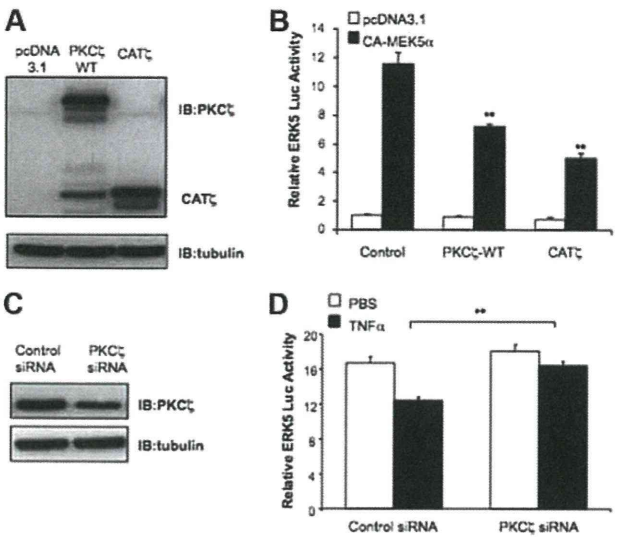

Figure 2. TNFα inhibits ERK5 transactivation by PKCζ. (A) HA-PKCζ-WT and CATζ overexpression was verified with the use of cell lysates probed with anti-PKCζ. (B) PKCζ-WT and CATζ inhibit ERK5 transactivation. HUVECs were transfected with pBIND-ERK5 and Gal4-dependent (pG5-Luc) reporter gene with or without pcDNA3-CA-MEK5α with 0.3 μg of control vector or expression plasmids for PKCζ-WT or CATζ. ERK5 transcriptional activity was evaluated by measuring luciferase activity after 24 hours. (C) PKCζ silencing was confirmed by Western blot analysis. (D) Depletion of PKCζ expression reverses TNFα-mediated inhibition of ERK5 transcriptional activity. HUVECs were pretreated for 24 hours with siRNA targeting PKCζ and then cotransfected with pBIND-ERK5 and Gal4-dependent (pG5-Luc) reporter gene with or without pcDNA3-CA-MEK5α. After 8 hours, cells were treated with 10 ng/mL TNFα, and ERK5 luciferase activity was determined 16 hours later. Results are expressed in arbitrary units normalized to the control (set to 1.0 for each experiment). Data are mean ± SD of 3 experiments performed in triplicate. $^{**}P < .05$.

PKCζ activation by TNFα inhibits ERK5 function

The critical role of the MEK5/ERK5/KLF2 pathway in inhibiting endothelial inflammation and stimulating eNOS expression is well described.[22,25,31-33] To investigate whether PKCζ activation negatively regulates ERK5, we cotransfected HUVECs with PKCζ-WT, CATζ, or the empty vector (pcDNA3.1) with Gal4-ERK5 and constitutively active MEK5 (CA-MEK5α) constructs as indicated (Figure 2), and then performed a luciferase assay. Both PKCζ-WT and CATζ significantly inhibited CA-MEK5α–mediated ERK5 transactivation (Figure 2B).

To inhibit PKCζ activity we used siRNA to reduce PKCζ expression (Figure 2C). To assay the effect of reducing PKCζ we measured ERK5-dependent luciferase activity as described in Figure 2B. ERK5 transactivation was inhibited by TNFα in control siRNA transfected cells and reversed in PKCζ siRNA transfected cells (Figure 2D). These data show a critical role for PKCζ in TNFα-induced inhibition of ERK5 transactivation.

We have previously reported that ERK5 SUMOylation at Lys6 and Lys22 inhibited its transcriptional activity.[25] These data suggest that the inhibition of ERK5 transactivation by TNFα may be due to ERK5 SUMOylation. To test this possibility, we studied the effect of TNFα on ERK5 transactivation in HUVECs expressing ERK5-K6R/K22R (K6/K22R mutant) which cannot undergo SUMOylation.[25] HUVECs were transfected with ERK5-K6/K22R, a Gal4-dependent luciferase reporter gene with or without CA-MEK5α, treated with TNFα, and assayed for luciferase activity. TNFα significantly inhibited ERK5 transactivation in a dose-dependent manner (supplemental Figure 1A, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article), but we observed no significant effects of the K6/K22R mutant on the TNFα-induced inhibition of ERK5 transactivation (supplemental Figure 1B). To determine whether ERK5 tyrosine phosphorylation at the TEY motif (Thr218/Tyr220; the dual-phosphorylation site) is involved in its down-regulation by TNFα, HUVECs were transduced with Ad.CA-MEK5α to activate ERK5. We found no inhibition of ERK5 phosphorylation at these sites by TNFα (supplemental Figure 1C). These results suggest that TNFα-mediated inhibition of ERK5 transactivation is not due to ERK5 SUMOylation or phosphorylation of the TEY motif.

PKCζ associates directly with ERK5

To determine whether PKCζ inhibition involved direct binding to ERK5, we tested their interaction. We cotransfected HeLa cells with HA-tagged PKCζ and Flag-tagged ERK5, immunoprecipitated with Flag antibody, and assayed for HA-PKCζ in the immunoprecipitates (Figure 3A). Under these conditions there was coprecipitation of PKCζ and ERK5. To investigate the interaction between endogenous PKCζ and ERK5, we stimulated HUVECs with TNFα at the indicated times, and the cell lysates were immunoprecipitated with ERK5 antibody (Figure 3B). We found that PKCζ coimmunoprecipitated with ERK5 under basal conditions (T = 0 hours) and that TNFα stimulation did not significantly increase PKCζ-ERK5 interaction (Figure 3B). These results suggest that PKCζ is constitutively associated with ERK5.

To determine the ERK5 binding site for PKCζ, we used a mammalian 2-hybrid assay in HUVECs. Plasmids containing GAL4-DBD and truncated forms of PKCζ were constructed with the use of the pBIND vector. A plasmid containing VP16-ERK5 was constructed with the use of the pACT vector. Transfection of HUVECs with these constructs, and assay for luciferase activity as a measure of interaction, showed that only cells expressing the C-terminal kinase domain (aa405-591) of PKCζ exhibited increased activity (Figure 3C), indicating that this is the ERK5 binding domain. To study further functional interaction, we transfected cells with CA-MEK5α and the truncated PKCζ constructs (Figure 3D). As expected, PKCζ fragment 3 (aa405-591), but not fragment 2 (aa201-400), significantly decreased ERK5 transactivation (Figure 3D).

To determine the PKCζ binding site for ERK5, we cotransfected HUVECs with Gal4-PKCζ and the truncated forms of VP16-ERK5 constructs. The interaction of these fragments was then assayed by luciferase activity. As shown in Figure 3E, we found that the C-terminal domain (aa571-806) of ERK5 was required for PKCζ-ERK5 association. Because this domain has multiple potential protein association motifs, future studies will be required to define the specific regions necessary for interaction.

PKCζ phosphorylates ERK5

To investigate whether PKCζ phosphorylates ERK5 and, if so, to determine the site(s) of phosphorylation, we generated GST-tagged ERK5 fragments and performed in vitro kinase assay with PKCζ. ERK5 fragments consisting of aa1-200 and aa400-600 were highly phosphorylated by PKCζ (Figure 4A). When we used NetPhosK1.0 software to search for possible phosphorylation sites in the ERK5 sequence, 2 serine residues (S31 and S468) were identified as the most likely phosphorylation targets. GST-ERK5 with either S31A or S486A point mutations were used in the in vitro kinase assay. We found that the S486A point mutation ablated PKCζ-mediated ERK5 phosphorylation (Figure 4C), whereas the S31A mutation did not (Figure 4B). These results indicate that PKCζ phosphorylates S486 of ERK5.

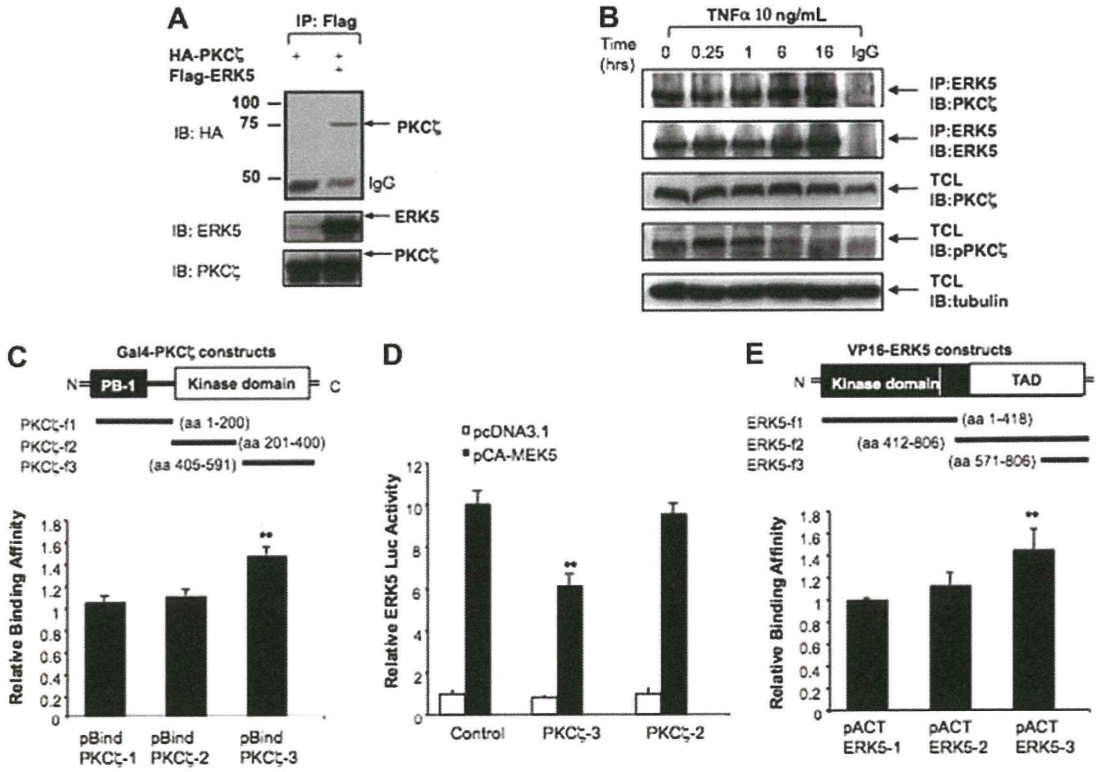

Figure 3. PKCζ interacts with ERK5. (A) ERK5 binds to PKCζ in vitro. HeLa cells were cotransfected with HA-PKCζ and pcDNA3 or Flag-ERK5 for 24 hours and subjected to immunoprecipitation with Flag antibody, followed by Western blot analysis with HA antibody. Expression of ERK5 and PKCζ was detected by Western blotting with specific antibodies. (B) TNFα slightly increases PKCζ-ERK5 binding. Subconfluent cocultures of HUVECs were treated for different time points with TNFα 10 ng/mL. The interaction of endogenous PKCζ with endogenous ERK5 was evaluated by immunoprecipitating 400 μg of total cell lysate with ERK5 antibody, and the immunoprecipitates were analyzed by immunoblotting with PKCζ antibody. Essentially identical results were obtained in 2 other experiments. (C,E) The COOH-terminus regions of PKCζ and ERK5 are critical for the ERK5-PKCζ interaction. HUVECs were transfected with plasmids expressing wild-type VP16-ERK5 with Gal4-PKCζ fragments (C) or wild-type Gal4-PKCζ with VP16-ERK5 fragments (E) as indicated, and luciferase activity was evaluated 24 hours after transfection. (D) PKCζ fragment able to bind ERK5 inhibits ERK5 transactivation. HUVECs were transfected with pBIND-ERK5 and Gal4-dependent (pG5-Luc) reporter gene with or without pcDNA3-CA-MEK5α with PKCζ-3 and PKCζ-2 fragments. Luciferase activity was measured after 24 hours of incubation. Data are mean ± SD of 3 experiments performed in triplicate. $^{**}P < .05$.

Role of PKCζ and PKCζ-mediated ERK5 phosphorylation in the regulation of eNOS protein stability

We showed earlier that s-flow–induced up-regulation of eNOS expression was inhibited by TNFα and that this inhibition was abrogated by expressing Ad.DN-PKCζ (Figure 1C-D), indicating that PKCζ is involved in down-regulating eNOS expression. Indeed, when we cotransfected Chinese hamster ovary cells with eNOS cDNA and the constitutively active PKCζ (CATζ), eNOS expression was dose-dependently inhibited (Supplemental Figure 2A-B), thus confirming the inhibitory role of PKCζ in eNOS expression. Because s-flow has been shown to induce eNOS mRNA expression by activating the ERK5/MEF2/KLF2 pathway[22] and because activation of PKCζ inhibits ERK5 transactivation (Figure 2B), it is possible that TNFα inhibits KLF2 and eNOS promoter activity by activating PKCζ. To test this possibility, HUVECs were transduced with Ad.DN-PKCζ and transfected with eNOS or pKLF2 promoter, exposed to TNFα or s-flow, and promoter activities were assayed by luciferase activity. In control cells, TNFα reduced eNOS and KLF2 promoter activity and mRNA expression under both static and flow conditions (supplemental Figure 3). Interestingly, expression of Ad.DN-PKCζ did not prevent the down-regulation of eNOS and KLF2 promoter activity and mRNA expression. These data suggest that the recovery of eNOS protein expression by Ad.DN-PKCζ shown in Figure 1C may be due to protein stabilization rather than increased transcription.

To determine the mechanism by which PKCζ regulates eNOS stability, we first examined TNFα-mediated eNOS degradation by PKCζ activation. HUVECs were transduced with Ad.LacZ or Ad.DN-PKCζ and treated with cycloheximide (CHX) plus TNFα for 0 to 12 hours as indicated (Figure 5A). Then, eNOS expression levels were analyzed by immunoblotting. In control cells expressing Ad.LacZ, TNFα decreased eNOS expression in the presence of the protein synthesis inhibitor CHX, but cells expressing Ad.DN-PKCζ, which inhibits PKCζ activity, significantly prevented the decrease in eNOS protein levels induced by TNFα (Figure 5B,D). These experiments show that PKCζ negatively regulates eNOS protein levels. Next, to determine the role of PKCζ-mediated ERK5 phosphorylation on eNOS stability, we transduced HUVECs with Ad.ERK5-WT or Ad.ERK5-S486 and repeated the same experiments described above. The expression of ERK5-WT and ERK5-S486A mutant was comparable throughout the time course of the experiment (Figure 5C second panel from top). In cells transduced with Ad.ERK5-WT, endogenous eNOS expression decreased after 12 hours of TNFα and CHX treatment. In contrast, in cells expressing the Ad.ERK5-S486A mutant, TNFα and CHX treatment did not significantly decrease eNOS expression by 12 hours (Figure 5C,E). These results suggest that the TNFα-mediated eNOS destabilization depends on PKCζ-mediated ERK5 phosphorylation.

In situ localization of PKCζ and ERK5 in ApoE$^{-/-}$ mouse aorta

Importantly, increased PKCζ activation at disturbed flow (d-flow) areas such as the lesser curvature of the aorta has been observed.[17]

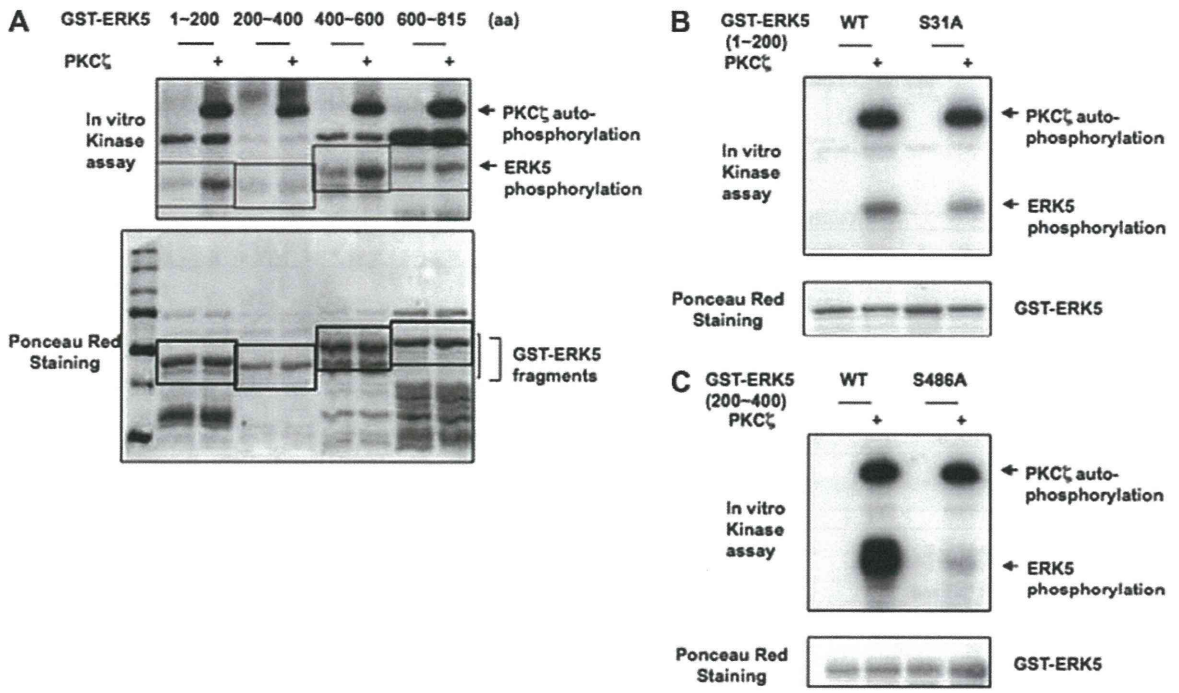

Figure 4. PKCζ directly phosphorylates ERK5 in vitro. (A) To determine direct PKCζ-induced ERK5 phosphorylation, we performed an in vitro kinase assay with 4 different GST-ERK5 fragments as substrate. In vitro kinase assay shows ^{32}P incorporation into 2 GST-ERK5 fragments (amino acids 100 ~ 200 and 400 ~ 600) but not into the others (amino acids 200 ~ 400 and 600 ~ 816). Ponceau staining shows the position of the proteins after separation by SDS-PAGE and near equal expression. (B-C) Characterization of PKCζ phosphorylation sites. In vitro kinase assay was performed with ERK5-WT fragment (aa1-200 or aa400-600) and ERK5 fragment with S31A (B) or S486A (C) mutations in the presence of recombinant PKCζ. Mutation of the serine 486 to alanine in GST-ERK5 200-600 ablated PKCζ-mediated ERK5 phosphorylation. Data presented are from a representative experiment of at least 3 independent experiments.

This athero-prone area is characterized by d-flow in contrast to the relatively protected greater curvature that is characterized by s-flow. Our data provide a novel insight into the mechanism by which eNOS expression is reduced in areas of d-flow as reported.[30] Our current study predicts that eNOS degradation in areas of d-flow is the result of phosphorylation of ERK5 by PKCζ. To further elucidate the importance of PKCζ activation in eNOS degradation, we studied an atherosclerotic disease model, the ApoE$^{-/-}$ C57BL/6J mouse. En face preparations of aortas were immunostained for p-PKCζ (Thr410) and PECAM-1 (as an EC marker) and studied with a confocal laser scanning microscope. Low-magnification (4×) images of representative mouse aortas are shown in Figure 6A. There was increased staining for p-PKCζ in atherosclerotic lesions present in the athero-prone region (lesser curvature) compared with athero-protected region (greater curvature). Increased magnification (10×) images show that p-PKCζ was specifically increased in ECs that exhibited abnormal morphology characterized by increased size, loss of polygonal shape, and appearance of gaps between cells (Figure 6B). p-PKCζ appeared to be localized to the perinuclear area and did not colocalize with PECAM-1 (Figure 6B). A direct comparison of the athero-prone region (Figure 6C) with the athero-protected region (Figure 6D) showed that cells with abnormal morphology were the ones that most highly expressed p-PKCζ. Analysis of PECAM-1 showed that it was also more highly expressed in the d-flow region (lesser curvature) than in the s-flow-region (greater curvature). This result is consistent with a recent study indicating that PECAM-1 contributes to atherosclerosis lesion formation in regions of d-flow in ApoE$^{-/-}$ mice.[34]

Unfortunately, we were unable to develop an antibody that could detect phospho-S486 ERK5, so we studied total ERK5 expression. Analysis of ERK5 expression showed that it was also more highly expressed in athero-prone regions compared with athero-protected regions (Figure 6E). Higher magnification images (Figure 6F) showed that cells that highly expressed ERK5 were the same cells that exhibited abnormal morphology and appeared to overlap with those that highly expressed p-PKCζ (compare Figure 6F with 6B). Interestingly ERK5 appeared to be cytoplasmic as well as nuclear in both normal- and abnormal-appearing ECs (Figure 6G). Direct comparison of the athero-prone region (Figure 6G) to the athero-protected region (Figure 6H) showed no obvious difference in ERK5 subcellular localization. There was clearly a significant increase in ERK5 expression in the d-flow region that corresponded to the increase in p-PKCζ (compare Figure 6G with 6C). These data suggest a potential proatherogenic role for endothelial PKCζ activation and PKCζ-mediated phosphorylation of ERK5.

Discussion

The main finding of the present study is that activation of PKCζ phosphorylates ERK5 and by inhibiting ERK5 function decreases eNOS protein expression in ECs. Specifically, we demonstrated that PKCζ binds and phosphorylates ERK5 at S486, and these events are required to increase eNOS protein degradation (Figure 7). Furthermore, we observed in vivo that PKCζ activity was up-regulated in athero-prone regions of the mouse aorta exposed to d-flow. These results define a new mechanism for endothelial dysfunction and atherosclerosis progression.

PKCζ activity was shown to be up-regulated in the d-flow region of the pig aorta by Magid and Davies,[17] but the mechanism by which PKCζ contributed to atherosclerosis susceptibility was not described. Previously, we found that PKCζ mediated TNFα-dependent EC apoptosis and was required for activation of

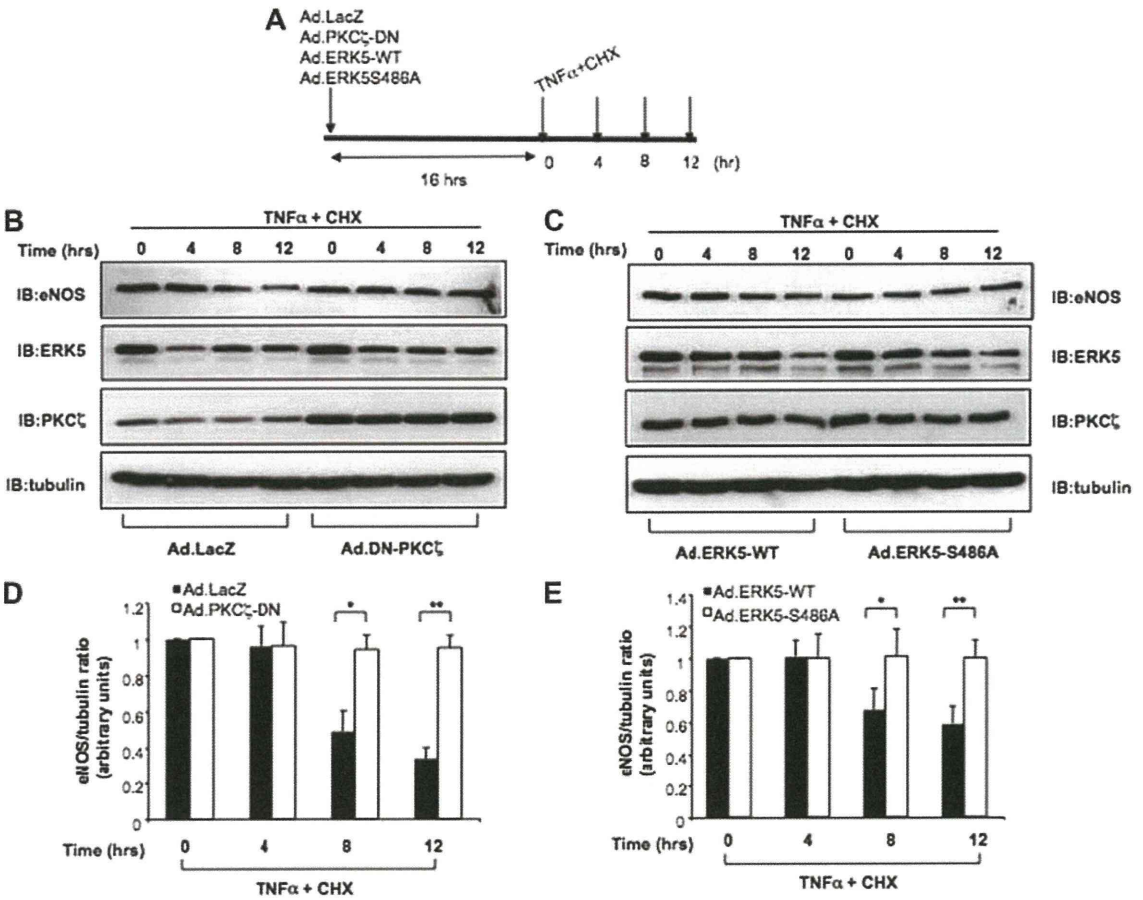

Figure 5. Role of PKCζ in eNOS protein stability. (A) Schematic diagram showing experimental protocol. (B-C) Ad.DN-PKCζ and Ad.ERK5-S486A increase eNOS protein stability. HUVECs were infected with Ad.LacZ (control) or Ad.DN-PKCζ (B) and Ad.ERK5-WT or Ad.ERK5-S486A (C). Sixteen hours later, the cells were incubated with TNFα 10 ng/mL plus the protein synthesis inhibitor cycloheximide (CHX; 10 μg/mL). HUVECs were harvested after 0, 4, 8 and 12 hours of treatment, and Western blots were performed with eNOS, PKCζ, ERK5, and tubulin antibodies. (D-E) Western blots were quantified by densitometry by setting the time zero to 1.0. Data presented are from a representative experiment of at least 3 independent experiments. $^{*}P < .1$; $^{**}P < .05$.

caspase-3.[16] Recently, eNOS expression was shown to be positively regulated in ECs by a MEK/ERK5/KLF2 pathway.[22] Because we demonstrated that ERK5 was activated by s-flow and protected ECs from apoptosis,[35] we hypothesized that PKCζ might negatively regulate ERK5 and thereby decrease eNOS expression. Furthermore, because both PKCζ and MEK5 contain a PB1 domain, a well-characterized protein-protein interaction domain, we expected that PKCζ inhibition might be by a direct effect on MEK5. However, we found that PKCζ bound directly to ERK5. Furthermore, the ERK5 binding site was within the catalytic domain of PKCζ, not the PB1 domain. Interestingly, we found that ERK5 is not only a binding partner of PKCζ but is also a substrate of this kinase. Our mutational analysis showed that PKCζ phosphorylates ERK5 at a previously uncharacterized phosphorylation site (S486). Phosphorylation of S486 was necessary for the decrease in eNOS protein stability, although the precise mechanism will require future studies.

We used TNFα as an agonist to stimulate ECs because TNFα has been shown to play an important role during the inflammatory process of atherosclerosis. In particular, TNFα deficiency retards fatty-streak lesion formation by down-regulating the expression of proatherogenic inflammatory factors.[36] TNFα has been detected in atherosclerotic lesions throughout all stages of human atherosclerosis[33,37] and was found to be associated with atherosclerosis in mouse models.[38,39] Mice deficient in both ApoE$^{-/-}$ and TNFα displayed less advanced atherosclerosis than ApoE$^{-/-}$ mice.[38,40] In addition, many features of EC dysfunction are mimicked by the inflammatory cytokine TNFα. For example, TNFα stimulation activates PKCζ,[15] induces adhesion molecule expression,[41] and decreases eNOS expression.[7-9] The mechanism for TNFα-mediated inhibition of eNOS expression has been thought to be primarily transcriptional.[7,8] However, a role for a decrease in mRNA stability has also been elucidated.[9] Surprisingly, our data predict that a posttranslational modification of eNOS protein by PKCζ targets eNOS for degradation. Several mechanisms have been proposed for eNOS degradation with calcium-dependent calpain-mediated degradation being most prominent.[42,43] Further studies will be required to define the molecular nature of the PKCζ-ERK5 pathway described here.

To understand the importance of the proposed PKCζ-ERK5 mechanism during early atherosclerotic events, we performed en face staining of aortas from ApoE$^{-/-}$ mice. Specifically, we showed that endothelial PKCζ phosphorylation and activation were increased in the athero-susceptible region of the aortic arch (d-flow region). In the in vivo system of the chow fed ApoE$^{-/-}$ mouse, the endothelium in the lesser curvature of the aorta had distinctively large ECs filled with vacuoles that were positive for p-PKCζ immunostaining. These EC changes, which were probably a consequence of intimal leukocyte accumulation, were not observed in regions that experienced s-flow. Note that the expression of ERK5, which has athero-protective effects, was also up-regulated in the same ECs that showed the highest level of p-PKCζ,

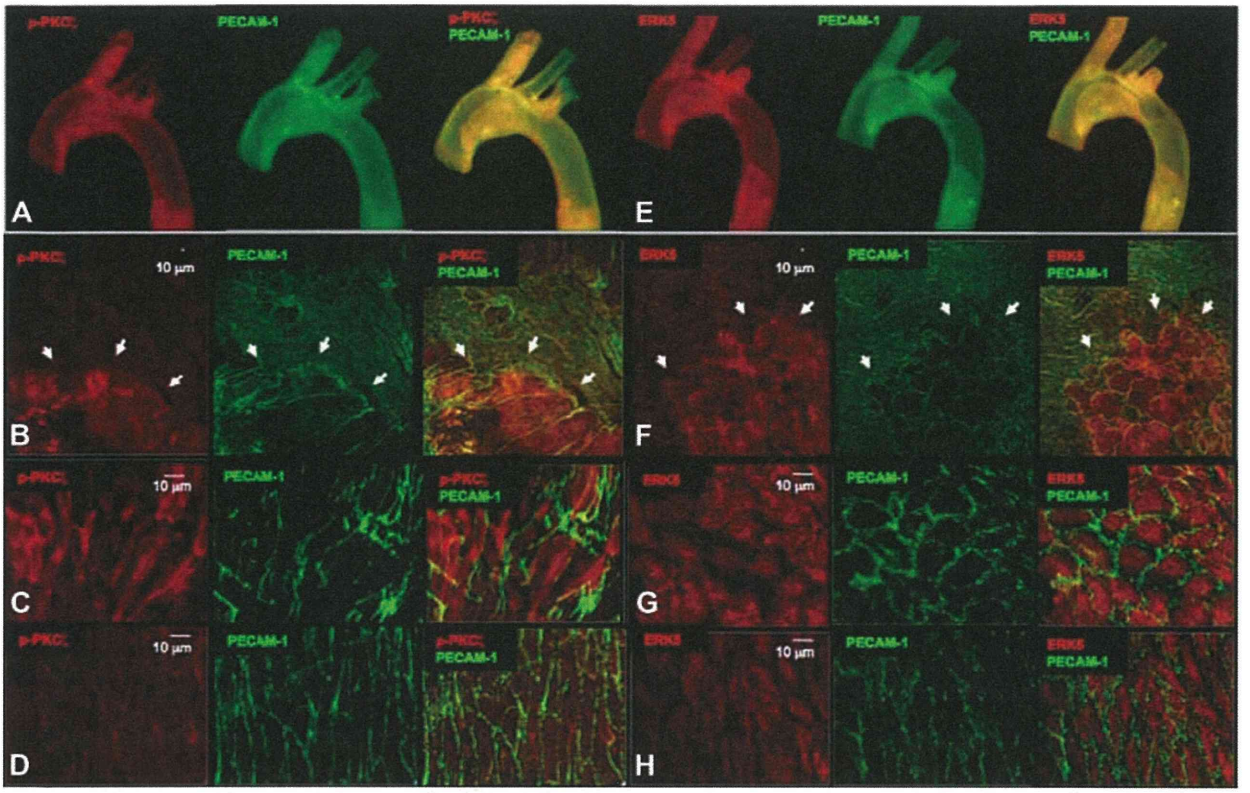

Figure 6. PKCζ activation and ERK5 expression in the ApoE$^{-/-}$ mouse. Aortas from 12-week-old ApoE$^{-/-}$ mice were harvested for qualitative analysis of PKCζ activation (A-D red) and ERK5 expression (E-H red). The differential PKCζ activation or ERK5 expression is evident in the images of the whole aortic mount (4× lens; A,E), in the en face analysis of the aortic arch (20× lens; B,F), and specifically in the athero-prone (C,G; 60× lens) and athero-protected (D, H; 60× lens) areas of the aortic arch. EC morphology was changed in the early atherosclerosis regions (B,F; arrowheads) where ECs are stretched and may have lost PECAM-1 staining (green) at some cell junctions. Bar = 10 μm.

suggesting a potential compensatory effect. This finding is consistent with the observations of Passerini et al[44] who observed by microarray analysis of athero-prone regions that both atherosclerosis-susceptible (proinflammatory, prothrombotic) and atherosclerosis-protective (antioxidant and antithrombotic) gene expression were up-regulated.

In conclusion, we believe that PKCζ is a proatherogenic effector in ECs and that it could be an innovative therapeutic target to improve endothelial dysfunction.

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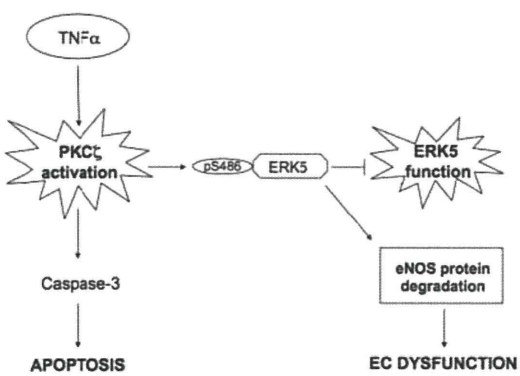

Figure 7. A scheme describing the PKCζ-mediated cross talk between the TNFα (proinflammatory) and ERK5 (anti-inflammatory) pathways.

Authorship

Contribution: P.N. contributed to the design of the experiments, performed the experiments, and generated the manuscript and figures; J.A. and K.F. contributed to discussions and design of experiments and critically edited the paper; C.-H.W. helped the design of the experiments and performed experiments; K.S., H.L., J.-D.L., and K.-S.H. contributed to discussions; C.M. and J.-H.L. performed experiments; M.R.O. performed mouse colony management and genotyping; and B.C.B. supervised the project, contributed to the design of the experiments, and edited the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Cyclophilin A enhances vascular oxidative stress and the development of angiotensin II–induced aortic aneurysms

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Inflammation and oxidative stress are pathogenic mediators of many diseases, but molecules that could be therapeutic targets remain elusive. Inflammation and matrix degradation in the vasculature are crucial for abdominal aortic aneurysm (AAA) formation. Cyclophilin A (CypA, encoded by *Ppia*) is highly expressed in vascular smooth muscle cells (VSMCs), is secreted in response to reactive oxygen species (ROS) and promotes inflammation. Using the angiotensin II (AngII)-induced AAA model in $Apoe^{-/-}$ mice, we show that $Apoe^{-/-}Ppia^{-/-}$ mice are completely protected from AngII–induced AAA formation, in contrast to $Apoe^{-/-}Ppia^{+/+}$ mice. $Apoe^{-/-}Ppia^{-/-}$ mice show decreased inflammatory cytokine expression, elastic lamina degradation and aortic expansion. These features were not altered by reconstitution of bone marrow cells from $Ppia^{+/+}$ mice. Mechanistic studies showed that VSMC-derived intracellular and extracellular CypA are required for ROS generation and matrix metalloproteinase-2 activation. These data define a previously undescribed role for CypA in AAA formation and suggest CypA as a new target for treating cardiovascular disease.

Inflammation and oxidative stress are pathogenic mediators of many diseases, especially cardiovascular diseases. In the vasculature, AAA formation requires inflammation and matrix degradation. Key mechanisms for AAA formation include VSMC senescence[1], oxidative stress[2,3], increased local production of proinflammatory cytokines[4] and increased activities of matrix metalloproteinases (MMPs)[5,6]. In experimental animal models of AAA, genetic and pharmacological inhibition of ROS production[7,8] and of MMPs[9,10] suppress aneurysm formation. A strong mechanistic link exists between increased ROS production and MMP activity[11–13], suggesting that therapies to limit ROS generation may be useful.

AngII induces ROS production through activation of NADPH oxidases[14] and activates MMPs[15]. AngII infusion into apolipoprotein E–deficient ($Apoe^{-/-}$) mice for 4 weeks promotes AAA formation[16,17].

CypA is a chaperone protein that binds cyclosporine[18] and is abundantly expressed in VSMCs[19]. We have shown that ROS stimulate secretion of CypA from VSMCs[19,20]. Extracellular CypA stimulates VSMC migration and proliferation[19,20], endothelial cell adhesion molecule expression and inflammatory cell chemotaxis[19,21,22]. Given these activities of CypA, we sought to determine its role in AngII-induced AAA[23,24]. We found that AAA formation in the AngII-induced $Apoe^{-/-}$ mouse model was completely prevented in the $Ppia^{-/-}$ genetic background. Mechanistically, CypA deficiency resulted in substantially decreased inflammatory cell recruitment, ROS production and MMP activation.

RESULTS

CypA deficiency blocks AngII-induced AAA formation *in vivo*

As previously reported[4,16,24,25], we found that treatment with AngII for 4 weeks promotes AAA formation in $Apoe^{-/-}$ mice (**Fig. 1a–c**). To determine the role of CypA in AAA formation, we established $Apoe^{-/-}Ppia^{-/-}$ (double-knockout) mice and treated them with AngII for 4 weeks. AngII treatment increased systolic blood pressure and total cholesterol levels, but these parameters did not differ between $Apoe^{-/-}$ mice and $Apoe^{-/-}Ppia^{-/-}$ mice (**Supplementary Table 1a** online). There were no gross differences in morphology between the aortas of control $Apoe^{-/-}$ and $Apoe^{-/-}Ppia^{-/-}$ mice (saline-infused mice, **Fig. 1a**). Notably, after AngII infusion, none of the $Apoe^{-/-}Ppia^{-/-}$ mice developed AAA, in contrast to 78% AAA incidence in $Apoe^{-/-}$ mice (**Fig. 1a,b**). There was also a significant decrease in maximal aortic diameter (**Fig. 1c**) and aortic weight (**Supplementary Table 1a**) in AngII-treated $Apoe^{-/-}Ppia^{-/-}$ mice compared to AngII-treated $Apoe^{-/-}$ mice. These results suggest that CypA is required for AAA formation induced by AngII.

Morphologically, the aortas of $Apoe^{-/-}Ppia^{-/-}$ mice infused with saline (**Fig. 1d–f**) did not differ from those of control $Apoe^{-/-}$ mice (data not shown). In $Apoe^{-/-}$ mice infused with AngII (**Fig. 1d–f**), there was a marked increase in the sizes of both the aortic lumen and wall. The aortic wall developed a tissue mass composed of organized thrombus, small blood vessels, extracellular matrix and spindle-shaped cells, as described previously[24]. Most of the cells that expressed CypA (**Fig. 1e**) concomitantly showed immunoreactivity for α-smooth muscle actin (α-SMA, **Fig. 1f**), suggesting that they were VSMCs[19].

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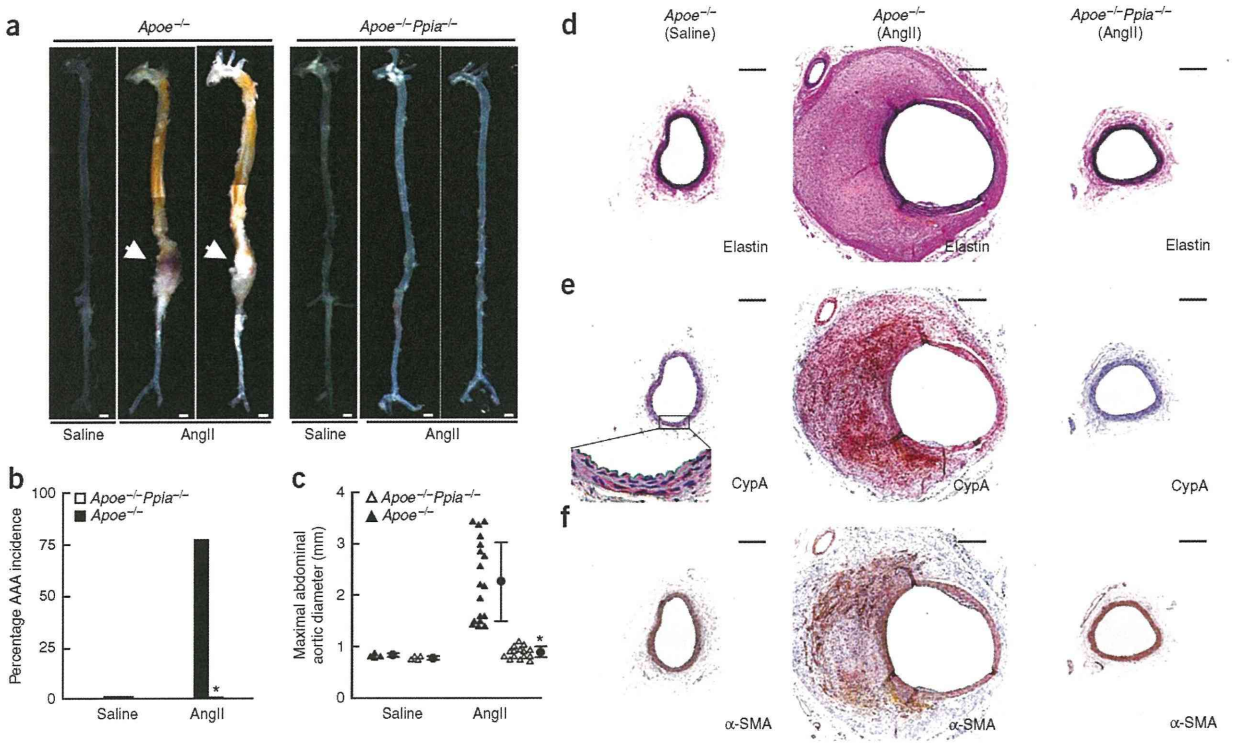

Figure 1 CypA deficiency prevents AngII-induced AAA formation. *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice were infused with AngII or saline for 4 weeks. (**a**) Representative photographs showing macroscopic features of aneurysms induced by AngII. The arrows indicate typical AAAs in *Apoe*$^{-/-}$ mice. Scale bars, 1 mm. (**b**) The incidence of AngII-induced AAA in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice ($n = 15$) compared with *Apoe*$^{-/-}$ mice ($n = 18$). There was no AAA formation in the control group (saline infusion) in both *Apoe*$^{-/-}$ ($n = 4$) and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice ($n = 4$). $^*P < 0.01$ compared with AngII-infused *Apoe*$^{-/-}$ mice. (**c**) Maximal abdominal aortic diameter in *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice after AngII infusion for 4 weeks. Triangles represent individual mice; circles represent the mean; error bars denote s.d. $^*P < 0.01$ compared with AngII-infused *Apoe*$^{-/-}$ mice. (**d**) Elastin van Gieson staining of aortic cross-sections of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice after AngII infusion for 4 weeks. (**e**,**f**) Representative immunostaining for CypA (**e**) and α-smooth muscle actin (α-SMA) (**f**) in serial sections. All aortic sections were from suprarenal aortas. Scale bars for **d**–**f**, 300 μm.

In contrast, the aortas of *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice infused with AngII showed no substantial increase in aortic wall thickness (**Fig. 1d–f**). These results suggest that CypA deficiency confers protection from the early stages of AAA formation.

Over the 4 weeks of the experiment, 35% of the *Apoe*$^{-/-}$ mice infused with AngII died, whereas none of the *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice did (**Supplementary Fig. 1a** online). Gross and histological examination of the dead mice revealed aortic rupture (**Supplementary Fig. 1b–d**). As expected, the elastic lamina was frequently disrupted and degraded in *Apoe*$^{-/-}$ mice (**Supplementary Fig. 1e**). In contrast, CypA deficiency completely prevented elastic lamina degradation (**Supplementary Fig. 1f**). On the basis of a semiquantitative analysis of elastin degradation (**Supplementary Fig. 1g**), CypA deficiency completely blocked elastin degradation after AngII treatment for 4 weeks (**Supplementary Fig. 1h**). These data suggest that protection from elastin degradation is a major mechanism for the inhibition of AAA formation in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice.

To ascertain whether AngII-induced vascular inflammation is CypA dependent, we examined inflammatory cell migration and microvessel formation. Inflammatory cell migration, as assessed by CD45$^+$ cell number, was significantly reduced in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice compared with *Apoe*$^{-/-}$ mice (**Fig. 2a–c**). The number of microvessels in the aortic wall was also markedly lower in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (**Fig. 2d–h**), consistent with the reduced inflammatory response.

To characterize the mechanisms by which CypA participates in the inflammatory response, we first analyzed the secretion of proinflammatory cytokines and chemokines *in vitro*. AngII treatment of VSMCs strongly induced the secretion of proinflammatory cytokines such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-6, as well as chemokines such as regulated CC motif chemokine ligand-5 (CCL5) and stromal cell–derived factor-1, whereas secretion of these factors was effectively blocked by CypA deficiency (**Supplementary Fig. 2a** online). We next showed that AngII stimulated CypA secretion in mouse aortic VSMCs (**Fig. 2i**). CypA secretion was maximal at 1 μM AngII (**Fig. 2i**). Pretreatment with Y27632 (a Rho kinase inhibitor) or simvastatin markedly reduced CypA secretion (**Fig. 2i**), which is consistent with our previous report[21]. We studied MCP-1 expression in the aortic wall because of the known role of MCP-1 in macrophage migration and AAA formation[24,26]. In the medial layer of saline-infused aortas, MCP-1 was more highly expressed in *Apoe*$^{-/-}$ aortas compared to *Apoe*$^{-/-}$*Ppia*$^{-/-}$ aortas (**Supplementary Fig. 2b,c**). MCP-1 was highly expressed in the aortas of AngII-treated *Apoe*$^{-/-}$ mice (**Supplementary Fig. 2d**), especially in the adventitia, but was not as highly expressed in aortas from AngII-treated *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (**Supplementary Fig. 2e**). The adventitial location of MCP-1 expression in response to AngII treatment is consistent with the function of MCP-1 as a chemoattractant for monocytes. Additionally, in cultured aortic VSMCs, AngII-stimulated MCP-1

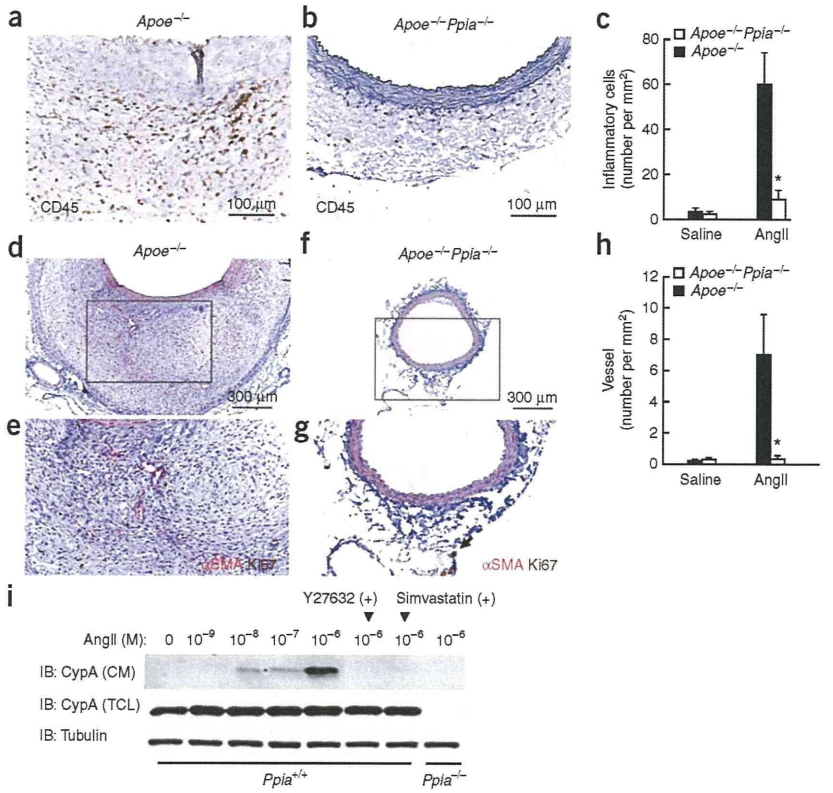

Figure 2 CypA deficiency reduces AngII-induced inflammatory cell accumulation and microvessel formation. (**a**,**b**) Representative CD45 staining of suprarenal aortas from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice infused with AngII for 4 weeks. (**c**) Number of migrating CD45^{+} cells in the aortic wall in *Apoe*$^{-/-}$ ($n = 9$) and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ ($n = 7$) mice. *$P < 0.01$ compared with *Apoe*$^{-/-}$ mice. (**d**–**g**) Representative immunostaining of α-smooth muscle actin (α-SMA) and Ki67 (a marker for proliferation) in suprarenal aortas from *Apoe*$^{-/-}$ (**d**,**e**) and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ (**f**,**g**) mice infused with AngII for 4 weeks. **e** and **g** are higher magnification images of the boxed areas in **d** and **f**, respectively. (**h**) Number of proliferating microvessels in the aortic wall. *$P < 0.01$ compared with *Apoe*$^{-/-}$ mice. (**i**) Immunoblots to assess the secretion of CypA from mouse VSMCs in response to AngII with or without pretreatment by the Rho kinase inhibitor Y27632 (30 μmol l^{-1}) or simvastatin (30 μmol l^{-1}) for 30 min. The experiment was repeated three times. IB, immunoblot; CM, conditioned medium; TCL, total cell lysate.

secretion was markedly decreased in *Ppia*$^{-/-}$ cells compared to *Ppia*$^{+/+}$ cells (**Supplementary Fig. 2f**), whereas other AngII signaling events such as activation of extracellular signal–related kinases 1 and 2 (commonly referred to as ERK1/2) did not differ.

Vascular CypA is essential for AAA formation

CypA has been reported to have a crucial role in regulating the survival, proliferation and differentiation of antigen-presenting cells by augmenting antigen uptake and presentation[27]. CypA has also been reported to stimulate migration of bone marrow–derived cells *in vitro*[22]. Hematopoietic cells, especially macrophages, are involved in AAA formation[4,24]. We hypothesized that CypA deficiency may impair macrophage differentiation and activation and thus prevent AAA formation by AngII. To test this possibility, we transplanted *Apoe*$^{+/+}$*Ppia*$^{+/+}$ GFP^{+} bone marrow cells into irradiated *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice. After 42 d of engraftment, we treated the mice with AngII. There was no significant difference in the reconstitution ratio (percentage of GFP^{+} cells in the peripheral blood) in GFP^{+} marrow–transplanted *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice compared with GFP^{+} marrow–transplanted *Apoe*$^{-/-}$ mice (99.5 ± 0.3% versus 99.6 ± 0.2%, respectively). There was no significant difference in the blood pressure among the transplanted mice groups (**Supplementary Table 1b**). However, the number of bone marrow–derived inflammatory cells (GFP^{+}CD45^{+} double-positive cells) in the aortic wall was significantly lower in chimeric AngII-treated *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice compared with chimeric AngII-treated *Apoe*$^{-/-}$ mice (**Fig. 3a–c**). We also measured the number of GFP^{+}CD45^{-} cells in AAA lesions after AngII infusion. The number of GFP^{+}CD45^{-} cells in the aortic wall was significantly lower in chimeric AngII-treated *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice compared with chimeric AngII-treated *Apoe*$^{-/-}$ mice (15.2 ± 15.3 cells per mm^2 versus 60.9 ± 28.9 cells per mm^2, respectively, $P < 0.05$). Recent studies have shown that both nonhematopoietic cells (CD45^{-}) and hematopoietic cells (CD45^{+}) are mobilized from the bone marrow and contribute to remodeling of the vascular wall[28]. The presence of GFP^{+}CD45^{-} cells in AngII-induced AAA lesions suggests that CypA has a crucial role in recruiting nonhematopoietic cells from the bone marrow. The number of bone marrow–derived macrophages (GFP^{+}Mac-1^{+}) found in AAA lesions was also significantly lower in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ recipient mice (**Supplementary Fig. 3a–c** online).

We frequently observed migration of bone marrow–derived cells into the media of the aorta in *Apoe*$^{-/-}$ recipient mice (**Fig. 3d**). In contrast, there were few GFP^{+} cells in the aortic media of *Apoe*$^{-/-}$*Ppia*$^{-/-}$ recipient mice (**Fig. 3e**,**f**), suggesting the importance of VSMC-derived CypA for inflammatory cell migration. Furthermore, microvessel density, as assessed by platelet/endothelial cell adhesion molecule-1 (PECAM-1) staining, was significantly reduced in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ recipient mice (**Fig. 3d**,**e**,**g**), supporting the concept that the decrease in inflammatory responses in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice is due to CypA deficiency in vascular cells. Consistent with this idea, the incidence of AAA was 56% in *Ppia*$^{+/+}$ marrow–transplanted *Apoe*$^{-/-}$ mice versus 0% in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice after transplantation of *Ppia*$^{+/+}$ bone marrow cells (**Fig. 3h**). Finally, we generated chimeric mice with *Ppia*$^{-/-}$ bone marrow (**Supplementary Fig. 3d**). The incidence of AAA was 60% in *Ppia*$^{-/-}$ marrow–transplanted *Apoe*$^{-/-}$ mice, similar to that in nontransplanted *Apoe*$^{-/-}$ mice, and the incidence of AAA was 0% in *Ppia*$^{-/-}$ marrow–transplanted *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice, as it was in nontransplanted *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (**Supplementary Fig. 3d**). Taken together, these data suggest that CypA expression by vascular cells, rather than bone marrow–derived cells, is crucial for the development of AAA.

CypA deficiency prevents AngII-induced MMP activation

AAA development and aortic rupture depend on macrophage-derived MMP-9 and VSMC-derived MMP-2 (refs. 24,29,30), which are enzymatically cleaved and activated by membrane type-1 MMP

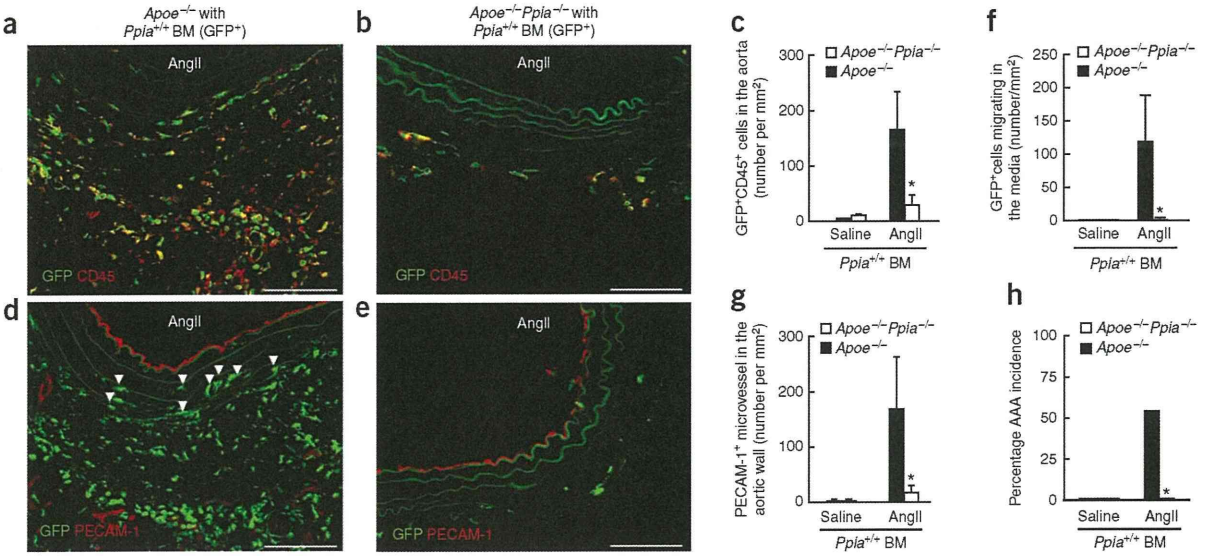

Figure 3 Bone marrow (BM) reconstitution shows a key role for vascular-derived CypA in AAA formation. *Ppia*$^{+/+}$ BM cells (GFP$^+$) were transplanted into irradiated *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice as described in the **Supplementary Methods** online. (**a**,**b**) Representative CD45 staining (Alexa Fluor 546, red) of suprarenal aortas from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice transplanted with *Ppia*$^{+/+}$ BM and infused with AngII for 4 weeks. (**c**) Number of GFP$^+$CD45$^+$ double-positive cells in the aortic wall in *Apoe*$^{-/-}$ ($n = 9$) and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ ($n = 8$) mice. (**d**,**e**) Representative PECAM-1 staining (Alexa Fluor 546, red) of suprarenal aortas from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice transplanted with *Ppia*$^{+/+}$ BM and infused with AngII for 4 weeks. Elastic lamina in the aortic wall shows green autofluorescence. Arrowheads in **d** indicate GFP$^+$ cells in the media. (**f**,**g**) Quantification of GFP$^+$ cells in the media (**f**) or PECAM-1$^+$ microvessels (**g**) in the aortic walls of *Apoe*$^{-/-}$ ($n = 9$) compared to *Apoe*$^{-/-}$*Ppia*$^{-/-}$ ($n = 8$) mice. (**h**) The incidence of AAA in *Apoe*$^{-/-}$ ($n = 9$) mice compared to *Apoe*$^{-/-}$*Ppia*$^{-/-}$ ($n = 8$) mice reconstituted with *Ppia*$^{+/+}$ bone marrow after AngII infusion for 4 weeks. $^*P < 0.01$ compared with *Apoe*$^{-/-}$ mice. Scale bars, 100 μm.

(MT1-MMP)[31]. Secreted CypA may activate MMPs through the extracellular MMP protein inducer (EMMPRIN)[32]. Therefore, we anticipated that MMP activity would be decreased in the absence of CypA. We performed western blotting for MMP-2 using a MMP-2–specific mouse monoclonal antibody that recognizes the 72-kDa latent and the 66-kDa active forms of MMP-2. We observed markedly reduced MMP-2 activity in AngII-treated *Ppia*$^{-/-}$ VSMCs compared to wild-type (WT, *Ppia*$^{+/+}$) VSMCs (**Supplementary Fig. 4a** online). MT1-MMP abundance in the membrane fraction was substantially increased in AngII-treated WT VSMCs compared to AngII-treated *Ppia*$^{-/-}$ VSMCs (**Supplementary Fig. 4b**), suggesting a key role for CypA in MT1-MMP translocation to the cell membrane. Consistent with these findings, AngII-induced activation of MT1-MMP was significantly ($P < 0.01$) elevated in WT VSMCs compared with *Ppia*$^{-/-}$ VSMCs (**Supplementary Fig. 4c**).

We next studied MMP levels in the aortas of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice. Basal expression of MT1-MMP was low in the aortas of mice of both genotypes (**Fig. 4a**). After AngII infusion, MT1-MMP expression was considerably increased in the aortas of both *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice, but this increase was markedly attenuated in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (**Fig. 4a**). In organ culture, conditioned medium obtained from aortas from AngII-treated *Apoe*$^{-/-}$ mice showed high levels of proMMP-9, proMMP-2 and activated MMP-2 by zymography (**Fig. 4b**). In contrast, conditioned medium from aortas from AngII-treated *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice lacked MMP expression or activity (**Fig. 4b**). *In situ* zymography supported these observations: MMP activity, which was negligible in saline-treated aortas, was much higher in the medial and adventitial layers of AngII-treated aortas from *Apoe*$^{-/-}$ mice as compared with *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (**Fig. 4c**,**d**). Notably, the ruptured aortas of *Apoe*$^{-/-}$ mice revealed a tremendously high level of MMP activity, especially in the false lumen (**Fig. 4c**).

To compare the properties of VSMCs in AAA-prone versus AAA-resistant areas, we collected and cultured VSMCs from the thoracic, suprarenal and infrarenal aortas and measured MMP activities in response to treatment with AngII (**Fig. 4e**). In cells from aortas treated with saline, there was no difference among the different types of VSMCs in MMP-2 activity, as assessed by gelatin zymography (**Fig. 4e**). AngII treatment substantially increased MMP-2 activity in *Apoe*$^{-/-}$ VSMCs, especially in VSMCs from the suprarenal aorta (**Fig. 4e**). In contrast, the induction of MMP-2 activity by AngII was substantially attenuated in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ VSMCs, regardless of the aortic location (**Fig. 4e**). Treatment of VSMCs with CypA augmented MMP activity by approximately twofold, as assessed by *in situ* zymography (**Fig. 4f**,**g**), demonstrating the importance of extracellular CypA for MMP activation in VSMCs. Consistent with these data, *in situ* zymography showed that active MMP expression was much greater in the media of suprarenal aorta than in that of infrarenal or thoracic aorta in AngII-treated mice (**Supplementary Fig. 5a–c** online). These *in vivo* and *in vitro* data demonstrate that CypA in VSMCs is crucial for activation of MMPs.

CypA deficiency prevents AngII-induced ROS production

We next investigated the mechanism by which CypA deficiency decreases MMP expression, secretion and activation. ROS have a crucial role in activating VSMC MMPs[33] via a pathway dependent upon the p47^{phox}-containing NADPH oxidase[34]. Therefore, we studied the effect of CypA deficiency on VSMC ROS production induced by AngII. Activation of ERK1/2 by AngII was not markedly different between *Ppia*$^{+/+}$ and *Ppia*$^{-/-}$ VSMCs (data not shown). In response

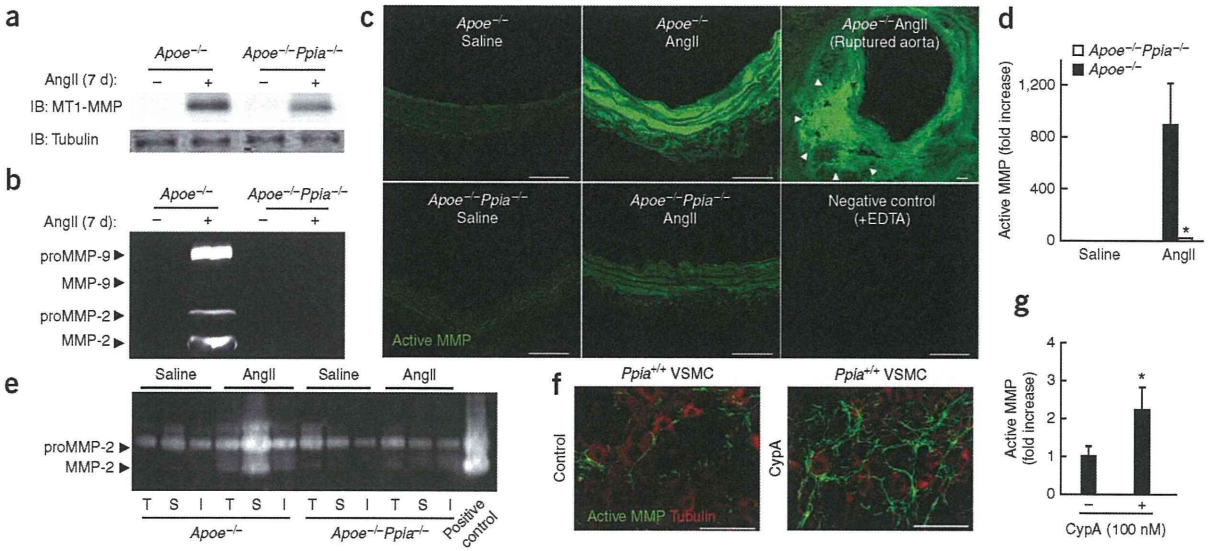

Figure 4 CypA is crucial for secretion and activation of MMPs. (**a**) Representative western blot of MT1-MMP expression in aortas of mice of the indicated genotypes after infusion of AngII for 7 d. $n = 3$ per group. (**b**) Gelatin zymography of conditioned medium from whole-aorta organ culture. Aortas from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice infused with saline or AngII ($n = 3$ per group) were incubated in culture medium for 20 h. (**c**) *In situ* zymography for gelatinase activity. Aortas from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice infused with saline or AngII for 7 d were analyzed. Arrowheads point to regions where the aorta has ruptured, as defined by destruction of the medial layer. Scale bars, 100 μm. (**d**) Densitometric analysis of MMP activity by DQ gelatin (a fluorogenic substrate used to detect protease activity) in aortas from *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice relative to that in control *Apoe*$^{-/-}$ mice (saline-infused). Addition of EDTA blocks the reaction and is used as a negative control. $n = 6$ per group. $^*P < 0.01$ compared with *Apoe*$^{-/-}$ mice. (**e**) Gelatin zymography to detect proMMP-2 and MMP-2 in VSMCs collected separately from the thoracic aorta (T), suprarenal aorta (S) and infrarenal aorta (I) of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice. All VSMCs were stimulated with saline or AngII (1 μM) for 24 h. Positive control, recombinant MMP-2. $n = 3$ per group. (**f**) Representative *in situ* zymography (DQ gelatin) and immunostaining to detect α-tubulin in *Ppia*$^{+/+}$ VSMCs after stimulation with CypA (100 nM) for 4 h. Control, saline. Scale bars, 30 μm. (**g**) Densitometric analysis of MMP activity relative to that in control VSMCs. $^*P < 0.01$ versus control VSMCs. Results are means ± s.d. of six independent experiments.

to treatment with AngII for 4 h, ROS production in *Ppia*$^{+/+}$ mouse VSMCs, as assessed by dichlorofluorescein staining, increased by 12-fold (**Fig. 5a,b**). *Ppia*$^{-/-}$ VSMCs showed significantly less ROS induction (**Fig. 5a,b**). Furthermore, treatment of *Ppia*$^{+/+}$ VSMCs with CypA for 4 h significantly ($P < 0.01$) augmented ROS production (**Fig. 5c,d**), suggesting that CypA secretion induced by AngII promotes ROS production.

To evaluate the effect of CypA deficiency on ROS generation *in vivo*, we incubated aortic sections with dihydroethidium, which, in the presence of superoxide, forms the highly fluorescent molecule oxyethidium. ROS production was very low in aortas from both saline-treated *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (**Fig. 5e**). After mice were treated with AngII for 7 d, oxyethidium fluorescence was markedly increased in *Apoe*$^{-/-}$ mouse aortas (**Fig. 5e,f**). In contrast, ROS production was not induced by AngII in aortas from AngII-treated *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (**Fig. 5e,f**). Taken together, these *in vivo* (**Fig. 5e,f**) and *in vitro* (**Fig. 5a,b**) data suggest that AngII-induced ROS production in VSMCs is enhanced by both intracellular and extracellular CypA.

VSMC-derived CypA promotes AAA formation *in vivo*

To provide further evidence that VSMC-derived CypA regulates ROS production and MMP activity, we used VSMC-restricted CypA–overexpressing mice (VSMC-Tg mice). We previously showed that CypA expression is approximately threefold higher in arteries of VSMC-Tg mice compared to WT mice[35]. In saline-infused mice, there was no difference in oxyethidium fluorescence between WT, *Ppia*$^{-/-}$ and VSMC-Tg aortas (**Fig. 6a**). However, after AngII-infusion for 7 d, oxyethidium fluorescence was markedly higher in VSMC-Tg aortas than in WT (intermediate) or *Ppia*$^{-/-}$ (lowest) aortas (**Fig. 6a**).

There was no difference in basal MMP activity between WT, *Ppia*$^{-/-}$ and VSMC-Tg aortas in saline-infused mice (**Fig. 6b**). However, after AngII infusion, MMP activity was considerably higher in VSMC-Tg aortas than in WT (intermediate) or *Ppia*$^{-/-}$ (lowest) aortas (**Fig. 6b**). We next assayed AngII-mediated activation of MMP-2 and MMP-9 by gel zymography (**Fig. 6c**). The abundance of active MMP-2 in the conditioned medium after organ culture of aorta was significantly augmented for VSMC-Tg aortas compared with WT aortas and significantly decreased for *Ppia*$^{-/-}$ aortas (**Fig. 6c,d**). These results were supported by a similar experiment using cultured VSMCs collected from mouse aorta: MMP-2 activity was substantially augmented in VSMCs from VSMC-Tg mice compared with those from WT or *Ppia*$^{-/-}$ mice (**Fig. 6e**). These data support the concept that VSMC-derived CypA is a key mediator of AngII-induced MMP-2 activation.

To provide additional support for the pathogenic role of CypA in AAA formation, we investigated the effects of AngII infusion in VSMC-Tg mice. We attempted to cross the VSMC-Tg onto the *Apoe*$^{-/-}$ background but did not obtain any viable pups, preventing us from directly testing the effect of CypA overexpression in VSMCs on AAA formation. In mice with an *Apoe*$^{+/+}$ genetic background, there was no marked difference in aortic weight and diameter between *Ppia*$^{-/-}$, WT and VSMC-Tg mice in control saline-infused mice (data not shown). In response to AngII infusion, the maximum aortic diameter increased markedly in VSMC-Tg mice by approximately twofold compared to *Ppia*$^{-/-}$ mice or WT mice (**Fig. 6f**), with a

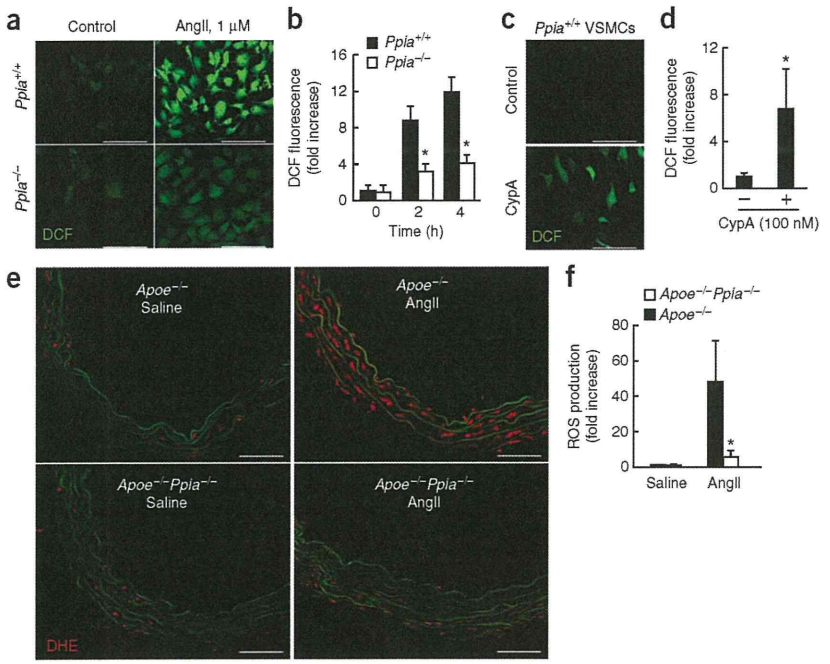

Figure 5 AngII-induced ROS formation in VSMCs requires CypA. (**a**) Representative dichlorofluorescein (DCF) staining of aortic VSMCs. Control, saline. Scale bars, 50 μm. (**b**) Densitometric analysis of DCF fluorescence in response to AngII. Results are means ± s.d. of five independent experiments. $*P < 0.01$ compared with *Ppia*$^{+/+}$ VSMCs. (**c**) Representative DCF staining of *Ppia*$^{+/+}$ VSMCs in response to 100 nM CypA. Scale bars, 50 μm. (**d**) Densitometric analysis of DCF fluorescence in *Ppia*$^{+/+}$ VSMCs in response to 100 nM CypA. Results are means ± s.d. of five independent experiments. $*P < 0.01$ compared with control VSMCs. (**e**) *In situ* dihydroethidium (DHE) staining of aortas from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Ppia*$^{-/-}$ mice infused with saline or AngII for 7 d. Green fluorescence in the media, observed in both control and AngII-treated aortas, is due to elastin fiber autofluorescence. All sections are shown with the lumen at the top. Scale bars, 100 μm. (**f**) Densitometric analysis of DHE fluorescence relative to control *Apoe*$^{-/-}$ mice (saline-infused). $n = 6$ per group; $*P < 0.01$ compared with *Apoe*$^{-/-}$ mice.

highly significant increase in AAA incidence (**Fig. 6g**). These results support the idea that VSMC-derived CypA is crucial for MMP-2 activation and AAA formation induced by AngII infusion.

Finally, we evaluated the role of CypA in human AAA lesions (**Supplementary Fig. 6** online). CypA was highly expressed throughout the aortic wall of AAA lesions, especially in areas that express active MMP (**Supplementary Fig. 6a,b**). We performed organ culture to determine the effect of AngII treatment on CypA secretion. AngII substantially increased secretion of CypA from human AAA lesions (**Supplementary Fig. 6c**). We next collected VSMCs from human AAA tissues and found that they expressed CypA at high levels (**Fig. 6d,e**). In response to AngII, MMP activity was also strongly increased (**Supplementary Fig. 6f,g**). Using gel zymography, this activity was shown to be mediated by MMP-2 (**Supplementary Fig. 6h,i**). Treatment with cyclosporine A markedly decreased MMP-2 activation, demonstrating a key role for the peptidyl-prolyl *cis/trans* isomerase activity of CypA (**Supplementary Fig. 6h,i**). These results suggest a crucial contribution by CypA to MMP activation in human AAA lesions.

DISCUSSION

Our major finding is that CypA is an essential mediator of AAA formation. We characterized four pathological mechanisms by which vascular CypA promotes AAA formation (**Supplementary Fig. 7**

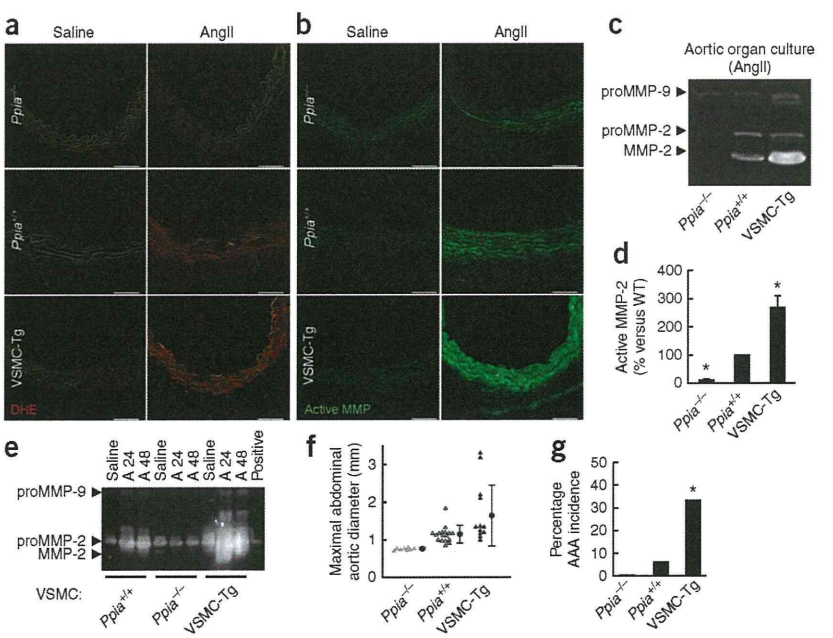

Figure 6 VSMC-derived CypA has a crucial role in aortic ROS production, MMP-2 activation and AAA formation. (**a,b**) DHE staining (**a**) and *in situ* zymography (**b**) of suprarenal aortas from mice of the indicated genotypes after treatment with saline or AngII for 7 d. All sections are shown with the lumen at the top. Scale bars, 100 μm. (**c**) Representative gelatin zymography of conditioned medium from mouse aorta after AngII infusion for 7 d. (**d**) Activity of MMP-2 in conditioned medium from AngII-treated aortic organ culture. $*P < 0.01$ versus *Ppia*$^{+/+}$ aorta. Results are means ± s.d. of three independent experiments. $n = 5$ per group. (**e**) Representative gelatin zymography of aortic VSMCs mice of the indicated genotypes after treatment with saline, AngII for 24 h (A 24) or AngII for 48 h (A 48). Positive; recombinant MMP-2 positive control. (**f**) Maximal abdominal aortic diameter in *Ppia*$^{+/+}$, *Ppia*$^{-/-}$ and VSMC-Tg mice 4 weeks after AngII infusion. Triangles represent individual mice; circles represent the mean; error bars denote s.d. (**g**) The incidence of AngII-induced AAA in *Ppia*$^{+/+}$ ($n = 17$), *Ppia*$^{-/-}$ ($n = 8$) and VSMC-Tg mice ($n = 12$). $*P < 0.01$ compared with AngII-infused *Ppia*$^{+/+}$ mice.

online). First, AngII-induced ROS promote secretion of CypA and proMMP-2. Second, secreted extracellular CypA contributes to ROS production synergistically with AngII in VSMCs. Third, CypA promotes activation of MMP-2 by inducing MT1-MMP activation and augmenting ROS generation. Last, CypA stimulates recruitment of $CD45^+$ inflammatory cells. The source of CypA responsible for AAA formation seems to be cells in the vessel wall, especially VSMCs, as no AAAs were observed in $Apoe^{-/-}Ppia^{-/-}$ mice after transplantation with $Ppia^{+/+}$ bone marrow cells. Also, overexpression of CypA specifically in VSMCs enhanced vascular ROS production, MMP activation and AAA formation. Therefore, we propose a key role for vascular CypA in AAA formation and other cardiovascular diseases associated with inflammation.

VSMCs seem to be essential for AngII-induced AAA formation. Expression of the AngII type 1a receptor, which is responsible for CypA secretion, ROS generation and MMP activity, is highest in VSMCs[36]. We found that *in situ* measurements of ROS generation and MMP activity were highest in medial cells that stained for α-SMA. Furthermore, cultured VSMCs from transgenic mice and human AAA lesions recapitulated our *in situ* findings of increased ROS and MMP activation. Finally, bone marrow transplantation experiments showed a minor role for hematopoietic cells in AAA formation in terms of CypA expression. Our data suggest that VSMC-derived CypA initiates AAA formation by promoting accumulation of macrophages. $Apoe^{-/-}Ppia^{-/-}$ mice had markedly attenuated vascular ROS production, MMP activation and MCP-1 secretion, resulting in decreased macrophage accumulation. Overexpression of CypA in VSMCs enhanced ROS production and MMP activation and caused AAA formation even in $Apoe^{+/+}$ mice. Finally, transplantation of bone marrow cells from $Ppia^{+/+}$ mice into $Apoe^{-/-}Ppia^{-/-}$ mice did not induce AAA formation, indicating that it is the *Ppia* status of cells resident in the vessel wall that affects AAA formation.

Our data show that extracellular CypA induces ROS production in VSMCs, which is consistent with our previous report that extracellular CypA stimulates at least three signaling pathways (ERK1/2 and Akt and Janus kinases) in VSMCs[19]. These signaling pathways have been shown to be crucial in ROS production[2,3]. Furthermore, ROS stimulate secretion of CypA from VSMCs[19,21]. These reports and our current data suggest that CypA has a crucial role in the matrix-degrading and proliferative functions of VSMCs by augmenting ROS generation. AngII is thought to induce the generation of ROS, thereby activating MMPs[26] and thus leading to the onset of vascular inflammatory cell migration and AAA formation[7,16,24].

In our experiments, CypA deficiency decreased both secretion of proMMP-2 and MMP-2 and expression of MT1-MMP, all of which can be explained by reduced ROS production. Additionally, AngII has been shown to generate ROS and activate MMP-2 in a $p47^{phox}$-dependent manner[7,15,37]. VSMC-derived MMP-2 promotes degradation of collagen and elastin, contributing to AAA formation[30,31]. Expression of MT1-MMP is important for activation of MMP-2 in AngII-induced AAA formation[38]. Aside from enzymatic cleavage and activation of MMP-2 by cell surface–expressed MT1-MMP[31], ROS have also been shown to directly activate MMP-2[33]. AAA formation results from the synergistic activation of ROS production, MT1-MMP and MMP-2. Therefore, CypA seems to be a central mediator of AngII-mediated AAA formation.

The identification of CypA as a mediator of tissue damage associated with inflammation and oxidative stress provides insight into the mechanisms underlying several therapeutic interventions. For example, the Rho kinase inhibitor Y27632 and simvastatin considerably reduced CypA secretion from VSMCs in this study. Rho kinase is a major therapeutic target in cardiovascular disease[39], and Rho kinase inhibition has been reported to reduce AngII-induced AAA formation[40]. AngII type 1 receptor blockers and angiotensin-converting enzyme (ACE) inhibitors have been shown to prevent AAA formation in mice[41–43]. In light of our results, reduced CypA secretion may partially contribute to the therapeutic effect of these drugs on AAA formation. Because inflammation and oxidative stress contribute to tissue damage in several situations, such as ischemia-reperfusion injury in the brain, heart and kidney, future studies of CypA-mediated function in appropriate models may reveal a major role for CypA in these conditions.

EMMPRIN, a putative CypA receptor, was identified as a tumor cell membrane protein that is expressed in VSMCs and activated by ROS and that stimulates MMP production[44]. A recent study showed ROS-dependent increases in EMMPRIN expression[45], which may be activated by binding of extracellular CypA[32]. Moreover, it has been shown that EMMPRIN is strongly expressed in human AAA lesions[46]. Therefore, it is logical to propose that agents that prevent CypA binding to its receptors may have therapeutic potential. In summary, these previous reports and our current study suggest that extracellular CypA and its receptor(s) represent new therapeutic targets, particularly for AAA progression.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

K.S. contributed to the design of the experiments, conducted and performed the experiments and generated the manuscript and figures. P.N. helped the design of the experiments and performed experiments. T.M., C.Y. and J.-i.A. contributed to generating VSMC-specific CypA–transgenic mice. M.R.O. and A.M. contributed to the *in vivo* experiments, including colony management, genotyping and hemodynamic measurements. Z.C. and X.S. contributed to preparation of recombinant CypA. K.A.I. contributed to the design of the experiments. B.C.B. supervised the project, contributed to the design of the experiments and wrote the manuscript.

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ONLINE METHODS

Analysis and quantification of abdominal aortic aneurysms. We conducted all mouse experiments in accordance with experimental protocols that were approved by the Institutional Animal Care and Use Committee at the University of Rochester. We used the AngII-infused AAA model to assess the effect of CypA deficiency on AAA development in *Apoe* $^{-/-}$ mice[16]. We infused 6- to 8-week-old male *Apoe* $^{-/-}$ *Ppia*$^{+/+}$ littermate control mice and *Apoe* $^{-/-}$ *Ppia* $^{-/-}$ mice on a normal chow diet with 1,000 ng min $^{-1}$ kg AngII (MP Biomedicals) or saline for 4 weeks. We purchased *Apoe* $^{-/-}$ mice and *Ppia* $^{-/-}$ mice from Jackson Laboratory. We backcrossed *Ppia* $^{-/-}$ to C57BL/6J mice for seven generations before crossing them with *Apoe* $^{-/-}$ mice. We dissolved AngII in sterile saline and infused it via Alzet osmotic pumps (model 2004, DURECT). We anesthetized the mice with an intraperitoneal injection of ketamine (80 mg per kg body weight) and xylazine (5 mg per kg body weight). We placed the pumps into the subcutaneous space of ketamine and xylazine–anesthetized mice through a small incision in the back of the neck that we closed by suturing. All incision sites healed rapidly without any infection. To determine the effect of CypA deficiency on AngII-induced aneurysm formation, we quantified AAA incidence and size[16,17]. We measured the maximum width of the abdominal aorta with Image Pro Plus software (Media Cybernetics). We quantified aneurysm incidence based on a definition of aneurysm as an external width of the suprarenal aorta that was increased by 50% or greater compared with aortas from saline-infused mice.

Reactive oxygen species analysis. We obtained mouse aortic VSMCs as previously described[35] and treated them with AngII (1 μM), washed them with PBS and loaded them with 2,7-dichlorofluorescein diacetate (H2DCF-DA) (5 μmol l $^{-1}$; Molecular Probes) for 30 min. We perfused aortas with PBS (pH 7.4) at 100 mm Hg for 5 min at 4 °C. We collected aortic tissue and embedded and snap-froze the abdominal aorta (suprarenal) in Optimal Cutting Temperature medium (Tissue-Tek). We topically applied dihydroethidine hydrochloride (5 μM, Molecular Probes) to the freshly cut frozen aortic sections (10 μm) for 30 min at 37 °C to reveal the presence of ROS as red fluorescence (585 nm) by confocal microscopy (Olympus, FLUOVIEW)[34].

Matrix metalloproteinase activity. We evaluated MMP activities in response to AngII as previously described[5,10,11]. To verify the role of CypA in AngII-induced MMP activation, we treated VSMCs with AngII (1 μM) in culture medium without serum (DMEM, sigma). We incubated aortas of mice infused with AngII for 7 d for 20 h in culture medium. Thereafter, we collected the medium and concentrated it to yield conditioned medium. We subjected the conditioned medium to electrophoresis in SDS-PAGE gels containing 0.8 mg ml $^{-1}$ gelatin (Sigma-Aldrich). We incubated the gels for 12 h (at 37 °C) in zymography buffer (50 mmol l $^{-1}$ Tris (pH 8.0), 10 mmol l $^{-1}$ $CaCl_2$ and 0.05% Brij 35 (Sigma)) and then stained them with Coomassie brilliant blue. For *in situ* zymography, we incubated freshly cut frozen aortic sections (suprarenal aorta, 10 μm) or VSMCs cultured on glass-bottom dishes with a fluorogenic gelatin substrate (DQ gelatin, Molecular Probes) according to the manufacturer's protocol. We detected proteolytic activity as green fluorescence by confocal microscopy (Olympus, FLUOVIEW). After fixation, we immunostained VSMCs with an antibody to α-tubulin (T9026, Sigma).

Statistical analyses. Quantitative results are expressed as means ± s.d. We made comparisons of parameters among two groups by the unpaired Student's *t* test. We made comparisons of parameters among three groups by one-way analysis of variance. We made comparisons of different parameters between two genotypes by two-way analysis of variance followed by a *post hoc* analysis using the Bonferroni test. We evaluated statistical significance with StatView (StatView 5.0, SAS Institute). We considered a value of $P < 0.05$ statistically significant.

Additional methods. Detailed methodology is described in the **Supplementary Methods** online.

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REVIEW

Cyclophilin A

– Promising New Target in Cardiovascular Therapy –

Kimio Satoh, MD, PhD; Hiroaki Shimokawa, MD, PhD; Bradford C. Berk, MD, PhD

Cyclophilin A (CyPA) has been studied as a multifunctional protein that is upregulated in a variety of inflammatory conditions, such as rheumatoid arthritis, autoimmune disease, and cancer. CyPA has been classified as an immunophilin and has a variety of intracellular functions, including intracellular signaling, protein trafficking, and the regulation of other proteins activity. Besides its intracellular functions, CyPA is a secreted molecule that has a physiological and pathological role in cardiovascular diseases, making it a potential biomarker and mediator in cardiovascular diseases, such as vascular stenosis, atherosclerosis, and abdominal aortic aneurysms. (*Circ J* 2010; **74:** 2249–2256)

Key Words: Immunophilin; Matrix metalloproteinase; Reactive oxygen species; Remodeling; Vasculature

The interaction between endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) plays an important role in regulating vascular integrity. ECs secrete a variety of vasoactive substances, including nitric oxide (NO) and prostacyclin, which protect against vascular remodeling.[1,2] VSMCs contain numerous sources of reactive oxygen species (ROS; ie, H_2O_2, O_2^-, and $^{\cdot}OH$), including NADPH oxidases, xanthine oxidase, the mitochondrial respiratory chain, lipoxygenases and NO synthases.

Oxidative stress, generated by excessive ROS, promotes cardiovascular disease. However, the precise mechanism of the deterioration in vascular function and promotion of vascular remodeling by ROS in vivo has not been clearly elucidated. VSMCs are among the most plastic of all cells in their ability to respond to different stimuli. Autocrine/paracrine growth factors from VSMCs have been mentioned for a long time as important mechanisms that mediate the varying cellular responses in vascular remodeling.[3–5] It has now become clear that almost all VSMC growth factors elicit auto/paracrine growth pathways. Recent evidence suggests that many other stimuli that modulate VSMC function, including ROS, promote VSMC growth by inducing auto/paracrine growth mechanisms (as reviewed by Taniyama and Griendling[6]). ROS increase cell proliferation, mediate agonist-induced hypertrophy, and also induce apoptosis in a concentration-dependent manner.[7]

Cyclophilin A (CyPA) is a 20-kD chaperone protein secreted from VSMCs in response to ROS, and it stimulates VSMC proliferation and inflammatory cell migration in vitro and in vivo. The major topics that will be addressed in this review are a series of projects that were performed in our laboratory. Does ROS-induced secreted CyPA actually contribute to several cardiovascular diseases in vivo? To answer this question, we used several genetic interventions, including the CyPA knockout mouse and the CyPA overexpressing transgenic mouse (VSMC-Tg). Thus, we elucidated that CyPA mediates a variety of cardiovascular diseases, including vascular stenosis, atherosclerosis, and abdominal aortic aneurysm (AAA). In this review we will discuss these recently revealed roles of CyPA in cardiovascular disease.

Intracellular CyPA as a Multifunctional Chaperone

In 1984, CyPA was identified as the main target for the immunosuppressive drug cyclosporine A (CsA).[8–11] Cyclophilins are a family of highly conserved and ubiquitous proteins, termed immunophilins.[12] The most abundant cyclophilin is CyPA,[13] which is widely distributed in almost all tissues in prokaryotes and eukaryotes. In humans, CyPA has been found in all organs and the CyPA concentration may account for as much as 0.1–0.4% of the total protein in a cell.[14–16] CyPA is abundant in the cytosolic extract from lymphocytes and has a high affinity for CsA.[8] CyPA was also shown to be a part of a cytosolic heat-shock protein–immunophilin chaperone complex that includes caveolin and cholesterol.[17] Because of its enzymatic properties, cellular localization, and role in protein folding, CyPA belongs to a diverse set of proteins known as molecular chaperones. Because CyPA catalyzes the cis-trans isomerization of the peptidyl-prolyl bonds of certain proteins (PPIase activity), CyPA acts as accelera-

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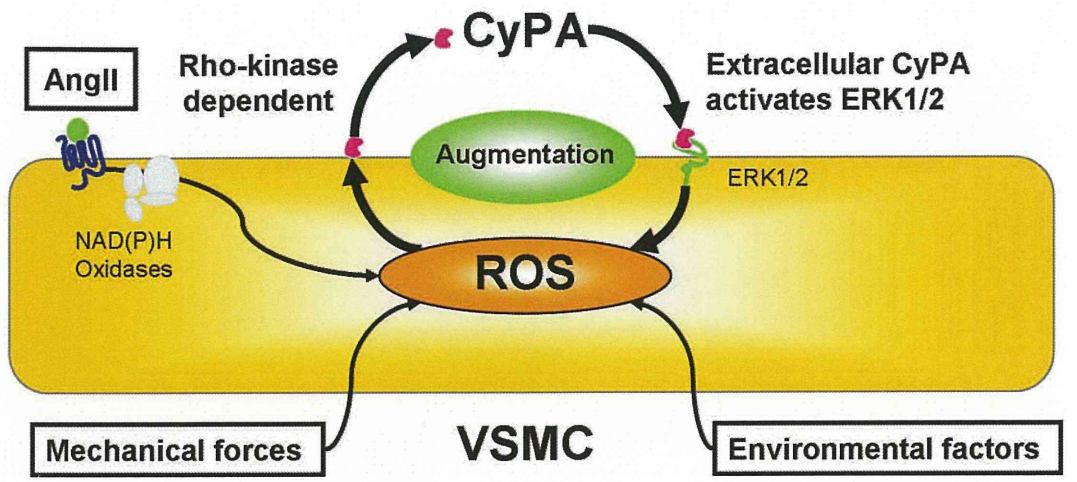

Figure 1. ROS-induced secretion of cyclophilin A (CyPA) synergistically augments ROS production. ROS inducers, such as angiotensin II (AngII), mechanical stress, and environmental factors, promote CyPA secretion from vascular smooth muscle cells (VSMC). Secreted CyPA activates ERK1/2 and promotes reactive oxygen species (ROS) production, thus augmenting it.

tion factor in protein folding and assembly. The first demonstration of this activity in vitro was delaying the maturation of collagen by blocking PPIase activity with CsA.[18] In addition to its role in protein folding, the PPIase activity of CyPA has recently been demonstrated to have other roles, including intracellular trafficking,[19] signal transduction, and transcription regulation.[20] Following the identification of CyPA, several other cyclophilins were cloned and characterized. Cyclophilin B (CyPB),[21] cyclophilin C (CyPC),[22] and cyclophilin D (CyPD)[23] were found to be less abundant and localized not only in the cytosol but also in membranes and subcellular organelles because of the presence of a hydrophobic N-terminal as well as C-terminal extensions. Human CyPB and murine CyPC are localized to the endoplasmic reticulum.[23] CyPD is localized to mitochondria and is an integral part of the mitochondrial permeability transition complex and plays a crucial role in apoptosis[24] and the pathogenesis of Alzheimer's disease.[25] A more detailed classification of the different cyclophilins has been reviewed recently.[13,26]

ROS in the Pathogenesis of Cardiovascular Disease

Production of intracellular ROS has been implicated in the pathogenesis of cardiovascular disease, in part by the promoting of VSMC proliferation.[27–29] Changes in vascular redox state are a common pathway involved in the pathogenesis of atherosclerosis, aortic aneurysms, and vascular restenosis after angioplasty. ROS target cellular biomolecules and cause severe damage, such as lipid peroxidation, protein oxidation/inactivation, and DNA damage/mutation. Although high levels of ROS might be hazardous to cells and their contents, controlled ROS levels (ie, physiological) are important in the regulation of cell functions and cell fate (proliferation/death). For example, H_2O_2 has also been implicated as important for EC function and vascular relaxation at very low concentrations.[30,31] In the vascular wall, ROS are generated by several mechanisms, including NADPH oxidases, xanthine oxidase, the mitochondrial respiratory chain, lipoxygenases and NO synthases.[32] Vascular ROS formation can be stimulated by mechanical stretch, pressure, shear stress, environmental factors such as hypoxia, and secreted factors such as angiotensin II (AngII).[33] We have demonstrated that ROS stimulate cultured VSMC proliferation and activate intracellular kinases such as ERK1/2 which is associated with cell growth.[29,34]

CyPA as a Secreted Oxidative Stress-Induced Factor (SOXF)

We found that activation of ERK1/2 by a ROS generator, napthoquinolinedione (LY83583), was biphasic (early and delayed activation). One explanation for the delayed ERK1/2 activation was the response to SOXF, which show autocrine/paracrine signals. In order to identify the presence of SOXF, we evaluated the ability of conditioned medium for ERK1/2 activation. The phosphorylation of ERK1/2 was significantly increased by conditioned medium from VSMCs treated with LY83583. Therefore, we analyzed the proteins released into the medium in response to LY83583 and finally found that CyPA is a major SOXF.[35] Furthermore, human recombinant CyPA stimulated ERK1/2 activity and DNA synthesis in VSMCs in a concentration-dependent manner.[36] Thus, we concluded that CyPA is a novel VSMC growth factor that contributes to the growth promoting activity of ROS in VSMCs.

Mechanism of CyPA-Induced VSMC Growth

Identification of the extracellular CyPA receptors is almost completely unexplored. We believe that further knowledge of the role played by extracellular CyPA receptors on vascular cell responses will help in designing therapeutics targeting inflammatory and cardiovascular diseases. In ECs, CyPA largely activates proinflammatory pathways, including increased expression of vascular cell adhesion molecule (VCAM)-1and E-selectin.[37] In VSMCs, ROS such as superoxide activate a pathway containing vesicles that results in secretion of CyPA.[38] Secreted extracellular CyPA stimulates ERK1/2, Akt and JAK in VSMCs, which contributes to ROS production again (**Figure 1**).[36] Despite the mounting evidence

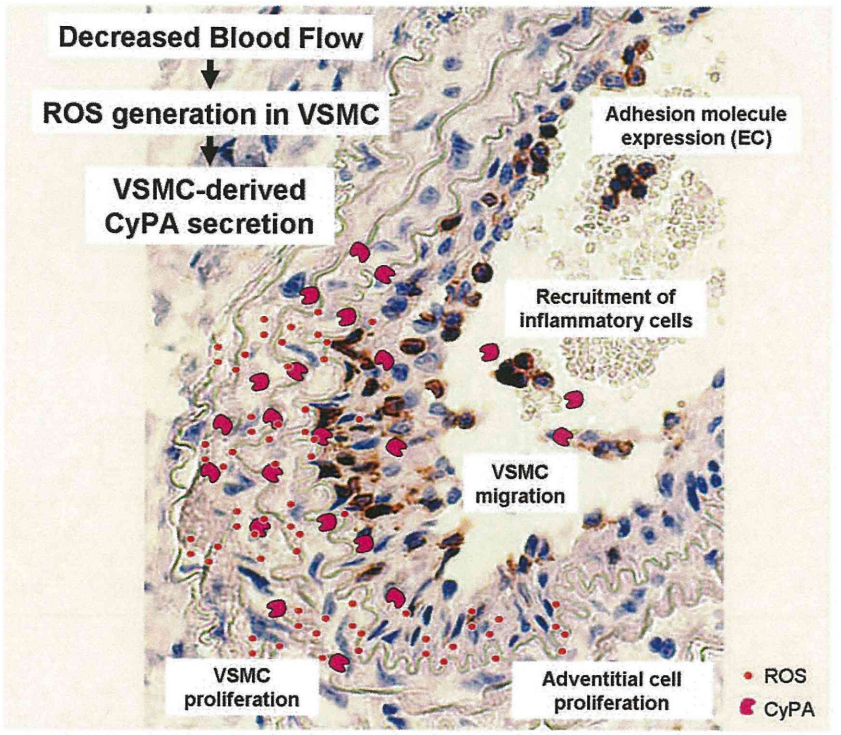

Figure 2. Cyclophilin A (CyPA) is a novel growth factor that mediates growth of vascular smooth muscle cells (VSMC) under oxidative stress. Decreased blood flow increases the generation of reactive oxygen species (ROS), which induces secretion of CyPA from VSMC. Secreted CyPA VSMC proliferation, endothelial cell (EC) adhesion molecule expression, and inflammatory cell migration (CD45-positive cells), resulting in vascular remodeling.

that cyclophilins serve multiple intracellular and extracellular functions, surprisingly little is known regarding their effect on specific receptors. Several molecules have been proposed as potential extracellular receptors for CyPA, including extracellular matrix metalloproteinase inducer (EMMPRIN).[39,40]

Mechanism of CyPA Secretion

It has been revealed that several growth factors are secreted from VSMCs in response to various stimuli.[41] CyPA is secreted from VSMCs via a highly regulated pathway that involves vesicle transport and plasma membrane binding.[38] Rho GTPases, including RhoA, Cdc42, and Rac1, are key regulators in signaling pathways linked to actin cytoskeletal rearrangement.[42] The Rho GTPases plays a central role in vesicular trafficking pathways by controlling the organization of the actin cytoskeleton. It has been reported that active participation of Rho GTPases is required for secretion of CyPA. We have shown consistently that the expression of dominant-negative mutants of RhoA and Cdc42 inhibited ROS-induced CyPA secretion, suggesting that both RhoA- and Cdc42-dependent signaling events regulate CyPA secretion.[38] Myosin II is involved in secretory mechanisms as a motor for vesicle transport.[43] Rho-kinase, a downstream effector of RhoA, mediates myosin II activation via phosphorylation and inactivation of myosin II light chain phosphatase.[44] We also demonstrated that a Rho-kinase inhibitor reduced ROS-induced CyPA secretion.[38,45] These results suggest that myosin II-mediated vesicle transport is required for CyPA secretion from VSMCs. CyPA is transported to the plasma membrane and colocalizes with VAMP in response to ROS stimulation. Therefore, CyPA is secreted from VSMCs through a process requiring ROS production and vesicle formation.

CyPA Promotes Intimal Thickness In Vivo

Increases in ROS represent a pathogenic mechanism for vascular disease.[46,47] ROS have been implicated in the pathogenesis of neointima formation, in part by promoting VSMC growth,[29,34] as well as by stimulating proinflammatory events.[48–51] We demonstrated that extracellular CyPA stimulates proinflammatory signals in ECs, including expression of E-selectin and VCAM-1.[37] In addition to the effects on vascular cells, CyPA has been shown to be a direct chemoattractant for inflammatory cells[52,53] and to promote matrix metalloproteinases (MMPs) activation.[54,55] Therefore, CyPA is a key mediator that affects ECs, VSMCs and inflammatory cell functions in vivo.

To confirm the role of CyPA in vascular remodeling, we observed the time course and distribution of its expression in carotid arteries after ligation.[56] We found that CyPA expression dramatically increased over a time course that paralleled neointimal formation, suggesting an important role for CyPA in the cellular response to oxidative stress induced by vascular injury. In parallel with CyPA expression, carotid ligation induced phosphorylation of ERK1/2 in wild-type carotids, which was significantly less in $CyPA^{-/-}$ carotids, consistent with the reduced number of $Ki67^{+}$ cells in ligated $CyPA^{-/-}$ carotids. The distribution of $Ki67^{+}$ cells closely overlapped with the areas of highest CyPA expression, especially in rapidly proliferating neointimal cells in WT mice. Colocalization of CyPA, α-smooth muscle actin (αSMA), and Masson-Trichrome staining revealed that CyPA expression was especially elevated in VSMCs. To prove further the contribution of VSMC-derived CyPA to vascular remodeling, we prepared VSMC-specific CyPA transgenic mice (VSMC-Tg). The observation that VSMC-specific CyPA overexpression not only increased the medial area but also the intimal area suggests that VSMC-derived extracellular CyPA promotes

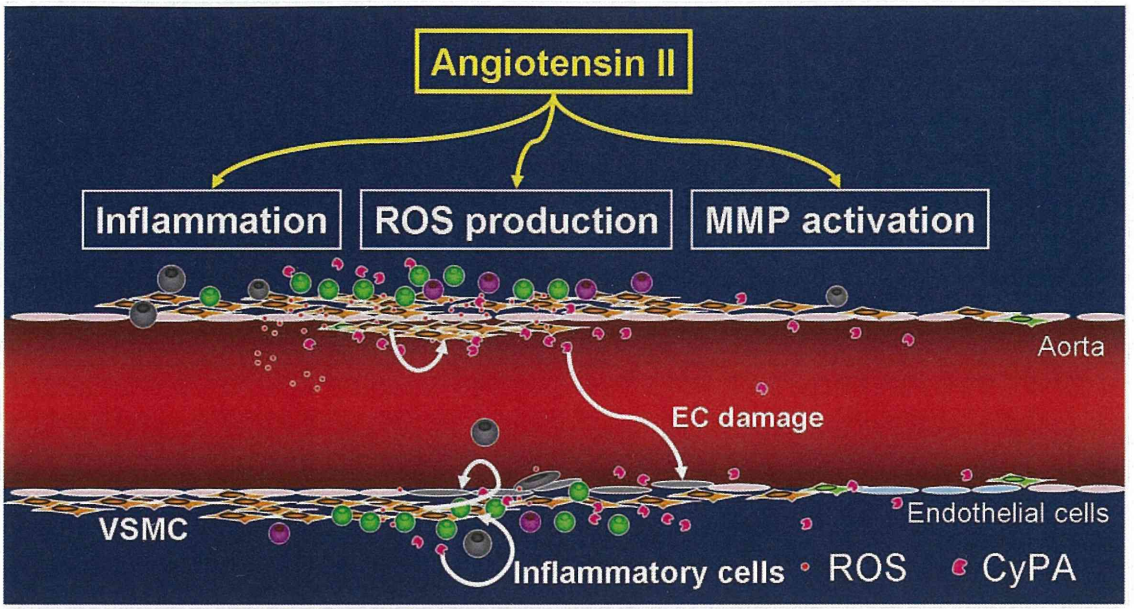

Figure 3. Proposed mechanisms for angiotensin II (AngII)-induced reactive oxygen species (ROS) production, cyclophilin A (CyPA) secretion, metalloproteinase (MMP) activation, and abdominal aorta aneurysm (AAA) formation. Secreted extracellular CyPA activates ERK1/2, Akt, and JAK in an autocrine/paracrine manner, which promotes proliferation and migration of vascular smooth muscle cells (VSMCs), MMP activation, and inflammatory cell migration.

the proliferation and migration of VSMCs via a paracrine manner. CyPA is expressed by all cell types participating in vascular pathology.[57] Additionally, extracellular CyPA has recently been found to induce interleukin (IL)-6 release in inflammatory cells.[58] Moreover, investigating CyPA function in monocyte/macrophage cell lines revealed that CyPA induces the expression of cytokines/chemokines such as tumor-necrosis factor α, monocyte chemotactic protein-1, IL-8, IL-1β and MMP-9 through a pathway that is dependent on nuclear factor-κB activation. In our carotid ligation model, we observed significant accumulation of CD45$^+$ inflammatory cells in the intima of ligated CyPA$^{-/-}$ carotids and the VSMC-specific overexpression of CyPA (VSMC-Tg) further enhanced the accumulation of inflammatory cells in the ligated carotids, supporting the important role of CyPA in mediating the recruitment of inflammatory cells (**Figure 2**).[56]

We propose that ROS generated locally by inflammatory cells cause VSMCs to release CyPA, which would then promote recruitment of inflammatory cells that release several proinflammatory cytokines. In addition, CyPA regulates the proteolytic activity necessary for the migration of inflammatory cells, through its activation of MMPs. Our study revealed 3 important pathologic consequences of CyPA activity in vivo. First, VSMC-derived secreted CyPA is mitogenic by virtue of its ability to promote VSMC proliferation. Second, secreted extracellular CyPA is proinflammatory because it stimulates the recruitment of inflammatory cells. Third, secreted CyPA promoted the pathological setting that exacerbated the generation of intracellular ROS in VSMC derived from mouse aorta (**Figure 2**).

CyPA Augments ROS Production and MMP Activation

As we have discussed, ROS stimulate secretion of CyPA from VSMCs, and that extracellular CyPA stimulates VSMC migration and proliferation (**Figure 1**).[35,36] Extracellular CyPA also stimulates EC adhesion molecule expression, and is a chemoattractant for inflammatory cells.[36,38,59] Furthermore, CyPA is upregulated in patients with rheumatoid arthritis and implicated because of its crucial role in MMP activation.[52] AngII infusion into ApoE$^{-/-}$ mice for 4 weeks promotes AAA formation.[60,61] In animal models of AAA, genetic and pharmacological inhibition of both ROS production[62,63] and MMPs[64,65] suppressed development of aneurysms. In that animal model, the AngII type 1 (AT_1) receptor in the vascular wall, but not in inflammatory cells, is required for the initiation of AngII-induced AAAs.[66] Furthermore, treatment with an AT_1 receptor blocker significantly suppressed aneurysm formation in ApoE$^{-/-}$ mice.[67] Therefore, we hypothesize that VSMC-derived CyPA augments AngII-induced ROS production, MMP activation, and inflammatory cell recruitment into the aortic VSMCs, contributing to AAA formation and progression.

CyPA Promotes AAA Formation and Aortic Rupture

In the cardiovascular system, AAA formation results from chronic inflammation of the aortic wall, associated with decreased medial VSMCs, and progressive destruction of structural components, particularly the elastic lamina.[68] Key mechanisms include VSMC senescence,[69] oxidative stress,[6,46] increased local production of proinflammatory cytokines[70] and increased activities of MMPs that degrade extracellular matrix.[71,72] As expected, AAA formation in the AngII-induced ApoE$^{-/-}$ model was completely prevented against a CyPA$^{-/-}$ background.[45] We also demonstrated that CyPA is highly expressed in the aorta of patients with AAA, and colocalizes with active forms of MMPs. Based on these find-

ings, we demonstrated that AngII induces ROS and MMP activation via a CyPA-dependent pathway, a novel mechanism for induction of AAA formation by AngII.

Our data suggest that extracellular CyPA and its signaling pathways are novel targets for AAA therapy and, potentially, other cardiovascular diseases associated with inflammation. In addition, extracellular CyPA induces ROS production in VSMCs, which is consistent with our previous report that extracellular CyPA stimulates at least 3 signaling pathways (ERK1/2, Akt and JAK) in VSMCs,[36] which has been shown to be important for ROS production.[6,46] All these data are proof-of-concept that CyPA plays a crucial role in VSMCs through ROS generation. AngII induces the generation of ROS and promotes the secretion of CyPA. ROS-induced CyPA secretion augments ROS production synergistically (**Figure 1**). Subsequently, secreted CyPA, acting as a proinflammatory cytokine, synergistically augments AngII-mediated ROS production, contributing to the onset of vascular inflammatory cell migration and AAA formation (**Figure 3**).[62]

CyPA as a Potential Atherogenic Cytokine

Numerous basic and clinical studies have clearly identified that ROS have a major role in endothelial damage and the development of atherosclerosis.[73–75] However, we still do not have a strong therapeutic strategy for the clinical benefits of antioxidant administration. One potential reason for this could be the crucial role of ROS (especially H_2O_2) at very low concentration in intracellular signaling pathways that are also important for vascular functions.[30,31,76,77] CyPA (both intracellular and extracellular) contributes to atherosclerosis by promoting EC apoptosis and EC expression of leukocyte adhesion molecules, stimulating inflammatory cell migration, enhancing ROS production, increasing proliferation of macrophages and VSMCs, and increasing proinflammatory signal transduction in VSMCs.[78,79] In the context of atherosclerosis, CyPA can be regarded as a proinflammatory and proatherogenic molecule. CyPA is highly expressed at sites of unstable atherosclerotic plaques, especially those associated with macrophages and foam cells. However, CyPA expression and its regulatory molecular mechanisms during the process of plaque destabilization remain elusive and further research into the role of CyPA in the progression of atherosclerosis is needed to identify potential CyPA-related therapeutic targets.

CyPA as a Potential Promoter of Cardiac Hypertrophy

AngII plays a key role in many physiological and pathological processes in cardiac cells, including cardiac hypertrophy.[80] Therefore, understanding the molecular mechanisms responsible for AngII-mediated myocardial pathophysiology is critical to the development of new therapies for cardiac dysfunction.[81] One important mechanism now recognized as involved in AngII-induced cardiac hypertrophy is ROS production,[82,83] but the precise mechanism by which ROS cause hypertrophy remains unknown.[84] Our recent study provides strong mechanistic evidence of synergy between CyPA and AngII to increase ROS generation.[45] Because ROS stimulate myocardial hypertrophy, matrix remodeling, and cellular dysfunction,[85] CyPA will potentially enhance AngII-induced cardiac hypertrophy.

CyPA as a Potential Promoter of Hypoxia-Induced Pulmonary Arterial Hypertension (PAH)

PAH is associated with hypoxic exposure, enhanced ROS, and proliferation of VSMCs. Erythropoietin (Epo) has long been regarded as a hypoxia-induced hormone that acts exclusively in the proliferation and differentiation of erythroid progenitors. However, recent studies have demonstrated expression of the Epo receptor (EpoR) in the cardiovascular system,[86] and the therapeutic potential of Epo has been noted in a variety of disorders, including cerebral infarction, myocardial ischemia–reperfusion, and congestive heart failure. Recently, we demonstrated that the endogenous Epo/EpoR system plays an important protective role against the development of hypoxia-induced PAH.[87] For this purpose, we used $EpoR^{-/-}$-rescued mice that express EpoR only in the erythroid lineage, but not in th ecardiovascular system.[86] Moreover, we demonstrated the important role of the endogenous Epo/EpoR system in ischemia-induced regeneration and angiogenesis.[88]

Considering the role of CyPA in augmentation of ROS and VSMC proliferation and migration in vivo and in vitro, CyPA may potentially promote hypoxia-induced PAH. We have reported that Rho-kinase is activated in patients with PAH.[89] In addition, the secretion of CyPA is regulated by the Rho/Rho-kinase system.[38,45] Therefore, we tested the hypothesis that CyPA contributes to Rho-kinase activation and pulmonary vascular remodeling in PAH patients.[90] A key aspect of the study that deserves comment is the strong CyPA expression on αSMA-positive cells in the lungs of patients with idiopathic PAH. It is reported that bone marrow-derived αSMA-positive cells contribute to the development of PAH[91] and promote atherosclerotic plaque stability.[92] Additionally, we have reported that statins and a Rho-kinase inhibitor reduced the secretion of CyPA from VSMCs,[38,45] and demonstrated that pravastatin ameliorates hypoxia-induced PAH in mice.[93] Based on this, inhibition of CyPA secretion by statins[93] or Rho-kinase inhibitor[94,95] may contribute to the therapeutic effect of these drugs in PAH patients.

Conclusions

The identification of CyPA as a mediator of tissue damage associated with inflammation and oxidative stress provides insight into the mechanisms of several therapies. For example, the Rho-kinase inhibitor, Y27632, and simvastatin significantly reduced CyPA secretion from VSMCs. Rho-kinase is an important therapeutic target in cardiovascular disease[96] and Rho-kinase inhibition has been reported to reduce AngII-induced AAA formation,[97] atherosclerosis, and cardiac hypertrophy.[98] Moreover, AT1a receptor blockers and angiotensin-converting enzyme inhibitors have been shown to prevent cardiovascular diseases,[66,67,99] and reduced CyPA secretion may partially contribute to the therapeutic effect of these drugs on AAA, atherosclerosis, and cardiac hypertrophy.[45] EMMPRIN, a putative CyPA receptor, was identified as a tumor cell membrane protein that is expressed in VSMCs, activated by ROS and stimulates MMP production.[100] A recent study demonstrated ROS-dependent increases in EMMPRIN,[101] which may be activated by binding of extracellular CyPA.[102] Moreover, it has been demonstrated that EMMPRIN is strongly expressed in human AAA lesions[103] and in cardiomyocytes.[104] Therefore, it is logical to propose that agents that prevent CyPA binding to its receptors may have therapeutic potential (**Figure 1**). Because inflammation