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

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Abstract

Inflammation and oxidative stress are pathogenic mediators of many diseases, but therapeutic targets remain elusive. In the vasculature, abdominal aortic aneurysm (AAA) formation critically involves inflammaton and matrix degradation. Cyclophilin A (CyPA, encoded by *Ppia*) is highly expressed in vascular smooth muscle cells (VSMC), 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 were completely protected from AngII–induced AAA formation, in contrast to $Apoe^{-/-}Ppia^{+/+}$ mice. $Apoe^{-/-}Ppia^{-/-}$ mice showed 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 demonstrated that VSMC-derived intracellular and extracellular CyPA were required for ROS generation and matrix metalloproteinase-2 activation. These data define a novel role for CyPA in AAA formation and suggest CyPA is a new target for cardiovascular therapies.

Introduction

Inflammation and oxidative stress are pathogenic mediators of many diseases, but therapeutic targets remain elusive. In the vasculature, abdominal aortic aneurysm (AAA) formation critically involves inflammaton and matrix degradation. Key mechanisms include vascular smooth muscle cells (VSMC) senescence1, oxidative stress2,3, increased local

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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.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.

production of proinflammatory cytokines4 and increased activities of matrix metalloproteinases (MMPs)5,6. In animal models of AAA, genetic and pharmacological inhibition of ROS production7,8 and MMPs9,10 suppressed aneurysm formation. There is a strong mechanistic link between increased ROS and MMP activity 11–13, suggesting that therapies to limit ROS generation may be useful.

Angiotensin II (AngII) induces ROS through NADPH oxidases14 and activates MMPs15. AngII infusion into apolipoprotein E–deficient (*Apoe*$^{-/-}$) mice for 4 weeks promotes AAA formation16,17.

Cyclophilin A (CyPA, encoded by *Ppia*) is a chaperone protein that binds cyclosporine18 and is abundantly expressed in VSMC19. Our lab showed that ROS stimulate secretion of CyPA from VSMC. Extracellular CyPA stimulates VSMC migration and proliferation19,20; endothelial cell adhesion molecule expression, and inflammatory cell chemotaxis19,21,22. Based on these CyPA functions we determined its role in AngII-induced AAA23. We found that AAA formation in the AngII-induced *Apoe*$^{-/-}$ mice model was completely prevented in the *Ppia*$^{-/-}$ background. Mechanistically CyPA deficiency significantly decreased inflammatory cell recruitment, ROS production and MMP activation.

Results

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

As previously reported4,16,24,25 we found that treatment with AngII for 4 weeks promoted AAA formation in *Apoe*$^{-/-}$ mice (Fig. 1a–c). To define the role of CyPA in AAA formation, we established *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (double-knockout) mice and treated these animals with AngII for 4 weeks. AngII increased systolic blood pressure and total cholesterol, but there were no differences between *Apoe*$^{-/-}$ mice and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (Supplement Table 1a). There were no gross differences in the aortas of control *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (saline-infused mice, Fig. 1a). Strikingly, after AngII infusion, *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice had no AAA incidence, 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 (Supplement Table 1a) in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice after treatment with AngII. 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 aortas of control *Apoe*$^{-/-}$ mice (not shown). In *Apoe*$^{-/-}$ mice infused with AngII (Fig. 1d–f) there was a dramatic increase in aortic size of both the 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 by Daugherty's group24. Most of the cells that were positive for CyPA (Fig. 1e) concomitantly exhibited immunoreactivity for α-smooth muscle actin (α-SMA, Fig. 1f), suggesting that these were VSMC19. In contrast, the aortas of *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice infused with AngII showed no significant tissue mass or enlargement. 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 while none of the *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice died (Supplement Fig. 1a). Gross and histological examination of the dead animals revealed aortic rupture (Supplement Fig. 1b–d). As expected, the elastic lamina was frequently disrupted and degraded in *Apoe*$^{-/-}$ mice (Supplement Fig. 1e). In contrast, CyPA deficiency completely prevented elastic lamina degradation (Supplement Fig. 1f). Based on a semi-quantitative analysis of elastin degradation (Supplement Fig. 1g), CyPA deficiency completely blocked elastin degradation after AngII treatment for 4 weeks (Supplement Fig. 1h). These data suggest that protection from elastin degradation is an important mechanism for inhibition of AAA in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice.

To ascertain whether AngII induced vascular inflammation was CyPA-dependent, we examined inflammatory cell migration and microvessel formation. Inflammatory cell migration, 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 dramatically reduced in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (Fig. 2d–h), consistent with the reduced inflammatory responses.

To characterize the mechanisms by which CyPA participates in the inflammatory response, we first analyzed the secretion of proinflammatory molecules by cytokine/chemokine array *in vitro*. AngII treatment strikingly induced the secretion of proinflammatory cytokines such as MCP-1 and IL-6, as well as chemokines such as RANTES and SDF-1; whereas CyPA deficiency effectively blocked the induction of these molecules (Supplement Fig. 2a). We next showed that CyPA secretion was stimulated by AngII in mouse aortic VSMC (Fig. 2i). CyPA secretion was maximal at 1 μM AngII (Fig. 2i). Pretreatment with Y27632 (Rho kinase inhibitor) or simvastatin significantly reduced CyPA secretion (Fig. 2i), consistent with our previous report21. We studied MCP-1 expression in the aortic wall because of its known role in macrophage migration and AAA formation24,26. In saline-infused aortas, MCP-1 appeared to be more highly expressed in *Apoe*$^{-/-}$ than in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ media (Supplement Fig. 2b,c). In response to AngII, MCP-1 was highly expressed in *Apoe*$^{-/-}$ aortas (Supplement Fig. 2d), especially in the adventitia. In contrast, MCP-1 was markedly decreased in the adventitia of *Apoe*$^{-/-}$*Ppia*$^{-/-}$ aortas (Supplement Fig. 2e). The adventitial location of MCP-1 in response to AngII is consistent with its function as a chemokine for monocytes. Additionally, in cultured aortic VSMC, AngII stimulated MCP-1 secretion was markedly decreased in *Ppia*$^{-/-}$ cells (Supplement Fig. 2f), while other AngII signal events such as ERK1/2 activation did not differ.

Vascular CyPA, not bone marrow-derived CyPA, is essential for AAA formation

CyPA has been reported to play a crucial role in regulating the survival, proliferation, and differentiation of antigen-presenting cells by augmenting antigen uptake and presentation27. CyPA has also been reported to stimulate migration of bone marrow-derived cells *in vitro*22. Hematopoietic cells, especially macrophages, are involved in AAA formation4,24. We hypothesized that CyPA deficiency may impair macrophage differentiation and activation and thus prevent AAA formation by AngII. To test this possibility, *Ppia*$^{+/+}$ GFP^{+} bone marrow cells were transplanted into irradiated *Apoe*$^{-/-}$ mice or *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice. After

42 d of engraftment the mice were treated with AngII. There was no significant difference in the reconstitution ratio (%GFP$^+$ cells in the peripheral blood) in GFP$^+$ marrow-transplanted *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice compared with GFP$^+$ marrow-transplanted *Apoe*$^{-/-}$ mice (99.5 ± 0.3% vs. 99.6 ± 0.2%, respectively). There was no significant difference in the blood pressure of chimeric mice (Supplement Table 1b). However, the number of bone marrow-derived inflammatory cells (GFP$^+$CD45$^+$ double-positive cells) present in the aortic wall was significantly less in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice compared with *Apoe*$^{-/-}$ mice (Fig. 3a–c). Parenthetically, we observed both GFP$^+$CD45$^+$ cells and GFP$^+$CD45$^-$ cells in the AAA lesions after AngII infusion. Recent papers have shown that both non-hematopoietic cells (CD45$^-$) and hematopoietic cells (CD45$^+$), are mobilized from the bone marrow, and contribute to remodeling of the vascular wall. The presence of GFP$^+$CD45$^-$ cells in AngII-induced AAA lesions suggested that CyPA plays a crucial role in recruiting non-hematopoietic cells from the bone marrow. The number of bone marrow-derived macrophages (GFP$^+$Mac-1$^+$) was also significantly less in the *Apoe*$^{-/-}$*Ppia*$^{-/-}$ recipient mice (Supplement Fig. 3a–c).

Migration of bone marrow-derived cells into the media was frequently observed in *Apoe*$^{-/-}$ recipient mice (Fig. 3d, arrows). In contrast, there were few GFP$^+$ cells in the media of *Apoe*$^{-/-}$*Ppia*$^{-/-}$ recipient mice (Fig. 3e,f), suggesting the importance of VSMC-derived CyPA for inflammatory cell migration. Furthermore, microvessel formation assessed by PECAM-1 staining was significantly less in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ recipient mice (Fig. 3d,e,g), supporting the concept that the reduced inflammatory responses in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice are due to CyPA deficiency. Consistent with this idea, the incidence of AAA was 56% in *Ppia*$^{+/+}$ marrow-transplanted *Apoe*$^{-/-}$ mice (Fig. 3h), versus 0% in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice after transplantation of *Ppia*$^{+/+}$ bone marrow cells. Finally, we prepared chimeric mice with *Ppia*$^{-/-}$ bone marrow (Supplement Fig. 3d). The incidence of AAA was 60% in *Ppia*$^{-/-}$ marrow-transplanted *Apoe*$^{-/-}$ mice, while the incidence of AAA in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice was still 0%. These data suggest that CyPA expression by vascular cells, rather than bone marrow-derived cells, is critical for development of AAA.

CyPA deficiency prevents AngII-induced MMP activation *in vivo* and *in vitro*

AAA development and aortic rupture depend on macrophage-derived MMP-9 and VSMC-derived MMP-224,28,29, which are enzymatically cleaved and activated by MT1-MMP30. Secreted CyPA may activate MMPs through the extracellular MMP protein inducer (EMMPRIN)31. Therefore, we anticipated decreased MMP activity in the absence of CyPA. We performed western blotting for MMP-2 using a MMP-2 mouse monoclonal antibody that recognizes the ~72 kDa latent and the 66 kDa active forms of MMP-2. Western blotting revealed significantly reduced MMP-2 activity in *Ppia*$^{-/-}$ VSMC after AngII treatment (Supplement Fig. 4a). MT1-MMP expression in the VSMC membrane fraction revealed a significant increase in WT VSMC compared with *Ppia*$^{-/-}$ VSMC in response to AngII treatment (Supplement 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 elevated in WT VSMC compared with *Ppia*$^{-/-}$ VSMC (Supplement Fig. 4c). We next studied MMP function in the aortas of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice. Basal expression of MT1-MMP was low in the aortas of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice

(Fig. 4a). While MT1-MMP expression was significantly increased in the aortas of both *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice after AngII infusion (Fig. 4a), the increase was significantly less in aortas from *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice. Organ culture of *Apoe*$^{-/-}$ mice aortas after AngII infusion showed high activities of proMMP-9, proMMP-2 and activated MMP-2 by zymography in conditioned media (Fig. 4b). In contrast, there was no MMP activity in conditioned media from *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice after AngII-treatment (Fig. 4b). In situ zymography supported these observations (Fig. 4c). MMP activity was negligible in saline-treated aortas (green fluorescence). Following AngII-treatment, the media and adventitia of *Apoe*$^{-/-}$ mice showed much higher MMP activity compared to *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (Fig. 4c). Interestingly, the ruptured aorta of *Apoe*$^{-/-}$ mice revealed tremendous MMP activity, especially in the false lumen.

To elucidate the biological properties of VSMC in AAA-prone versus AAA-resistant areas, we harvested and cultured VSMC from thoracic, suprarenal, and infrarenal aorta, and compared MMP activities in response to AngII (Fig. 4e). There was no difference in the activities of MMP-2 in cells from aortas treated with saline, assessed by gelatin zymography (Fig. 4e). AngII treatment significantly increased activities of MMP-2 in *Apoe*$^{-/-}$ VSMC, especially in VSMC from the suprarenal aorta (Fig. 4e, AngII-S). In contrast, MMP-2 activity induced by AngII was significantly less in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ VSMC regardless of the aortic location (Fig. 4e). Treatment of VSMC with CyPA augmented MMP activity by ~2-fold, assessed by in situ zymography (Fig. 4f,g), demonstrating the importance of extracellular CyPA for MMP activation in VSMC. Consistent with these data, in situ zymography showed that active MMP was much greater in the media of suprarenal aorta than infrarenal and thoracic aorta (Supplement Fig. 5a–c). These *in vivo* and *in vitro* data demonstrate that CyPA in VSMC is crucial for activation of MMPs.

CyPA deficiency prevents AngII-induced ROS production *in vivo* and *in vitro*

We next investigated the mechanism by which CyPA deficiency decreases MMP expression, secretion and activation. ROS play a crucial role in activating VSMC MMPs in 32 in a p47phox-dependent manner33. Therefore, we studied the effect of CyPA deficiency on VSMC ROS production induced by AngII. First, we compared activation of ERK1/2 by AngII and found no significant difference between *Ppia*$^{+/+}$ and *Ppia*$^{-/-}$ VSMC (data not shown). In response to AngII for 4 h, *Ppia*$^{+/+}$ mouse VSMC increased ROS production by 12-fold as assessed by dichlorofluorescein (DCF) (Fig. 5a,b). *Ppia*$^{-/-}$ VSMC showed significantly less ROS induction (Fig. 5a,b). Additionally, treatment of *Ppia*$^{+/+}$ VSMC with CyPA significantly augmented ROS production after 4 h (Fig. 5c,d) suggesting that AngII-mediated CyPA secretion contributes to ROS production.

To evaluate the effect of CyPA deficiency on ROS generation *in vivo*, aortic sections were incubated with dihydroethidium (DHE), which in the presence of superoxide forms oxy-ethidium. In saline-infused aorta, ROS production (red fluorescence) was very low in both *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (Fig. 5e). After 7 d of AngII treatment, oxyethidium fluorescence was markedly increased in *Apoe*$^{-/-}$ mice aorta (Fig. 5e,f). In contrast, in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice ROS production was not induced by AngII (Fig. 5e,f). These *in vivo*

and *in vitro* data suggest that AngII-induced ROS production in VSMC 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 created VSMC-specific CyPA overexpressing mice (VSMC-Tg). We previously showed that CyPA expression is ~3-fold greater in arteries of VSMC-Tg mice versus WT ($Ppia^{+/+}$) mice34. In saline-infused mice, there was no basal difference in oxy-ethidium fluorescence (red fluorescence) between WT, $Ppia^{-/-}$ and VSMC-Tg aorta (Fig. 6a). However, after AngII-infusion for 7 d, oxy-ethidium fluorescence was significantly higher in VSMC-Tg aorta (Fig. 6a) than in WT (intermediate) and $Ppia^{-/-}$ (lowest) aortas.

There was no basal difference in MMP activity (green fluorescence) between WT, $Ppia^{-/-}$ and VSMC-Tg aorta in saline-infused mice (Fig. 6b). However, after AngII-infusion MMP activity was significantly less in $Ppia^{-/-}$ compared with WT aorta, and significantly greater in VSMC-Tg aorta (Fig. 6b). We next assayed AngII-mediated activation of MMP-2 and MMP-9 by gel zymography (Fig. 6c). Active MMP-2 in the conditioned media after organ culture of aorta was significantly augmented in VSMC-Tg compared with WT aorta, and significantly decreased in $Ppia^{-/-}$ aorta (Fig. 6c,d). These results were supported by a similar experiment using cultured VSMC harvested from mouse aorta (Fig. 6e). MMP-2 activity was significantly augmented in VSMC from VSMC-Tg mice compared with those from WT or $Ppia^{-/-}$ mice, (Fig. 6e). These data support the concept that VSMC-derived CyPA is an important 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 tried to cross VSMC-Tg onto the $Apoe^{-/-}$ background, but did not obtain any viable pups. There was no significant difference in the aortic weight and diameter between $Ppia^{-/-}$, WT, and VSMC-Tg mice in control, saline-infused, mice (not shown). In response to AngII infusion on $Apoe^{+/+}$ background, the maximum aortic diameter increased significantly in VSMC-Tg by ~2-fold compared to $Ppia^{-/-}$ and WT (Fig. 6f), with a highly significant increase in AAA incidence (Fig. 6g). These results support the concept that VSMC-derived CyPA is critical for MMP-2 activation and AAA formation induced by AngII infusion.

Finally, we evaluated the role of CyPA in human AAA lesions (Supplement Fig. 6). CyPA was highly expressed throughout the aortic wall of AAA lesions, especially in areas that express active MMP (Supplement Fig. 6a,b). We performed organ culture to determine the effect of AngII treatment on CyPA secretion. AngII significantly increased secretion of CyPA from human AAA lesions (Supplement Fig. 6c). We next harvested VSMC from human AAA tissues and characterized them as highly expressing CyPA (Fig. 6d–e). In response to AngII, MMP activity was also strongly increased (Supplement Fig. 6f–g). This activity was shown to be MMP-2 by gel zymography (Fig. 6h–i). A key role for CyPA PPIase activity was shown by the marked decrease in MMP-2 activation by treatment with CsA (Supplement Fig. 6h–i). These results suggest a crucial role for CyPA in MMP activation in human AAA lesions.

Discussion

The major finding of the present study is that CyPA is a novel mediator of abdominal aortic aneurysm (AAA) formation. We characterized four pathologic mechanisms by which vascular CyPA promotes AAA formation (Supplement Fig. 7). First, AngII-induced ROS promoted secretion of CyPA and proMMP-2. Second, secreted extracellular CyPA contributed to ROS production synergistically with AngII in VSMC. Third, CyPA promoted activation of MMP-2, by inducing MT1-MMP and augmenting ROS generation. Fourth, CyPA stimulated recruitment of $CD45^+$ inflammatory cells. The source of CyPA responsible for AAA formation appeared to be cells resident in the vessel wall, especially VSMC since no AAA were observed in $Apoe^{-/-}Ppia^{-/-}$ mice after transplantation with $Ppia^{+/+}$ bone marrow cells. Also, overexpression of CyPA in VSMC 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.

Daugherty's group characterized the temporal events in AngII-induced AAA formation24. First, they described medial accumulation of macrophages in regions of elastin degradation. Second, medial dissection occurred with dilation of the lumen and thrombus formation. Third, an inflammatory response comprised of macrophages and T and B lymphocytes was observed. Fourth, a repair response including elastin fiber regeneration and reendothelialization occurred; and finally there was neovascularization of the thrombus and vascular wall.

VSMC appear to be essential for AngII-induced AAA formation. Expression of the AT1a receptor, responsible for CyPA secretion, ROS generation and MMP activity, is highest in VSMC. In situ measurements of ROS generation and MMP activity were greatest in medial cells that stained for αSMA. Cultured VSMC from transgenic mice and human AAA lesions recapitulated the findings of increased ROS and MMP activation. Finally, bone marrow transplantation showed a minor role for hematopoietic cells. Specifically, our data suggest that VSMC-derived CyPA initiated AAA formation by promoting accumulation of macrophages. $Apoe^{-/-}Ppia^{-/-}$ mice had significantly attenuated vascular ROS production, MMP activation, and MCP-1 secretion resulting in decreased macrophage accumulation. Overexpression of CyPA in VSMC enhanced ROS production and MMP activation, and caused AAA formation even in $Apoe^{+/+}$ mice (Fig. 6f). Finally, transplantation of bone marrow cells from $Ppia^{+/+}$ mice into $Apoe^{-/-}Ppia^{-/-}$ mice did not induce AAA formation, indicating that cells resident in the vessel wall were essential for AAA formation.

Our novel data show that extracellular CyPA induces ROS production in VSMC, which is consistent with our previous report that extracellular CyPA stimulates at least 3 signaling pathways (ERK1/2, Akt and JAK) in VSMC19. These signaling pathways have been shown to be important for ROS production2,3. Furthermore, ROS stimulate secretion of CyPA from VSMC19,21. These reports and the present data suggest that CyPA plays a crucial role in VSMC through ROS generation. AngII is thought to induce the generation of ROS and thereby activates MMPs26, thus leading to the onset of vascular inflammatory cell migration and AAA formation7,16,24.

In the present study, CyPA deficiency reduced secretion of proMMP-2 and MMP-2 as well as MT1-MMP expression, all of which can be explained by reduced ROS production. Additionally, AngII has been shown to generate ROS and activate MMP-2 in a p47phox-dependent manner in the same model7,15,35. VSMC-derived MMP-2 promotes degradation of collagen and elastin, contributing to the AAA formation29,30. Expression of MT1-MMP is important for activation of MMP-2 in AngII-induced AAA formation36. Besides enzymatic cleavage and activation of MMP-2 by cell surface expressed MT1-MMP30, ROS have been shown to directly activate MMP-232. AAA formation results from the synergistic activation of ROS production, MT1-MMP, and MMP-2. Therefore, CyPA appears 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 of several therapies. For example, the Rho-kinase inhibitor Y27632, and simvastatin significantly reduced CyPA secretion from VSMC. Rho-kinase is an important therapeutic target in cardiovascular disease37 and Rho-kinase inhibition has been reported to reduce AngII-induced AAA formation38. AT1a receptor blockers and ACE inhibitors have been shown to prevent AAA formation in mice39–41. Based on the present study, 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 significant role in other diseases.

EMMPRIN, a putative CyPA receptor, was identified as a tumor cell membrane protein that is expressed in VSMC, activated by ROS and stimulates MMP production42. A recent paper demonstrated ROS-dependent increases in EMMPRIN43, which may be activated by binding of extracellular CyPA31. Moreover, it has been demonstrated that EMMPRIN is strongly expressed in human AAA lesions44. Therefore, it is logical to propose that agents which prevent CyPA binding to its receptors may have therapeutic potential. In summary, these reports and the present study suggest that extracellular CyPA and its receptor(s) represent novel therapeutic targets, particularly for AAA progression.

Methods

Analysis and quantification of AAAs

All animal experiments were conducted in accordance with experimental protocols that were approved by the Institutional Animal Care and Use Committee at the University of Rochester. AngII-infused AAA models were employed to assess the effect of CyPA deficiency on AAA development in *Apoe*$^{-/-}$ mice16. Six- to 8 week old male *Apoe*$^{-/-}$*Ppia*$^{+/+}$ littermate control mice and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice on a normal chow diet were infused with 1,000 ng min^{-1} kg AngII (MP Biomedicals) or saline for 4 weeks. AngII was dissolved in sterile saline and infused using Alzet osmotic pumps (Model 2004, DURECT Corp.). Mice were anesthetized with an intraperitoneal injection of ketamine (80 mg kg^{-1}) and xylazine (5 mg kg^{-1}). Pumps were placed into the subcutaneous space of ketamine and xylazine anesthetized mice through a small incision in the back of the neck that was closed with suture. 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 size16,17. The maximum width of the abdominal aorta was measured with Image Pro Plus software (Media Cybernetics Inc.). Aneurysm incidence was quantified based on a definition of an external width of the suprarenal aorta that was increased by 50% or greater compared with aortas from saline-infused mice.

ROS analysis

After treatment with AngII (1 μM), VSMC were washed with PBS and loaded with 2,7-dichlorofluorescein diacetate (H2DCF-DA) (5 μM; Molecular Probes) for 30 min. Aortas were perfused with PBS (pH 7.4) at 100 mmHg for 5 minutes at 4°C. Aortic tissue was harvested, and the abdominal aorta (supra renal) were embedded in OCT (Tissue-Tek; Miles Inc., Elkhart, Illinois, USA) and snap-frozen. Dihydroethidine hydrochloride (5 μM, Molecular Probes) was topically applied 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)33.

MMP activity

The evaluation of MMP activities in response to AngII was performed as described5,10,11. To verify the role of CyPA in AngII-induced MMPs activation, VSMC were treated with AngII (1 μM) in culture medium. Likewise, aortas of mice infused with AngII for 7 d were incubated for 20 h in culture medium. Thereafter medium was collected and concentrated to yield conditioned medium (CM). CM was electrophoresed in SDS-PAGE gels containing 0.8 mg ml^{-1} gelatin (Sigma-Aldrich). Gels were incubated for 12 h (37°C) in zymography buffer (50 mmol l^{-1} Tris (pH 8.0), 10 mmol l^{-1} CaCl2, 0.05% Brij 35), and stained with Coomassie brilliant blue. For in situ zymography, freshly cut frozen aortic sections (supra renal aorta, 10 μm) or VSMC cultured on glass bottom dish were incubated with a fluorogenic gelatin substrate (DQ gelatin, Molecular Probes) according to the manufacturer's protocol. Proteolytic activity was detected as green fluorescence by confocal microscopy (Olympus, FLUOVIEW). After fixation, VSMC were immunostained with α-tubulin.

Statistical analyses

Quantitative results are expressed as mean ± SD. Comparisons of parameters among 2 groups were made by the unpaired Student's *t*-test. Comparisons of parameters among the 3 groups were made by one-way analysis of variance (ANOVA), and comparisons of different parameters between the 2 genotypes were made by two-way analysis of variance (ANOVA), followed by a post hoc analysis using the Bonferroni test. Statistical significance was evaluated with StatView (StatView 5.0, SAS Institute Inc.). A value of $P<0.05$ was considered to be statistically significant.

Additional methods

Detailed methodology is described in the Supplementary Methods online.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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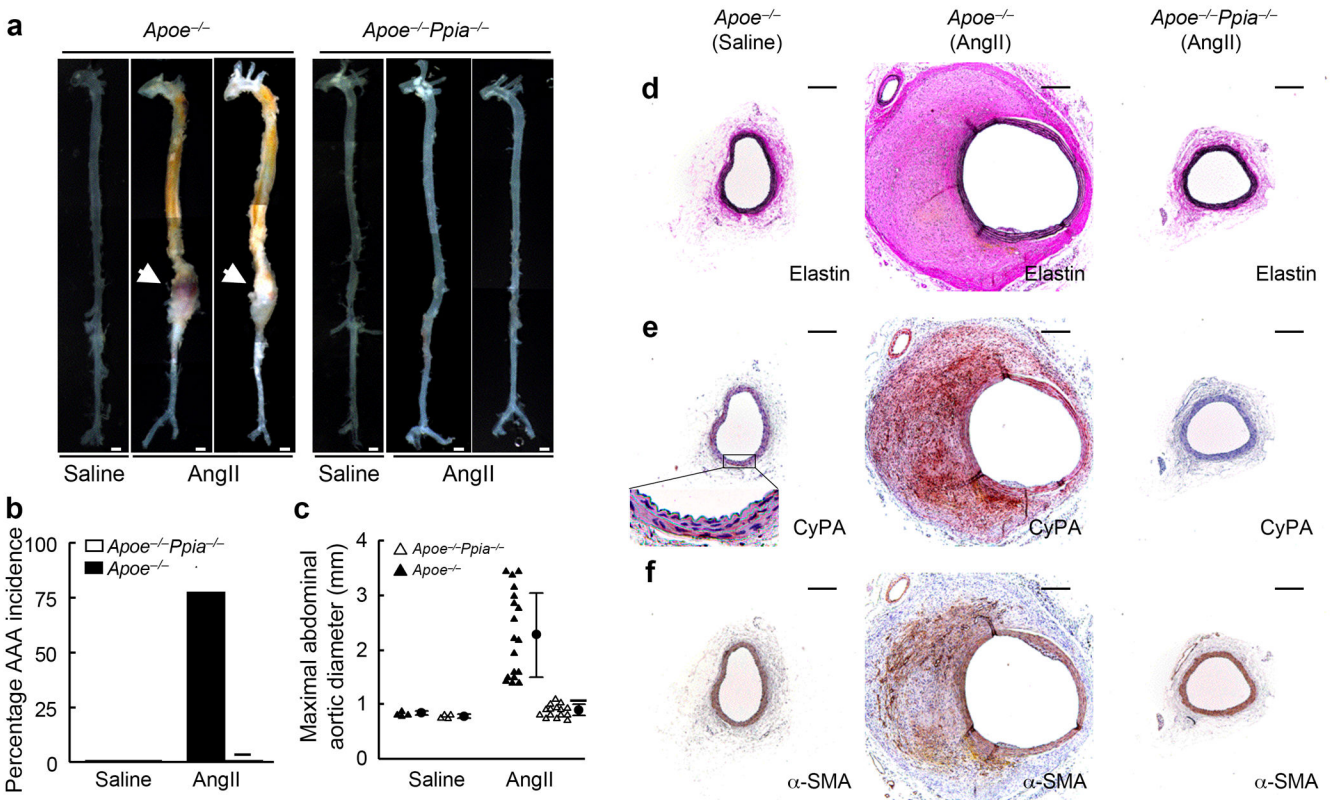

Figure 1.

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 AAA in *Apoe*$^{-/-}$ mice. Scale bars, 1 mm. (**b**) The incidence of AngII-induced AAA was significantly reduced 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*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (n=4, respectively). (**c**) Maximal abdominal aortic diameter was significantly reduced in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice after AngII infusion for 4 weeks. Triangles represent individual mice; circles represent the mean; error bars denote SD. ★$P < 0.01$ 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**) The predominant cellular component in the AAA expressing CyPA was VSMC as revealed by immunostaining for CyPA (**e**) and α-smooth muscle actin (α-SMA) (**f**), in serial sections. All aortic sections were from the suprarenal aorta. Scale bars, 300 μm.

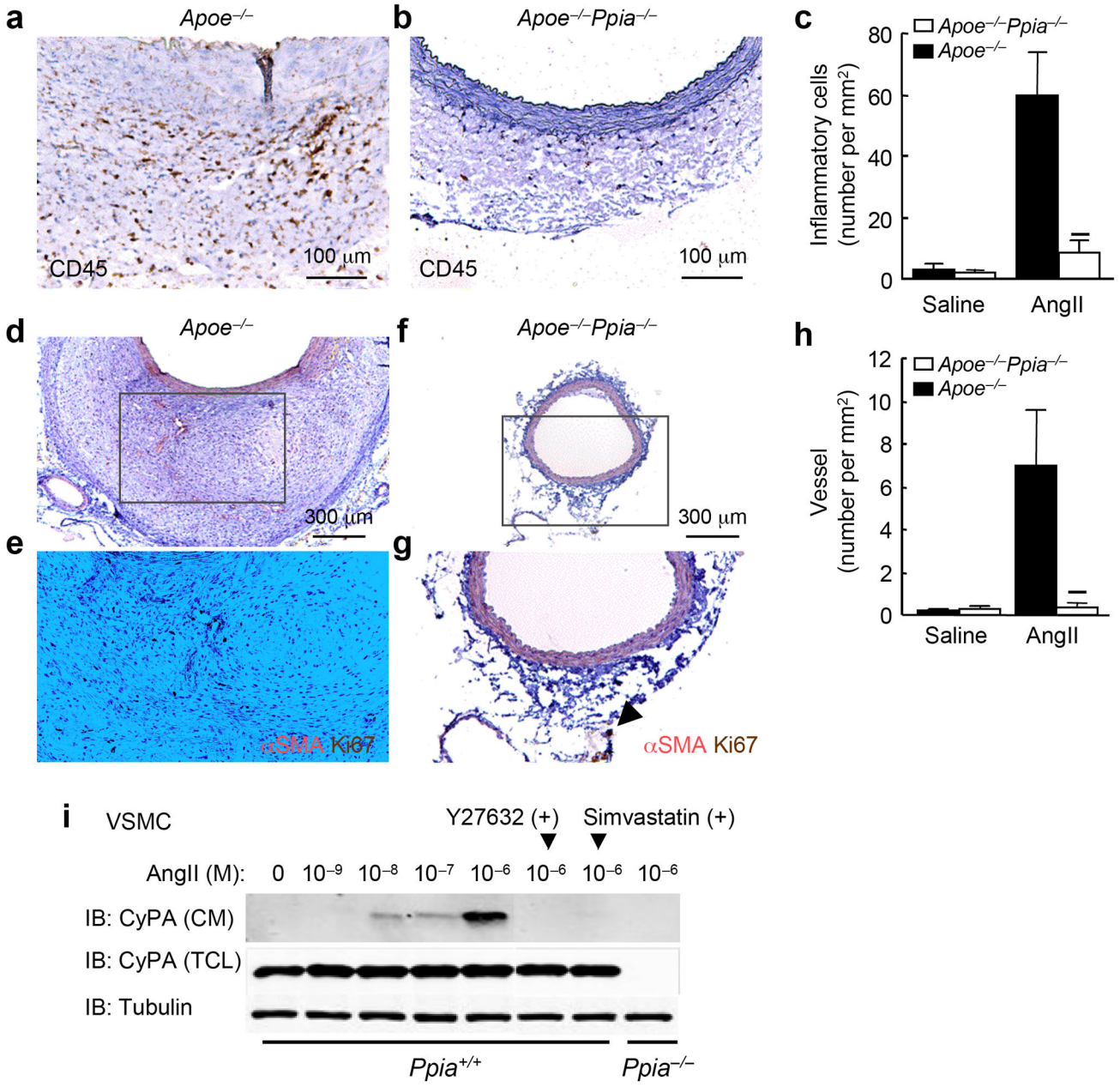

Figure 2.

CyPA deficiency reduces AngII-induced inflammatory cell accumulation and microvessel formation. (**a,b**) Representative CD45 staining of suprarenal aorta 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. (**d**–**g**) Representative immunostaining of α-smooth muscle actin (α-SMA) and Ki67 in suprarenal aorta. (**h**) Number of proliferating microvessels in the aortic wall. Results are mean ± SD. ★$P < 0.01$ compared with *Apoe*$^{-/-}$ mice. (**i**) CyPA is secreted from mouse VSMC in response to AngII. Pretreatment with Rho kinase inhibitor Y27632 (30 μM) and simvastatin (30 μM) for 30 min reduced CyPA secretion. CM, conditioned media; TCL, total cell lysate.

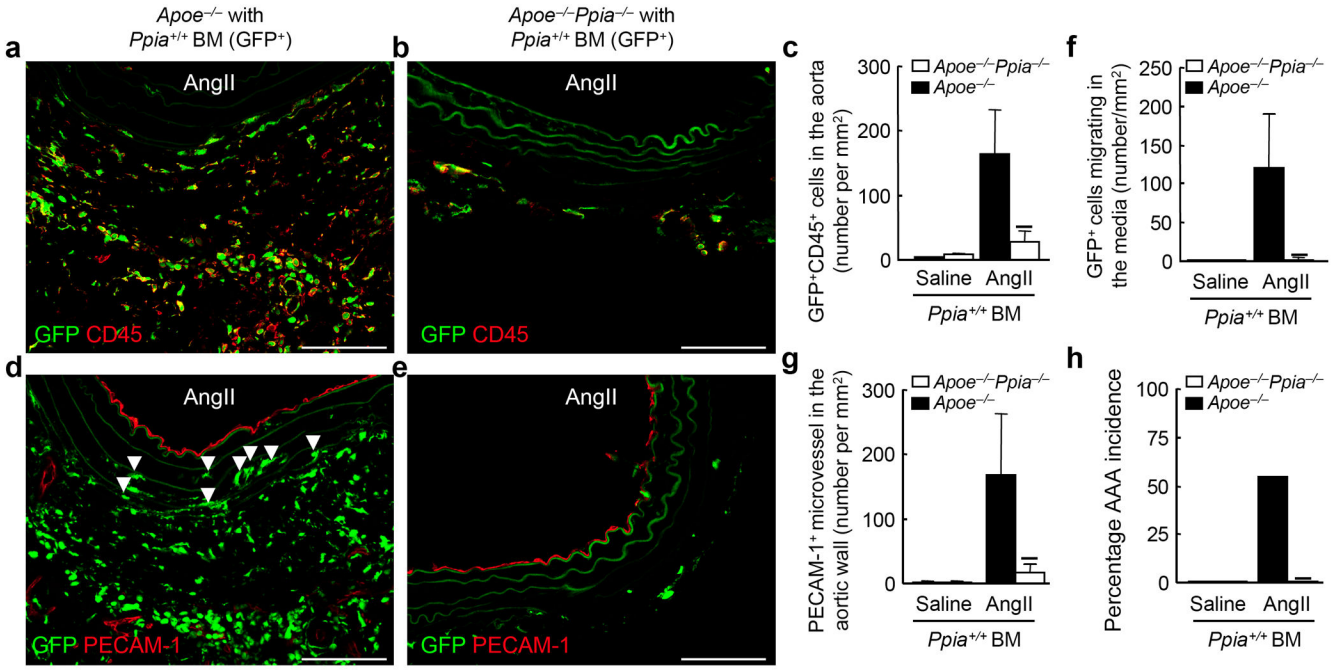

Figure 3.

Bone marrow (BM) reconstitution shows key role for vascular-derived CyPA in AAA formation. *Ppia*$^{+/+}$ BM cells (GFP^{+}) were transplanted into irradiated *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice as described. (**a,b**) Representative CD45 staining (Alexa Fluor 546, red) of suprarenal aorta from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice transplanted with *Ppia*$^{+/+}$ BM, and infused with AngII for 4 weeks. (**c**) Number of migrating GFP^{+}CD45^{+} double-positive cells in the aortic wall in *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice. (**d,e**) Representative PECAM-1 staining (Alexa Fluor 546, red) of suprarenal aorta from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice transplanted with *Ppia*$^{+/+}$ BM and infused with AngII for 4 weeks. Elastic lamina in the aortic wall demonstrate green auto-fluorescence. Arrows indicate migrating GFP^{+} cells in the media (**d**). Number of migrating GFP^{+} cells in the media (**f**) or PECAM-1^{+} microvessels (**g**) were dramatically higher in the aortic wall of *Apoe*$^{-/-}$ compared to *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice. (**h**) The incidence of AAA in *Apoe*$^{-/-}$ (n = 9) was much higher than *Apoe*$^{-/-}$*Ppia*$^{-/-}$ (n = 8) mice reconstituted with *Ppia*$^{+/+}$ bone marrow after AngII infusion for 4 weeks. Results are mean ± SD. ★$P < 0.01$ compared with *Apoe*$^{-/-}$ mice. Scale bars, 100 μm.

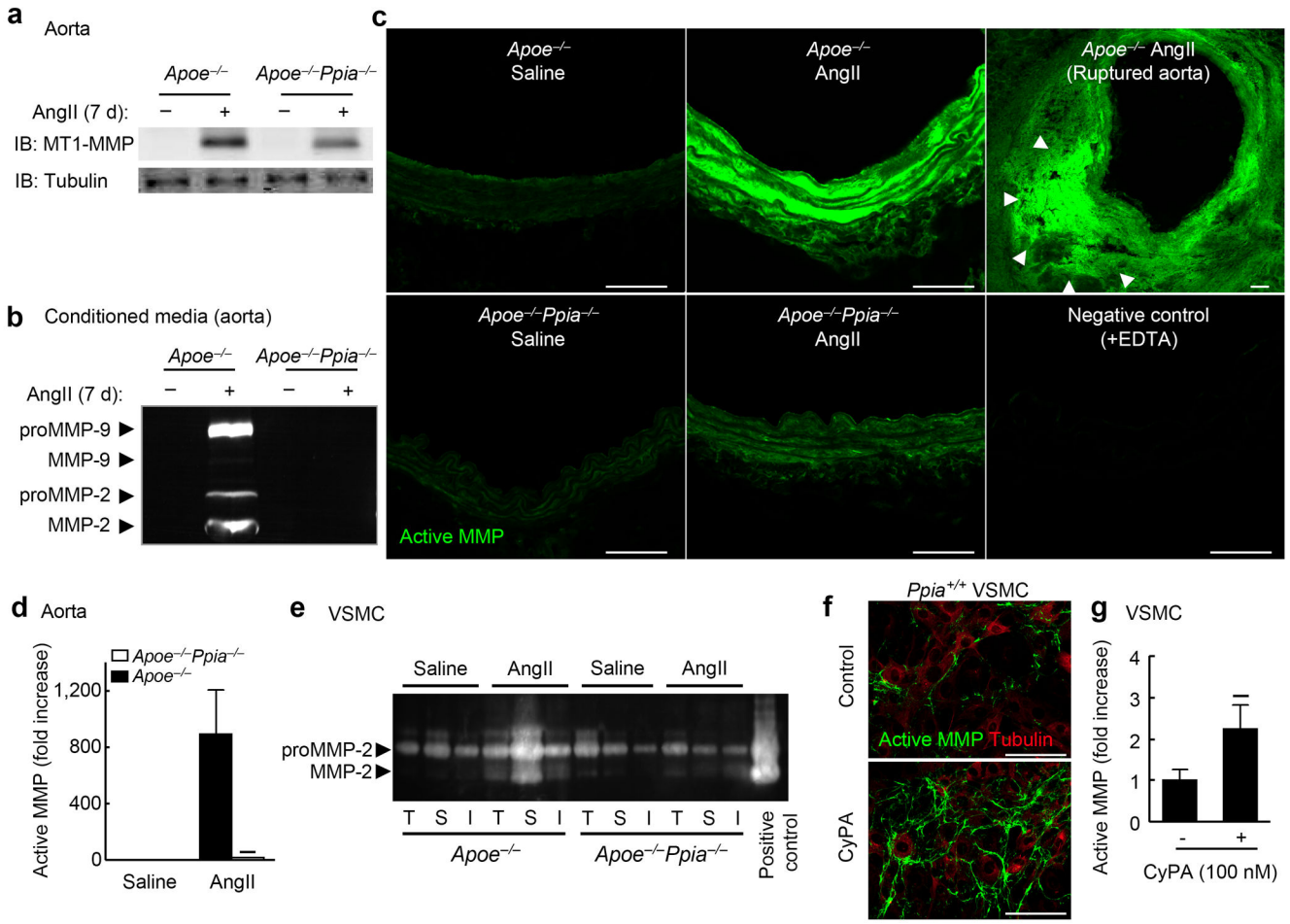

Figure 4.

CyPA is crucial for secretion and activation of MMPs. (**a**) Representative western blot of MT1-MMP expression in mouse aorta after 7 d infusion of AngII. (**b**) Gelatin zymography for conditioned media from whole aorta organ culture. Aortas from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice infused with saline or AngII were incubated in media for 20 h. (**c**) In situ zymography for gelatinase activity. Aortas from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice infused with saline or AngII for 7 days were analysed. Scale bars, 100 μm. (**d**) Densitometric analysis of MMP activity (DQ gelatin) changes relative to the density of MMP activity in control *Apoe*$^{-/-}$ mice (saline-infused). Results are mean ± SD. ★$P < 0.01$ compared with *Apoe*$^{-/-}$ mice. (**e**) Gelatin zymography for VSMC harvested separately from the thoracic aorta (T), suprarenal aorta (S), and infrarenal aorta (I) of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice. VSMC from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice harvested from different portions of aorta were stimulated with AngII (1 μM) for 24 h. (**f**) Representative in situ zymography (DQ gelatin) of *Ppia*$^{+/+}$ VSMC and immunostaining with α-tubulin after stimulation with CyPA (100 nM) for 4 h. (**g**) Densitometric analysis of MMP activity changes relative to the density of MMP activity in control VSMC. ★$P < 0.01$ vs. control VSMC. Results are mean ± SD of 6 independent experiments.

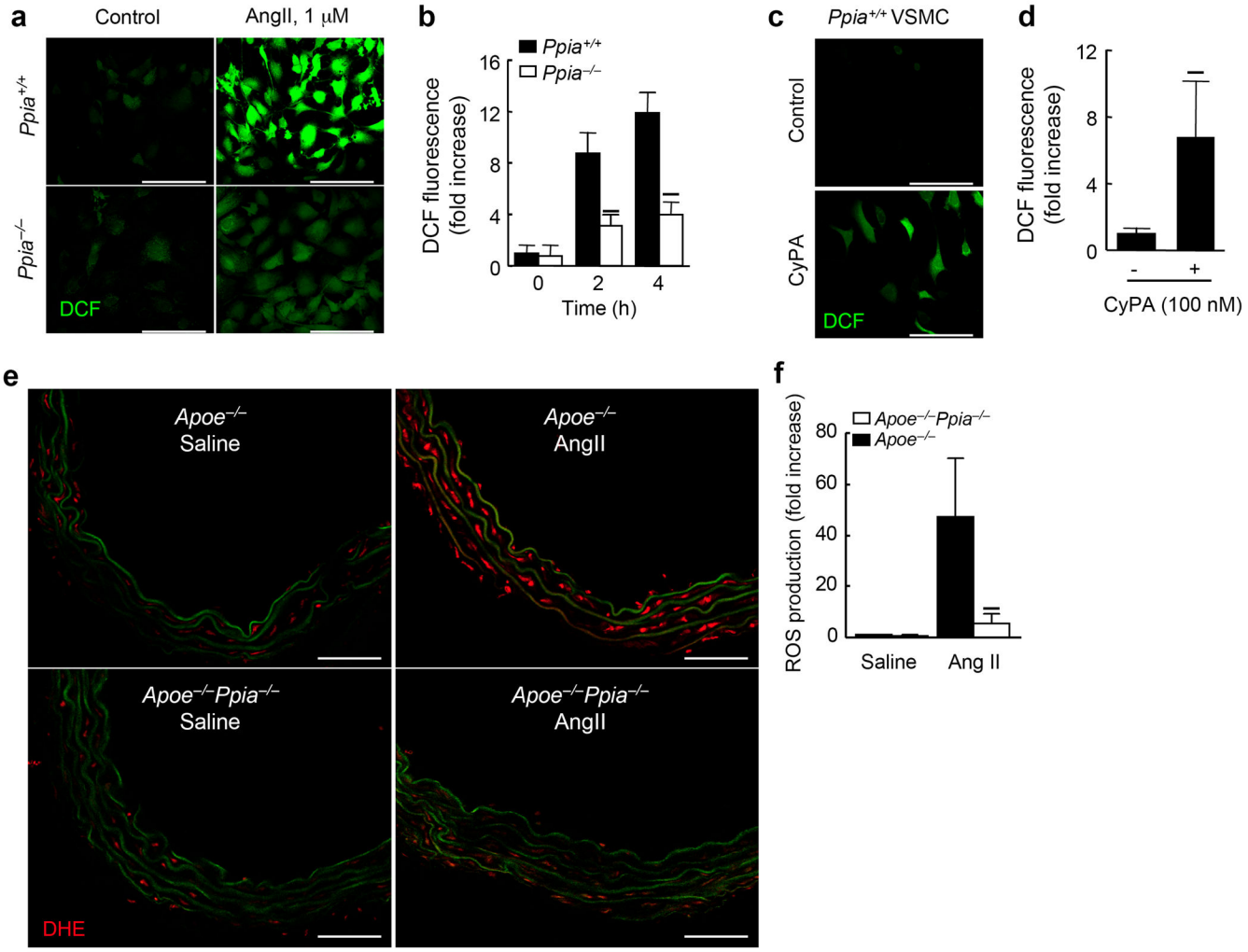

Figure 5.

AngII-induced ROS formation in VSMC requires CyPA. (**a**) Representative DCF staining of mouse aortic VSMC. AngII-induced ROS generation was decreased in CyPA-deficient VSMC. (**b**) Densitometric analysis of DCF fluorescence in response to AngII shows ~60% reduction in *Ppia*$^{-/-}$ VSMC at 4 h. Results are mean ± SD of 5 independent experiments. ★$P < 0.01$ compared with *Ppia*$^{+/+}$ VSMC. (**c**) Representative DCF staining of *Ppia*$^{+/+}$ VSMC in response to 100 nM CyPA. (**d**) Densitometric analysis of DCF fluorescence in *Ppia*$^{+/+}$ VSMC in response to 100 nM CyPA. Results are mean ± SD of 5 independent experiments. ★$P < 0.01$ compared with control VSMC. (**e**) In situ dihydroethidium (DHE) staining of mouse aorta showed decreased DHE staining in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ aortas. Aortas from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice infused with saline or AngII for 7 d were analysed. Media green fluorescence is from elastin fiber autofluorescence, which appeared both in control and AngII-treated aorta. All sections are shown with the lumen above. Scale bars, 100 μm. (**f**) Densitometric analysis of DHE fluorescence relative to control *Apoe*$^{-/-}$ mice (saline-infused). Results are mean ± SD. ★$P < 0.01$ compared with *Apoe*$^{-/-}$ mice.

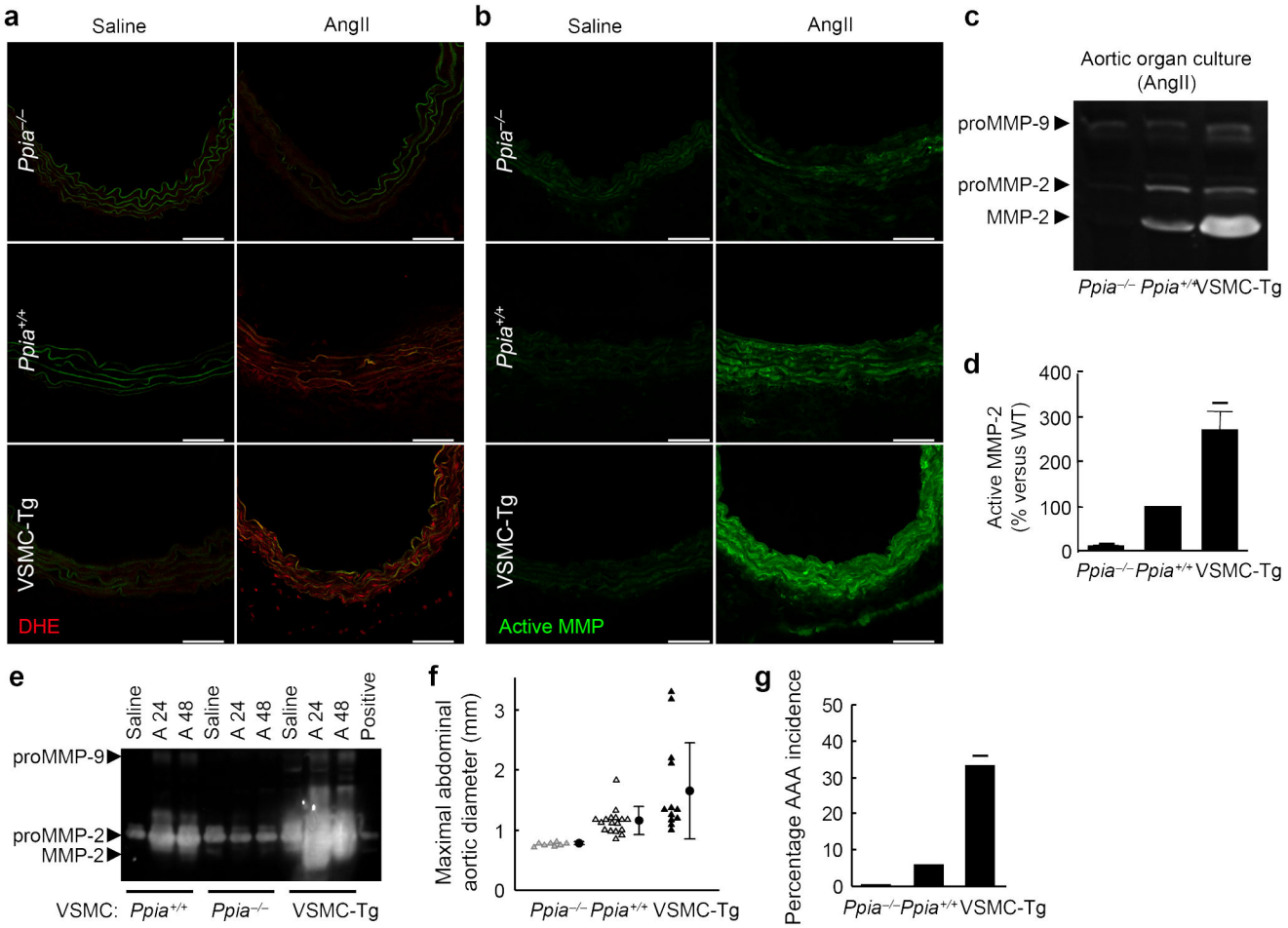

Figure 6.

VSMC-derived CyPA plays a crucial role for aortic ROS production, MMP-2 activation, and AAA formation. (**a,b**) DHE staining and in situ zymography of supra-renal aorta after treatment with saline or AngII for 7 d. There was increased DHE fluorescence in response to AngII with relative levels: VSMC-Tg > *Ppia*$^{+/+}$ > *Ppia*$^{-/-}$. All sections are shown with the lumen above. Scale bars, 100 μm. (**c**) Representative gelatin zymography of conditioned media from mouse aorta after AngII-infusion for 7 d. (**d**) Active MMP-2 in conditioned media from AngII-treated aortic organ culture shows relative activity: VSMC-Tg > *Ppia*$^{+/+}$ > *Ppia*$^{-/-}$. ★$P < 0.01$ vs. *Ppia*$^{+/+}$ aorta. Results are mean ± SD of 3 independent experiments. (**e**) Representative gelatin zymography of aortic VSMC from *Ppia*$^{+/+}$, *Ppia*$^{-/-}$, VSMC-Tg mice after treatment with saline, AngII for 24 h (A24), or AngII for 48 h (A48). Positive; MMP-2 positive control. (**f**) Maximal abdominal aortic diameter significantly increased in VSMC-Tg mice 4 weeks after AngII infusion. Triangles represent individual mice; circles represent the mean; error bars denote SD. (**g**) The incidence of AngII-induced AAA was significantly increased in VSMC-Tg mice ($n = 12$) compared with *Ppia*$^{+/+}$ mice ($n = 17$). There was no AAA induction in *Ppia*$^{-/-}$ mice ($n = 8$). ★$P < 0.01$ compared with AngII-infused *Ppia*$^{+/+}$ mice.