA. In the absence of this master mediator, apoptosis would be inhibited even when aortic SMCs are surrounded by infiltrating inflammatory cells, their inflammatory byproducts, and degraded elastin fibers. Based on rescue experiments presented here, as well as other data from the current and earlier reports, we propose a model in which PKCδ-mediated MCP-1 functions as a molecular link through which apoptotic SMCs stimulate the inflammatory process. Importantly, our model suggests that SMC apoptosis may contribute to aneurysm development primarily through the induction of inflammatory cytokines. That is, in the presence of abundant proinflammatory cytokines, such as the environment created by delivery of exogenous MCP-1 protein, the inflammatory and proteolytic events can proceed in full force without the participation of apoptosis.

Using a rat carotid balloon injury model of intimal hyperplasia, our group recently showed that PKCδ mediated the expression of MCP-1, which was critical for the migration of adventitial fibroblasts to the media and neointima. In the CaCl₂-treated aorta, we noted a marked expansion of the adventitia associated with abundant infiltration of macrophages and other inflammatory cells. Although the presence of macrophages in the adventitia is a prominent feature of AAA, and the role of adventitial fibroblasts in aneurysm has been explored to some extent by several groups, the precise relationship between adventitial fibroblasts, SMCs, and inflammatory cells in the context of AAA remains a highly interesting subject for future study. Evidence presented here shows the localization of IL-6 and macrophages predominantly in the adventitia, whereas MCP-1 production and apoptosis appears to occur primarily, though not exclusively, in the medial layer. It is also important to note that PKCδ, being a ubiquitously expressed protein, is also found in the adventitia. Whether PKCδ also contributes to aneurysm pathogenesis through adventitia cells should be explored in future studies given the prominent inflammatory response in the adventitia. However, several key questions remain to be addressed in models of AAA, for example, how adventitial fibroblasts may respond to medial SMC depletion, matrix degradation, and inflammatory cell infiltration.

Being a major signaling molecule, PKCδ can be activated by multiple extracellular and intracellular signals, including growth factors, inflammatory cytokines, mechanical stimuli, and oxidative stress. Not all of these signals are able to induce apoptosis or the production of MCP-1. It remains to be determined whether the proapoptotic and proinflammatory functions of PKCδ are exerted through the same or partially overlapping pathways. We have previously shown that mitogen-activated protein (MAP) kinase pathways are affected by PKCδ gene deficiency. Although the involvement of MAP kinases in the regulation of MCP-1 expression has been demonstrated, the precise molecular interaction between PKCδ and MAP kinases and how this interaction may influence MCP-1 expression remains to be determined. Additionally, Liu et al. recently demonstrated that PKCδ mediates the stability of MCP-1 mRNA in vascular SMCs using a chemical inhibitor of PKCδ. Our group previously described the role of MAP kinases in regulation of mRNA stability in vascular SMCs, leading us to speculate that PKCδ may control MCP-1 mRNA turnover through a MAP kinase–mediated mechanism.

Taken together, our data show that the stress response regulating apoptosis and inflammatory signaling in the arterial wall may be largely dependent upon PKCδ upregulation. Accordingly, inhibition of PKCδ attenuated vascular inflammation and preserved tissue integrity, resulting in the prevention of aneurysm development in a CaCl₂-induced model of AAA. Further, PKCδ gene deficiency appears to protect mice from developing aneurysm in the elastase model of AAA, as shown in Figure III in the online-only Data Supplement. Unfortunately, the potential role of PKCδ in the Angiotensin II model is yet to be explored, as our attempts to breed PKCδ–/– apolipoprotein E–deficient, double KO mice were unsuccessful. However, we did show that levels of PKCδ were significantly elevated in aortas of apolipoprotein E–deficient mice treated with Angiotensin II. Taken together, we believe the elevated expression of this stress gene in human aneurysmal tissues, as well as the role we have shown it to play in mouse models, suggest it to be an attractive candidate for therapeutic target(s).
Acknowledgments

We thank Drs K. Craig Kent and Ion Mutsumura of the University of Wisconsin, Madison for intellectual inputs, Dr Keiichi I. Nakayama of Kyushu University in Japan for the generous gift of PKCδ KO mice, Dr T. Biden at the Garvan Institute of Australia for dominant negative PKCδ mutant, and Drew Roenemburg of the University of Wisconsin, Madison for technical assistance in histology.

Sources of Funding

This work was supported by National Institutes of Health grant R01HL088447 (Dr Lou), American Heart Association grant 10GRNT3020052 (Dr Lou), Howard Hughes Medical Institute grant MSN135276 (S. Seelidal), and Ruth L. Kirschstein National Research Service Award T32 HL 07936 from the National Heart, Lung, and Blood Institute to the University of Wisconsin–Madison Cardiovascular Research Center (S. Morgan).

Disclosures

None.

References


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Arterioscler Thromb Vasc Biol. 2012;32:2493-2502; originally published online August 9, 2012;
doi: 10.1161/ATVBAHA.112.255661
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplemental Methods

Mouse Models of AAA

The generation of PKCδ target deletion in mice was described elsewhere. PKCδ knockout mice and their wildtype littermates were generated by mating heterozygous pairs. C57BL/6 mice and apoE−/− mice were purchased from Harlan Laboratories (Madison, WI) and Jackson Laboratory (Bar Harbor, ME), respectively.

Male mice, 12 weeks of age, underwent a CaCl₂- or elastase-induced abdominal aortic aneurysm model as described previously. Briefly, animals were anesthetized using continuous flow of 1-2% Isofluorane. For the CaCl₂, the infrarenal region of the aorta was isolated and treated with 0.5M CaCl₂ perivascularly via gauze for 20 minutes. Control mice were similarly treated with 0.5M of sodium chloride (NaCl). For the elastase model, the infrarenal region of the aorta was isolated temporary silk ligatures were placed around proximal and distal portions of the aorta. An aortotomy was created near the bifurcation using a 30-gauge needle and heat-tapered polyethylene tubing (Baxter Healthcare Corp., Illinois, USA) was introduced through the aortotomy and secured with a silk tie. The aorta was filled with saline containing 0.295 U/mL Type I porcine pancreatic elastase (Sigma, St. Louis, MO) at a constant pressure of 100mm Hg. For control, the elastase solution was heat-inactivated (100°C) before use.

In CaCl₂ and Elastase model surgical procedures, Buprenorphine was administered subcutaneously at a dose of 0.05mg/kg immediately after surgery. Subsequently, a 2.5% Xylocaine topical ointment was applied to the suture site. Additional doses of Buprenorphine were given via intraperitoneal injection every 8-12
hours after surgery for the first 48 hours. The maximum external diameter of the infrarenal aorta was measured using a digital caliper (VWR Scientific, Radnor, PA) prior to treatment and at the time of tissue harvest.

For the Angiotensin II model, male, 24-week-old, apoE-deficient mice with a C57BL/6 background were implanted with a Alzet osmotic minipump (model 2004; Alzet, CA) delivering Angiotensin II (1000ng/kg/min) or saline subcutaneously for up to 4 weeks. The external aortic diameter was measured at the region showing maximum dilatation with a digital caliper (VWR, PA).

At selective time points, mice were killed by an overdose of isoflourane and tissues were perfusion-fixed with 4% formaldehyde in phosho-buffered saline (PBS). Tissues meant for immunohistochemical analyses were imbedded in O.C.T. Compound (Sakura Tissue Tek, Netherlands), and tissues meant for morphological analyses were processed for paraffin embedding. All frozen sections were cut to 6μm thick using a Leica CM3050S cryostat and paraffin sections were cut to 8μm thick using a Reichert-Jung 2050 SuperCut Microtome. All experiments were conducted in accordance with experimental protocols that were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison (Protocol M02284).
Van Geison stains were carried out using Chromaview Van Gieson kit (Richard Allan Scientific, Kalamazoo, MI) according to provided protocol. Elastin integrity was evaluated using a semi-quantitative methodology described previously: (1, no elastin degradation or mild elastin degradation; 2, moderate; 3, moderate to severe; and 4, severe elastin degradation)\(^6,7\). Representative images for each grading score are provided in Supplemental Figure X. Each section was numbered and photographed at 10x and 20x magnification, maintaining their respective numbers. Then, an objective participant graded the photographs according to the aforementioned scale and recorded the grade with the section number.
Arterial sections were permeabilized with 0.1% TritonX for 10 minutes at room
temperature. Non-specific sites were blocked using 5% bovine serum albumin (BSA),
3% normal donkey serum in Tris-buffered Saline and Tween 20 (TBS-T) for 1 hour at
room temperature. Primary antibodies to CD3, MCP-1, and CD68 were purchased from
Santa Cruz (Santa Cruz, CA), IFN-γ, IL-6, MOMA2, CD45, and anti-smooth muscle
specific Myosin heavy chain 11 (MHC) antibodies from Abcam (Cambridge, MA), and
Cleaved Caspase 3 from Cell Signaling (Danvers, MA). All antibodies were diluted in
previously described blocking solution and incubated overnight at 4°C. Fluorescent
stains were completed using secondary antibodies purchased from Invitrogen Molecular
Probes (Carlsbad, CA) and 4′6-diamidino-2-phenyl-indole, dihydrochloride (DAPI, Invitrogen, CA) was used to detect nuclei. Control images, included in Supplemental
Figure 11A and B, show primary and secondary only antibody stains, respectively.
Conventional stains were developed using secondary antibodies purchased from Bio Rad
(Hercules, CA) with hematoxylin counter-stain. TUNEL staining kit was purchased from
Roche (Madison, WI) and carried out according to the provided protocol. Staining was
visualized with a Nikon Eclipse E800 upright microscope or Nikon A1RSi Confocal
system, and digital images were acquired using a RetigaEXi CCD digital camera.

Microscope exposure settings were held constant for all images taken amongst
experimental group sets. For example, the representative images shown in Figure 5C
were all taken with ISO Sensitivity ISO400, with exposure time for MOMA (FITC-
HYQ) at 1/7s, TUNEL (G-1A) at 1/3s, and DAPI (UV) at 1/180s. Similarly,
representative values for confocal images shown in Figure 1C were Gain 5.95B and
Offset 127 for green (PKCδ) and Gain 6.65B and Offset 127 for red (TUNEL).
Quantification of stains was performed in a manner to that previously described \(^8\) using Image J Software as provided by the National Institutes of Health. Data quantification was performed using at least 3 sections per artery.

*Fresh Tissue Western Blot*

Aorta was harvested from NaCl or CaCl\(_2\)-treated WT and KO mice and the tissue was flash-frozen in liquid nitrogen to be processed at a later time. Aortic tissue was ground to a fine powder in a small amount of liquid nitrogen, and protein was extracted from the powder using radioimmunoprecipitation assay (RIPA) buffer consisting of 50mM TRIZMA HCl, 150mM NaCl, 1% Nonidet-P40, 0.5% sodium dioxycholate, 0.1% SDS (all reagents from Sigma-Aldrich, St. Louis, MO). Equal amounts of protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The membranes were incubated with rabbit polyclonal antibodies to Protein Kinase C\(\delta\), C-17 fragment from Santa Cruz (Santa Cruz, CA) and mouse monoclonal antibodies to \(\beta\)-actin (Sigma, MO) followed by horseradish peroxidase labeled goat anti-rabbit or anti-mouse IgG (Bio-Rad, Hercules, CA). Labeled proteins were visualized with an enhanced chemiluminescence system (Perkin-Elmer, Boston, MA).

*Cell Culture*

The murine macrophage cell line RAW264.7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Primary mouse aortic SMCs from the thoracic aorta of both PKC\(\delta\) KO and WT mice were isolated based on a protocol
described by Clowes et al. Briefly, aortas were perfused with phospho-buffered saline supplemented with 2% penicillin/streptavidin antibiotics. The aorta was isolated from the aortic arch to the iliac bifurcation and incubated 30 minutes in digestion buffer (DMEM, Bovine serum albumin, Collagenase, Soybean trypsin inhibitor, and Elastase Type III) at 37°C. Adventitia was pulled away from the medial layer, tissues were minced, and further digested for 4 hours at 37°C. Tissue was spun to a pellet by centrifugation and washed with 10%FBS DMEM once, before suspension in a small volume of 10%FBS-DMEM and left undisturbed for 48 hours to allow cells to migrate from tissue. All cell types were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin, and 100µg/mL streptomycin in a 5% CO2/water-saturated incubator at 37°C.

**MCP-1 ELISA**

The BD OptEIA ELISA kit was obtained from BD Biosciences (San Jose, CA), and carried out according to the manufacturer’s protocol.

**Real-Time PCR Analysis**

Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen, CA) according to manufacturer’s protocol. For fresh tissue, aortic tissue was ground to a fine powder in a small amount of liquid nitrogen, and RNA was isolated from the powder using the RNeasy Plus Mini Kit (Qiagen, CA). cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA) on a Veriti 96-well Thermal Cycler (Applied Biosystems, CA). Primers
were purchased from Invitrogen and amplification was detected using SYBR Green PCR Master Mix (Applied Biosystems, CA). Real-time PCR was carried out using a 7500 Fast Real-time PCR System Machine (Applied Biosystems, CA). RQ value, where $RQ = \frac{E_{\text{target}}^{\Delta C_{P \text{target(ctl-sample)}}}}{E_{\text{reference}}^{\Delta C_{P \text{ref(ctl-sample)}}}}$, the reference gene was GAPDH, and CP is defined as a ‘crossing point’, was used to compare expression of target cytokines.

**Adenovirus Infection**

The adenoviral vector that expresses wildtype PKCδ was created as described previously \(^\text{10}\). A recombinant adenoviral vector containing the dominant negative PKCδ (AdPKCδDN) \(^\text{11}\) was obtained from Dr. T. Biden (Garvan Institute, Australia). *In vitro* adenovirus infection was carried out as described previously \(^\text{12}\). For perivascular adenovirus delivery, a small piece of latex was inserted underneath the aorta to create a ‘cup’ cradling the infrarenal aorta. After removal of the CaCl₂–containing gauze from the artery, adenovirus suspended in saline (2.5x10⁹ particle forming units (PFU)/mL) was added to the latex cup to bathe the artery for 20 minutes. Precautions were taken to avoid direct contact of viral solution with surrounding tissues.

**Migration Assay**

*In vitro* migration assay was carried out as previously described \(^\text{13}\). RAW 264.7 macrophages, or CD11b⁺ cells isolated from bone marrow harvested from PKCδ KO or WT mice, were placed in a 5µm pore transwell insert (Corning Inc, Wilkes Barre, PA) to a density of 500,000 cells/mL. Media conditioned by TNFα (R&D Systems, Minneapolis,
MN) stimulated aortic SMCs, or recombinant MCP-1 protein (R&D Systems, Minneapolis, MN) were used as chemoattractants. Following 6 hour incubation at 37°C, inserts were removed and washed with PBS, fixed with ice cold 70% Ethanol and stained with hematoxylin for nuclei visualization. The mean value of migrated cells counted in eight high-power fields per membrane was used as a measurement of migration.

Bone Marrow Isolation and Sorting

Bone marrow cells from both PKCδ KO and WT animals were isolated from long bones, washed with PBS, and counted. Monocytes were isolated from bone marrow by magnetic sorting using anti-CD11b microbeads (Miltenyi Biotec, Auburn CA). Purity of the resulting CD11b+ cells was assessed by flow cytometry using antibodies to CD3 (FITC), CD11b (APC), and CD45/B220 (APC-Cy7) (BD Pharmigen, San Diego, CA). Flow cytometric data was collected on a BD FACS Calibur Flow Cytometer equipped with a Cytek 633nm laser (Freemont, CA) and analysis was performed using Flow Jo software (TreeStar, Inc.).

Migration Assay

Chemotaxis assay was performed as described previously (cite). 2x10^5 macrophages (RAW264.7) were placed in the upper chamber of Costar 24-well transwell plates with 5um pore filters (Corning, Inc., Corning, NY). Cultured conditioned medium or control media was placed into the lower chambers or wells. Anti-MCP-1 antibody (Biolegend, CA) was used for neutralization of MCP-1. After incubating plates for 6h at 37C,
migrated cells were collected from the lower chambers and on the bottom of the filters were counted.

Statistical analysis

Values were expressed as mean ± standard error. Experiments were repeated at least three times unless stated otherwise. Differences between 2 groups were analyzed by Student’s $t$ test. For time course comparison, one-way ANOVA analysis was followed by Bonferroni correction to adjust for multiple comparisons. Values of $p<0.05$ were considered significant.


**Supplemental Table I**

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Supplemental Figure II

A. 

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**Supplementary Table I.** Complete Blood Count (CBC) results of PKCδ WT and KO mice. Red blood cells (RBC) and white blood cells (WBC) are shown; n=4.

**Supplementary Figure I.** Perivascular application of CaCl2 induces progressive dilatation of abdominal aorta. (A) Representative photos of abdominal aortas of C57B/6 mice treated with 0.5M CaCl2 or equal concentration of NaCl, taken 42 days after surgery. (B) Van Gieson stain to identify elastin continuity in aneurysmal (CaCl2) and control (NaCl) aortas. Scale Bar=50μm. (C) Aortic expansion over the time course of 42 days measured by fold change of maximum diameters as calculated by dividing aortic diameter at time of sacrifice by aortic diameter prior to treatment.

**Supplementary Figure II.** PKCδ expression correlates with apoptosis in an experimental aneurysm model. (A) Infusion of AngII in ApoE−/− animals leads to induction of both PKCδ (green) and TUNEL (red) in the aortic wall, overlay with DAPI (blue).

**Supplemental Figure III.** PKCδ expression moderates aneurysmal expansion in an Elastase perfusion model of murine AAA. Aortic diameter measured prior to (Pre, white bars), and 14 days after (Post, black bars), Elastase perfusion treatment.

**Supplemental Figure IV.** CaCl2 treatment causes similar early elastin damage in both PKCδ WT and KO aortas. Representative Van Gieson stains of arterial sections harvested 7 days after surgery.

**Supplemental Figure V.** The lack of PKCδ causes an apoptosis-resistant phenotype in vivo and in vitro. (A) Representative photos of immunohistochemistry for cleaved caspase-3 at 7 days, scale bar=100μm. (B) Cleaved caspase 3 index. *p<0.05, n=6. (C) PKCδ KO SMCs were resistant to apoptosis induced by TNFα (50ng/mL, for 6h). (D) Adenovirus-mediated expression
of exogenous PKCδ in PKCδ KO SMCs rescued the apoptotic phenotype. *p<0.05 compared to AdLacZ, n=3.

**Supplemental Figure VI. PKCδ gene deficiency has a broad inhibitory effect on inflammatory infiltration.** (A) IHC for neutrophils (Ly6G), T lymphocytes (CD3), and leukocytes (CD45) at 7 days; Scale Bar=200μm. Quantification of immunohistochemistry stains expressed as ratio of positive cells divided by nuclei. *p<0.05, n=6.

**Supplementary Figure VII. PKCδ mediates expression of cytokines and chemokines in aortas.** Total RNA was isolated from aortas of PKCδ WT or KO mice 7 days after surgery. mRNA levels of selected cytokines and chemokines were determined by RT-PCR. , *p<0.05, n=4.

**Supplementary Figure VIII. PKCδ gene deficiency does not significantly affect the number or function of monocytes.** (A) Bone marrow cells isolated from PKCδ WT or KO mice were stained with CD11b (monocytes), B220 APC-CY7 (B lymphocytes), and CD3 (T lymphocytes) for analysis by flow cytometry. (B) Purified monocyte population derived from whole bone marrow using CD11b⁺ magnetic bead sorting technique compared to whole bone marrow and CD11b-depleted bone marrow. (C) CD11b⁺ monocytes were isolated from bone marrow of PKCδ WT and KO mice. Migration of monocytes toward recombinant MCP-1 was analyzed by the trans-well assay, n=4.

**Supplementary Figure IX. Perivascular application of adenovirus leads to aorta-specific gene transfer.** (A&B) AdGFP or AdLacZ were administered perivascularly following CaCl₂ treatment. Green fluorescence was evaluated by fluorescent microscopy for GFP expression in
the aortic wall, Scale bar=500μm, (A) or by flow cytometry for GFP expression in peripheral blood, GFP<sup>+/+</sup> and GFP<sup>-/-</sup> mice were used as positive and negative controls, respectively (B). (C&D) PKC<sub>δ</sub> KO mice were treated with Ad PKC<sub>δ</sub> or AdLacZ following the CaCl<sub>2</sub> procedure. Aortic sections were co-stained with antibodies specific to PKC<sub>δ</sub> (green) and smooth muscle cell-specific α-actin (SMA, red), overlay with DAPI (blue). Scale bar=200μm.

**Supplemental Figure X. Elastin degradation scaling.** Representative images depicting the grading scale used to evaluate elastin degradation in mouse aneurysmal tissue. Detailed description in Supplemental Methods.

**Supplemental Figure XI. Representative control images for immunofluorescent staining.** (A) Primary antibody alone for PKC<sub>δ</sub> and TUNEL (diluent only, no enzyme), overlay with DAPI. Scale bar = 200μm. (B) Secondary antibody only for green fluorescent anti-mouse (Anti-Mouse 488) and red fluorescent anti-rabbit (Anti-Rabbit 546), overlay with DAPI. Scale bar = 200μm.