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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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Aab Cardiovascular Research Institute and Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, NY (K.S., B.C.B.), USA; Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai (K.S., H.S.), Japan

Mailing address: Kimio Satoh, MD, PhD, Assistant Professor, Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan. E-mail: satoh-k@cardio.med.tohoku.ac.jp or Bradford C. Berk, MD, PhD, Aab Cardiovascular Research Institute, University of Rochester, Box CVRI, 601 Elmwood Avenue, Rochester, NY 14642, USA. E-mail: bradford_berk@urmc.rochester.edu

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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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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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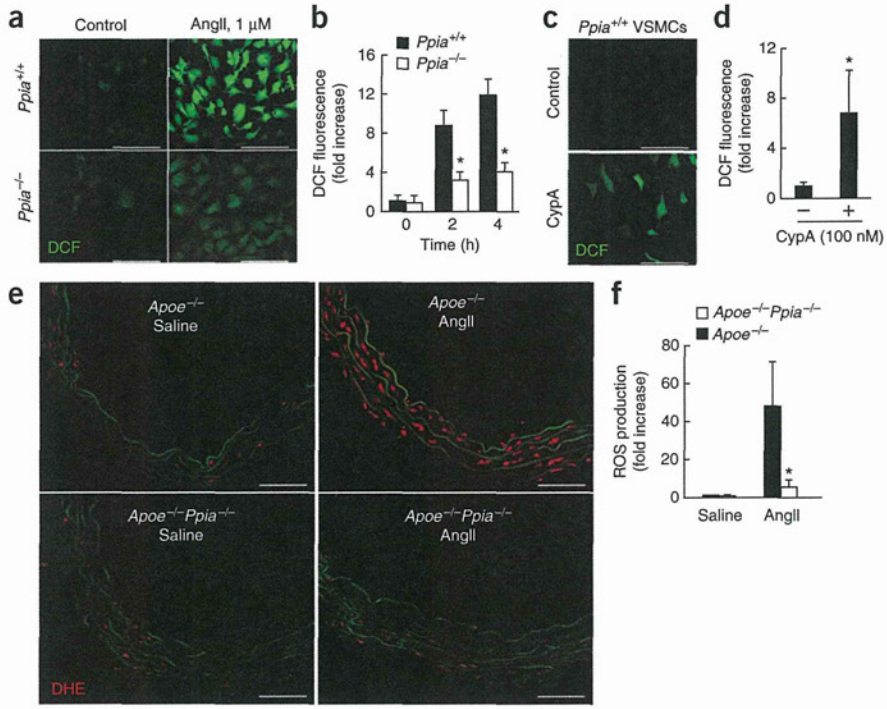

Figure 5 AngII-induced ROS formation in VSMCs requires CypA. (**a**) Representative dichlorofluorescein (DCF) staining of aortic VSMCs. Control, saline. Scale bars, 50 μm. (**b**) Densitometric analysis of DCF fluorescence in response to AngII. Results are means ± s.d. of five independent experiments. $*P < 0.01$ 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**

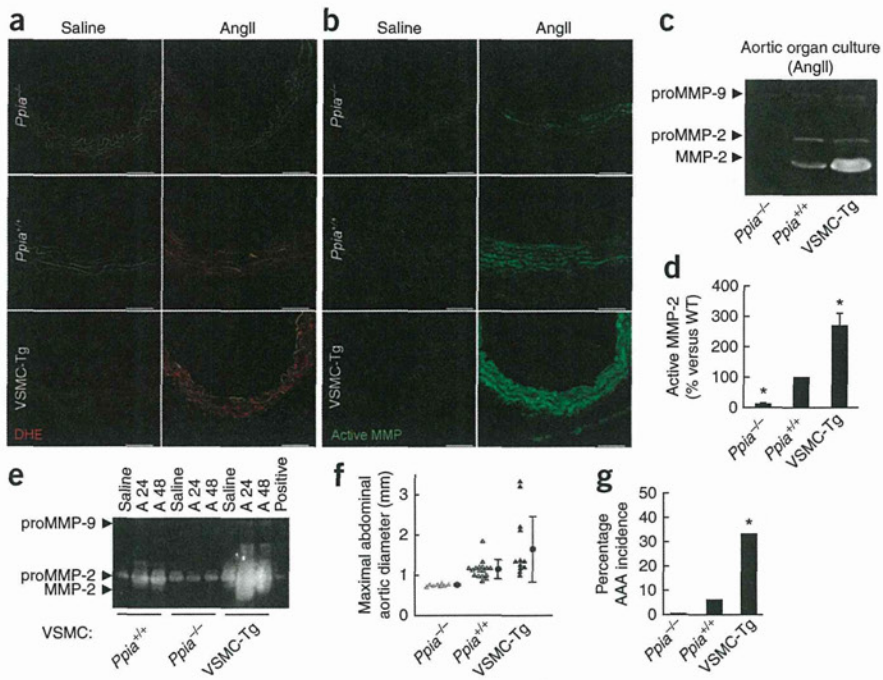

Figure 6 VSMC-derived CypA has a crucial role in aortic ROS production, MMP-2 activation and AAA formation. (**a**,**b**) DHE staining (**a**) and *in situ* zymography (**b**) of suprarenal aortas from mice of the indicated genotypes after treatment with saline or AngII for 7 d. All sections are shown 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.

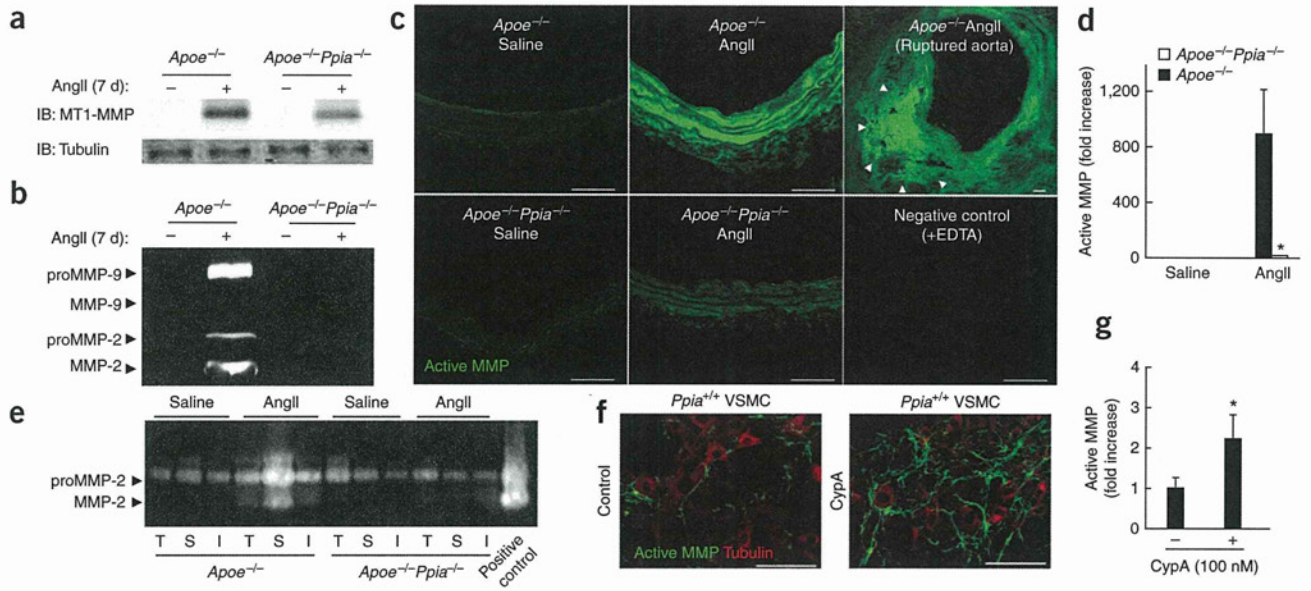

Figure 4 CypA is crucial for secretion and activation of MMPs. (**a**) Representative western blot of MT1-MMP expression in aortas of mice of the indicated genotypes after infusion of AngII for 7 d. $n = 3$ per group. (**b**) Gelatin zymography of conditioned medium from whole-aorta organ culture. Aortas 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

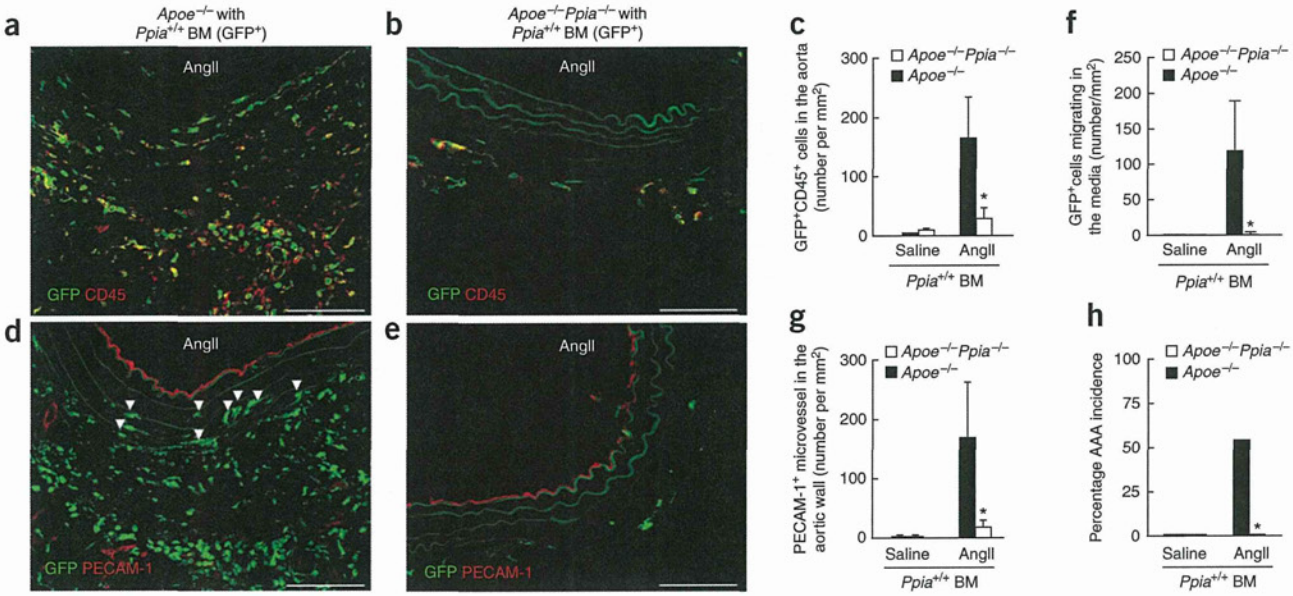

Figure 3 Bone marrow (BM) reconstitution shows a key role for vascular-derived CypA in AAA formation. *Ppia*$^{+/+}$ BM cells (GFP$^+$) were transplanted into irradiated *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice as described in the **Supplementary Methods** online. (**a**,**b**) Representative 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

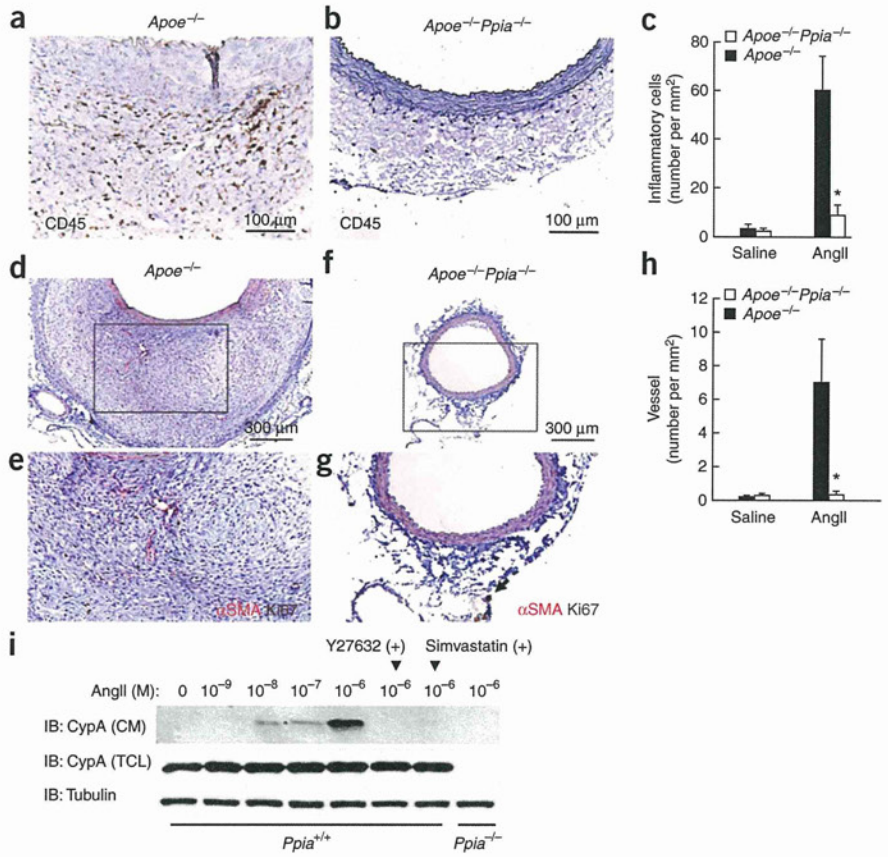

Figure 2 CypA deficiency reduces AngII-induced inflammatory cell accumulation and microvessel formation. (**a**,**b**) Representative CD45 staining of suprarenal aortas from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *Ppia*$^{-/-}$ mice infused with AngII for 4 weeks. (**c**) Number of migrating CD45^{+} cells 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

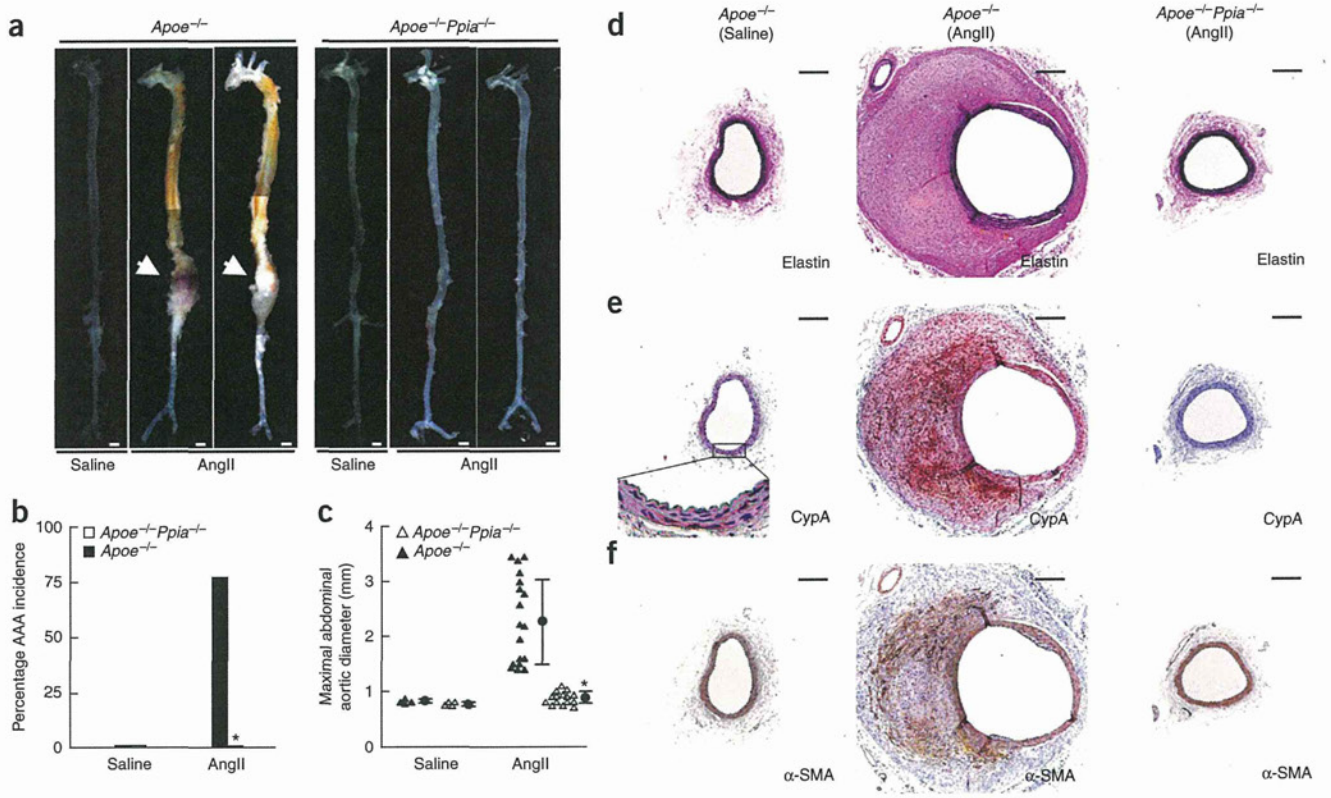

Figure 1 CypA deficiency prevents AngII-induced AAA formation. *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice were infused with AngII or saline for 4 weeks. (**a**) Representative photographs showing macroscopic features of aneurysms induced by AngII. The arrows indicate typical AAAs in *Apoe*$^{-/-}$ 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

nature medicine

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

Kimio Satoh[1,3], Patrizia Nigro[1], Tetsuya Matoba[1], Michael R O'Dell[1], Zhaoqiang Cui[1], Xi Shi[1], Amy Mohan[1], Chen Yan[1], Jun-ichi Abe[1], Karl A Illig[2] & Bradford C Berk[1]

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].

[1]Aab Cardiovascular Research Institute and Department of Medicine and [2]Division of Vascular Surgery, University of Rochester School of Medicine and Dentistry, Rochester, New York, USA. [3]Present address: Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Aoba-ku, Sendai, Japan. Correspondence should be addressed to B.C.B. (bradford_berk@urmc.rochester.edu).

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