厚生労働科学研究費補助金

創薬基盤推進研究事業

大動脈瘤治療薬開発を目指した基礎的・臨床的基盤研究

平成 22 年度　総括研究報告書

研究代表者　佐藤　公雄

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総括研究報告書

大動脈瘤治療薬開発を目指した基礎的・臨床的基盤研究

研究代表者　佐藤　公雄　東北大学大学院医学系研究科循環器内科学・助教

研究要旨

【目的】動脈硬化や大動脈瘤は血管内皮機能、血管平滑筋細胞活性化、炎症細胞浸潤などが複雑に相互作用して進展し、最終的に破綻する。進展や破綻には、慢性炎症が重要因子として働いていると考えられているが、そのメカニズムは不明な点が多い。我々はこれまでRho-kinaseが心血管疾患を促進する重要なシグナル伝達系であることを証明してきた。一方我々は、酸化ストレス下で血管平滑筋細胞より分泌される18kDaの炎症促進蛋白**サイクロフィリンA（CyPA）**を同定し、心血管疾患促進の重要因子であることを証明した。さらに近年、酸化ストレスによるCyPAの分泌はRho-kinase依存性であり、**両者が密接に絡み合って相加・相乗効果を生む酸化ストレス増幅系を形成する**ことを発見した。すなわち、新規炎症蛋白CyPAとRho-kinaseとの相互作用は、**「酸化ストレス増幅機構」**の基盤を担い、大動脈瘤を初めとするあらゆる心血管疾患の発症機構の根幹に関わる可能性がある。本研究では、**大動脈瘤の進行におけるRho-kinase/ CyPA系に焦点**し、その制御・破綻機構の解明および**バイオマーカーとしての可能性**を探る。

【方法】本研究は、サイクロフィリンAをターゲットとした活動性大動脈瘤の評価法開発および活動性制御を目指した実際の治療を視野にいれた詳細な研究を行う。研究方法としては、**大動脈瘤手術サンプル**を用いた解析に加え、**血清濃度測定法（ELISA）の開発**、**in vivo イメージング**（マウス）、PET（ヒト）による**病変部位検出法**の開発を行う。既に開発済みである当科独自の臓器特異的Rho-kinase遺伝子欠損マウス、臓器特異的Rho-kinase遺伝子過剰発現マウス、CyPA遺伝子欠損マウス、CyPA受容体遺伝子欠損マウス（ApoE欠損背景）を駆使した動物モデルの検討に加えて、当科が保有するヒト冠動脈硬化病変検体、心疾患ごとにライブラリー化を進めている患者血清を用いて、臨床的意義も平行して検討する。

【期待される成果】サイクロフィリンAは、まさに心臓や血管の生理的恒常性を失わせる、酸化ストレス増幅の重要蛋白であることが、動物実験で証明された。この知見をヒトの医療に応用する必要がある。サイクロフィリンAは**大動脈瘤発症・動脈硬化破綻の必須蛋白**であり、血清サイクロフィリンA濃度はその早期発見や活動性評価に有効である可能性が高い。また、サイクロフィリンAと免疫抑制薬シクロスポリンとの結合性に着目した**分子修飾薬開発**により、**体内での部位診断**も可能である。仮に、血清サイクロフィリンA濃度が心筋梗塞や大動脈瘤破裂の発症前予測に少しでも情報を与えてくれるならば、急増するメタボ症例（脳梗塞・心筋梗塞・大動脈瘤予備軍）の中から、積極的治療介入すべき患者をより効率的に発見することができる。そして、医師不足の現実、膨らみ続ける医療費、そして経済的メリットの全ての目的を達成できる可能性がある。サイクロフィリンAの分泌抑制もしくは細胞外受容体阻害に着目した治療薬の開発も期待でき、**日本発の新規動脈性疾患治療薬開発**に繋がる可能性がある。

分担研究者氏名・所属機関名および所属機関における職名

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A. 研究目的

我が国では、生活の欧米化により動脈硬化性疾患が増加しており、特に心筋梗塞や大動脈瘤の患者数が年々増加している。また、その予備軍とも言えるメタボリック症候群の患者数が激増しており、経済的・効率的にそうした患者を早期発見・重症度評価する方法の開発が待ち望まれている。

血管内皮・血管平滑筋と炎症細胞の相互作用が血管の恒常性維持に重要であり、酸化ストレスはその恒常性の破綻に重要であることが注目され始めているが、その詳細なメカニズムは未解明であった。我々は最近、酸化ストレス下で血管平滑筋細胞より分泌される18kDaの新規蛋白サイクロフィリンAを同定し、これが血管内皮障害・血管平滑筋細胞増殖・サイトカイン分泌・炎症細胞活性化・MMP活性化の全てを制御する重要因子であり、大動脈瘤破裂に必須であることを報告した。

これらの発見を踏まえて、本研究では実際の臨床応用を目指したトランスレーショナルリサーチを開始することを目的としている。具体的には、サイクロフィリン Aをターゲットとした活動性大動脈瘤の早期発見、活動性評価、およびその内科的治療法の開発を目指している。ひいては、我が国の活力のある社会の実現に大きく貢献することを目指す。

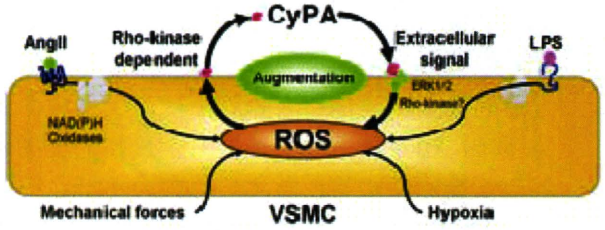

図１． 血管平滑筋への酸化ストレス刺激と細胞外サイクロフィリンＡによる増幅回路の形成　(Satoh K, et al. ***Circulation J*** 74:2249-2256, 2010)。

B. 研究方法

本研究は新しい大動脈瘤治療法を目指した 基礎研究と臨床研究から成る（**図２**）。

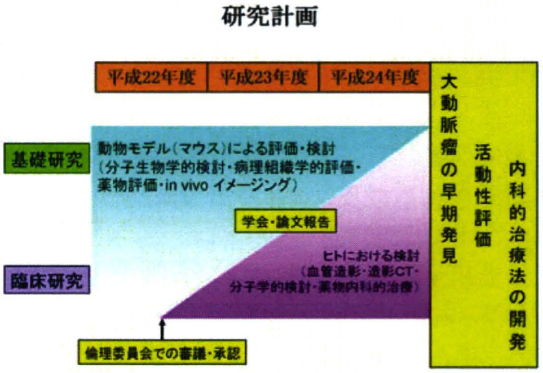

図２．本研究のスケジュール

本研究は、サイクロフィリンAをターゲットとした活動性大動脈瘤の評価法開発および活動性制御を目指した詳細な研究

を行う。研究方法としては、大動脈瘤手術病変サンプルを用いた解析に加え、サイクロフィリンA測定法(ELISA)の開発、in vivo イメージング（マウス）、PET（ヒト）による病変部位検出法の開発を行う。

1. 末梢血 CyPA 濃度の測定法開発

実際の大動脈瘤患者由来血清を用いてサイクロフィリンA濃度を測定する。日常診療において、大動脈瘤術前術後の心臓カテーテル検査を日常的に行っており、手術前後のサイクロフィリンA血中濃度測定を行う。

2. 大動脈瘤発症・進展の制御機構の解明

動脈硬化の進行は喫煙・脂質異常症・糖尿病など酸化ストレス促進因子との密接な関係があり、腹部大動脈瘤発症の基盤となることが分かっている。当科が保有する遺伝子改変マウスを用いた詳細な解析を行う。

3. ヒト動脈硬化病変と血清サイクロフィリンA濃度の関連

冠動脈狭窄を有する動脈硬化患者での血清サイクロフィリンA濃度測定を追加し、動脈硬化との相関関係を評価する。

4. 血管平滑筋におけるサイクロフィリンA受容体の網羅的探索

サイクロフィリンAが大動脈瘤発症に必須であり、細胞外サイクロフィリンAによって細胞内シグナル伝達機構が活性化されることや酸化ストレス産生が増幅されることを報告したが、その特異的受容体の発見には至っていない。そこで、大動脈瘤手術サンプルを用いたサイクロフィリンA受容体の網羅的探索を行う。

5. サイクロフィリンA特異的センサー分子の開発

サイクロフィリンAと結合する新規トレーサー分子を開発する。この新規トレーサーを用いれば、サイクロフィリンAの発現が活性化している活動性大動脈瘤の検出に用いることができ、さらには動脈硬化不安定プラークの検出にも応用できる可能性がある。

6. サイクロフィリンA特異的センサー分子による活動性大動脈瘤 In vivoイメージング

サイクロフィリンA特異的センサー分子を用いてin vivoでの大動脈瘤への集積を確認する。我々が開発したマウス大動脈瘤モデルを用いて、病変検出能の評価を行う。

（倫理面での配慮）

本課題で現在進行中の臨床研究については、いずれも、既に東北大学医学系研究科倫理委員会の承認を得た上で行っている。また、臨床研究の実施に際しては、十分な説明の上、全例から書面で同意を得ている。

現在進行中の動物実験については、いずれも、既に東北大学動物実験専門委員会の承認を得た上で、実験動物に対する

十分な動物愛護上の配慮の下、実験を行っている。

C. 研究結果

1. 末梢血 CyPA 濃度の測定法開発

当科で心臓カテーテル検査を施行する患者の血清サンプルのライブラリー化を進めた。基礎心疾患ごとにサンプル集積が順調に進み、約１０００サンプルを得ることができた。これらの血清サイクロフィリンA濃度をELISA法で測定し、疾患ごとに評価した。本年度は、２０名の腹部大動脈瘤症例の測定を行った。健常者に比して上昇していることが示唆された（**図３**）。ヒト血清中の濃度測定が可能であることが確認できた。

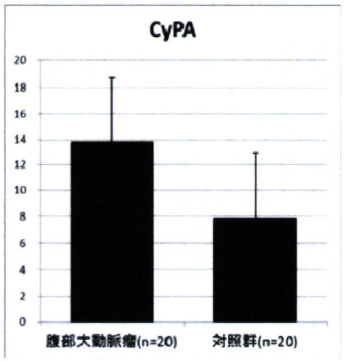

図3. 腹部大動脈瘤患者の末梢血における血清サイクロフィリンA濃度

初期の２０例の解析結果をまとめて循環器学会総会（シンポジウム）で発表した（Circ J. 2011 suppl.）（**図３**）。さらに症例数を増やし、血清サイクロフィリンA濃度の大動脈瘤患者における意義について検証していく。

2. 大動脈瘤発症・進展の制御機構の解明

動脈硬化は酸化ストレス促進因子との密接な関係があり、腹部大動脈瘤発症の基盤となる。高脂肪食負荷 12 週間による動脈硬化モデルを用いた基礎検討により、サイクロフィリン A 欠損マウス (*Apoe-/-Ppia-/-*)では対照マウス(*Apoe-/-*)に比して、著明な動脈硬化抑制効果を認めた（Nigro P, Satoh K, ***J ExpMed*** 208:53-66, 2011）。

図4. 高脂肪食負荷による動脈硬化（サイクロフィリン A 欠損マウスで-65%の抑制効果）

3. ヒト動脈硬化病変と血清サイクロフィリン A 濃度の関連

上記の結果を踏まえ、冠動脈狭窄を有する動脈硬化患者における血清サイクロフィリン A 濃度測定の準備を開始した（既に 450 人の試料採取終了。目標試料数は 1000 とする）。

4. 血管平滑筋におけるサイクロフィリンA受容体の網羅的探索

細胞外サイクロフィリンAによって血管平滑筋細胞内シグナル伝達機構が活性

化することをマウス由来平滑筋細胞を用いて検討してきた。受容体探索に当たり、まずはヒト由来平滑筋細胞を用いて実際にシグナルが入るかどうかの検討を行った。リコンビナント・ヒト・サイクロフィリンA蛋白で刺激すると、ヒト平滑筋細胞のシグナル伝達が活性化することが確認され、アメリカ心臓協会(AHA)年次集会で発表した(***Circulation*** 2010, suppl.)。

5. サイクロフィリンA特異的センサー分子の開発

サイクロフィリンAと結合する新規トレーサー分子を開発する為に、準備を進めてきた。しかし、2011年3月11日の東日本大震災で東北大学アイソトープセンターが壊滅的被害を受けた。復旧に向けた準備が開始されたところである。

6. サイクロフィリンA特異的センサー分子による活動性大動脈瘤 In vivoイメージング

上記に記載したが、想定外の震災の影響で、計画に遅れが生じる。サイクロフィリンA特異的センサー分子を用いてin vivoでの大動脈瘤への集積を確認する予定にしているが、施設の復旧次第では計画通りの病変検出能の評価は難しい可能性がある。

D. 考察

大動脈瘤症例を対象とした臨床研究において、血清サイクロフィリンA濃度測定が早期発見や重症度評価に有効である可能性を示唆する結果を得ており、虚血性心疾患においても有効性が期待できる。今後、現在進行中の臨床研究においてはさらなる解析を追加する。また、基礎研究を加速することにより、サイクロフィリンAに着目した画期的な新規治療法の開発を目指す。

E. 結論

心筋梗塞や大動脈瘤の患者数が年々増加しており、その予備軍とも言えるメタボリック症候群の患者数は激増している。そうした患者を早期発見・重症度評価する方法の開発は、医療経済的にも待ち望まれている。大動脈瘤や虚血性心疾患症例を対象とした初期の評価において、有効性を示唆する結果を得ており、今後、幅広い心血管疾患への応用が期待される。安全で負担のない検査法の開発は、患者の肉体的・精神的負担の軽減や医療費の大幅な削減、我が国の活力のある社会の実現に大きく貢献することが期待される。

F. 健康危険情報

特になし

G. 研究発表

1．論文発表　１１件

1) **Satoh K**, Fukumoto Y, Shimokawa H. Rho-kinase: important new therapeutic target

in cardiovascular diseases. ***Am J Physiol Heart Circ Physiol.*** 2011 (in press).

2) **Satoh K**, Berk BC, Shimokawa H. Vascular-derived reactive oxygen spiecies for homeostasis and diseases. ***Nitric Oxide*** 2011 (in press).

3) **Satoh K**, Fukumoto Y, Nakano M, Kagaya Y, Shimokawa H. Emergence of the Erythropoietin /Erythropoietin Receptor System as a Novel Cardiovascular Therapeutic Target. ***Journal of Cardiovascular Pharmacology*** 2011 (in press).

4) Nigro P,* **Satoh K**,* O'Dell MR, Soe NN, Cui Z, Mohan A, Abe J, Alexis J, Sparks JD, Berk BC. Cyclophilin A is an inflammatory mediator that promotes atherosclerosis in apolipoprotein E-deficient mice. ***Journal of Experimental Medicine.*** 208:53-66, 2011. (*equal contribution)

5) **Satoh K**, Nigro P, Zeidan A, Soe NN, Jaffré F, Oikawa M, O'Dell MR, Cui Z, Menon P, Lu Y, Mohan A, Yan C, Blaxall BC, Berk BC. Cyclophilin A promotes cardiac hypertrophy in apolipoprotein E-deficient mice. ***Arterioscler Thromb Vasc Biol.*** 31:1116-1123, 2011.

6) **Satoh K**, Shimokawa H, Berk BC. Cyclophilin A: a promising new target in cardiovascular therapy. ***Circulation J*** 74:2249-2256, 2010 (review).

7) **Satoh K**, Nigro P, Berk BC. Oxidative stress and vascular smooth muscle cell growth: A mechanistic linkage by cyclophilin A. ***Antioxidants & Redox Signaling*** 12:675-682, 2010.

8) **Satoh K**, Nigro P, Matoba T, O'Dell MR, Mohan A, Yan C, Illig KA, Berk BC. Cyclophilin A enhances vascular oxidative stress and the development of angiotensin II-induced aortic aneurysms. ***Nature Medicine*** 15:649-656, 2009.

9) **Satoh K**, Fukumoto Y, Nakano M, Ishii N, Sugamura K, Shimokawa H. Statin ameliorates hypoxia-induced pulmonary hypertension associated with down-regulated stromal cell-derived factor-1. ***Cardiovascular Research*** 81:226-234, 2009.

10) **佐藤公雄**. 『血管と酸化ストレス』先端医学社 野出孝一編 ***Angiotensin Research*** 8: 2011.

11) **佐藤公雄**, 下川宏明. 『病態把握と治療戦略にバイオマーカーを活かす』エリスロポエチンと酸化ストレス応答性サイクロフィリンA 先端医学社 ***Vascular Medicine*** 2010.

２．学会発表　９件

（１）国内

1) **佐藤公雄**「新しい心血管疾患バイオマーカー」Cyclophilin A Is a Novel Biomarker for Aortic Aneurysms and Atherosclerotic Plaque Instability. （日本循環器学会・シンポジウム 2011 年 8 月 3 日、横浜）

2) **佐藤公雄** Cyclophilin A mediates endothelial damage and promotes recruitment of inflammatory cells and atherosclerosis. 6th International Conference on the Biology, Chemistry, and Therapeutic Applications of Nitric Oxide. Young Investigator's Award 最優秀賞 （国際 Nitric Oxide 学会賞・受賞講演 2010 年 6 月 14 日、京都）

3) **佐藤公雄** Emerging Importance of the Erythropoietin/Erythropoietin Receptor System and Cyclophilin A as Novel Therapeutic Targets in Cardiovascular Medicine. Young Investigator's Award. （日本循環器学会賞・受賞講演 2010 年 3 月 5 日、京都）

4) **佐藤公雄** Cyclophilin A enhances vascular oxidative stress and the development of angiotensin II-induced aortic aneurysms. Young Investigator Award 最優秀賞 （日本血管生物医学会・受賞講演、2009 年 10 月 9 日、東京）

5) **佐藤公雄** Cyclophilin A enhances vascular oxidative stress and the development of angiotensin II-induced aortic aneurysms. Young Investigator Award 最優秀賞（日本血管生物医学会・受賞講演、2009 年 10 月 9 日、東京）

（２）海外

1) **佐藤公雄** Cyclophilin A is an inflammatory mediator that promotes atherosclerosis. Young Investigator's

Award. （アジア血管生物学学会･受賞講演 2010 年 11 月 21 日、香港）

2) **佐藤公雄** Emerging Importance of the Erythropoietin/Erythropoietin Receptor System and Cyclophilin A as Novel Therapeutic Targets in Cardiovascular Medicine. 4th Scientific Meeting of Asian Society for Vascular Biology 招待講演（2010 年 11 月 20 日、香港）

3) **佐藤公雄** Cyclophilin A: a potential molecular marker of instability of atherosclerotic plaque and abdominal aortic aneurysm. Visiting AstraZeneca Mölndal R&D, Sweden 招待講演（2010 年 9 月 2 日、ストックホルム）

4) **佐藤公雄** Cyclophilin A Augments ROS production and Angiotensin II–induced Cardiac Hypertrophy in Mice. American Heart Association, Melvin L. Marcus Young Investigator's Award. （アメリカ心臓協会学会賞･受賞講演、2009 年 11 月 15 日、米国オーランド）

H. 知的所有権の出願・取得状況（予定を含む）

1. 特許取得

肺高血圧症のモデル動物作成法、肺高血圧症治療法、および治療薬物" 特許 国内特許出願;特願 2006-008242 機構整理番号 P038P02, 外国特許出願;独立行政法人科学技術振興機構 機構整理番号 P038P02US(PCT) 佐藤公雄, 下川宏明, 小野栄夫, 菅村和夫, 石井直人

2. 実用新案登録

なし

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
佐藤公雄	血管と酸化ストレス	野出孝一	Angiotensin Research	先端医学社	東京	２０１１	１－１０
佐藤公雄	エリスロポエチンと酸化ストレス応答性サイクロフィリンＡ		Vascular Medicine	先端医学社	東京	２０１０	３５－４５

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Satoh K, et al.	Rho-kinase: important new therapeutic target in cardiovascular diseases.	***Am J Physiol Heart Circ Physiol.***	300	In press	2011
Satoh K, et al.	Vascular-derived reactive oxygen spiecies for homeostasis and diseases.	***Nitric Oxide***		In press	2011
Satoh K, et al.	Emergence of the Erythropoietin /Erythropoietin Receptor System as a Novel Cardiovascular Therapeutic Target.	***Journal of Cardiovascular Pharmacology***		In press	2011
Nigro P, Satoh K, et al.	Cyclophilin A is an inflammatory mediator that promotes atherosclerosis in apolipoprotein E-deficient mice.	***Journal of Experimental Medicine.***	208	53-66	2011
Satoh K, et al.	Cyclophilin A promotes cardiac hypertrophy in apolipoprotein E-deficient mice.	***Arterioscler Thromb Vasc Biol.***	31	1116-1123	2011
Satoh K, et al.	Cyclophilin A: a promising new target in cardiovascular therapy.	***Circulation J***	74	2249-2256	2010
Satoh K, et al.	Oxidative stress and vascular smooth muscle cell growth: A mechanistic linkage by cyclophilin A.	***Antioxidants & Redox Signaling***	12	675-682	2010
Satoh K, et al.	Cyclophilin A enhances vascular oxidative stress and the development of angiotensin II-induced aortic aneurysms.	***Nature Medicine***	15	649-656	2009
Satoh K, et al.	Statin ameliorates hypoxia-induced pulmonary hypertension associated with down-regulated stromal cell-derived factor-1.	***Cardiovascular Research***	81	226-234	2009

研究成果の刊行物・別刷

nature medicine

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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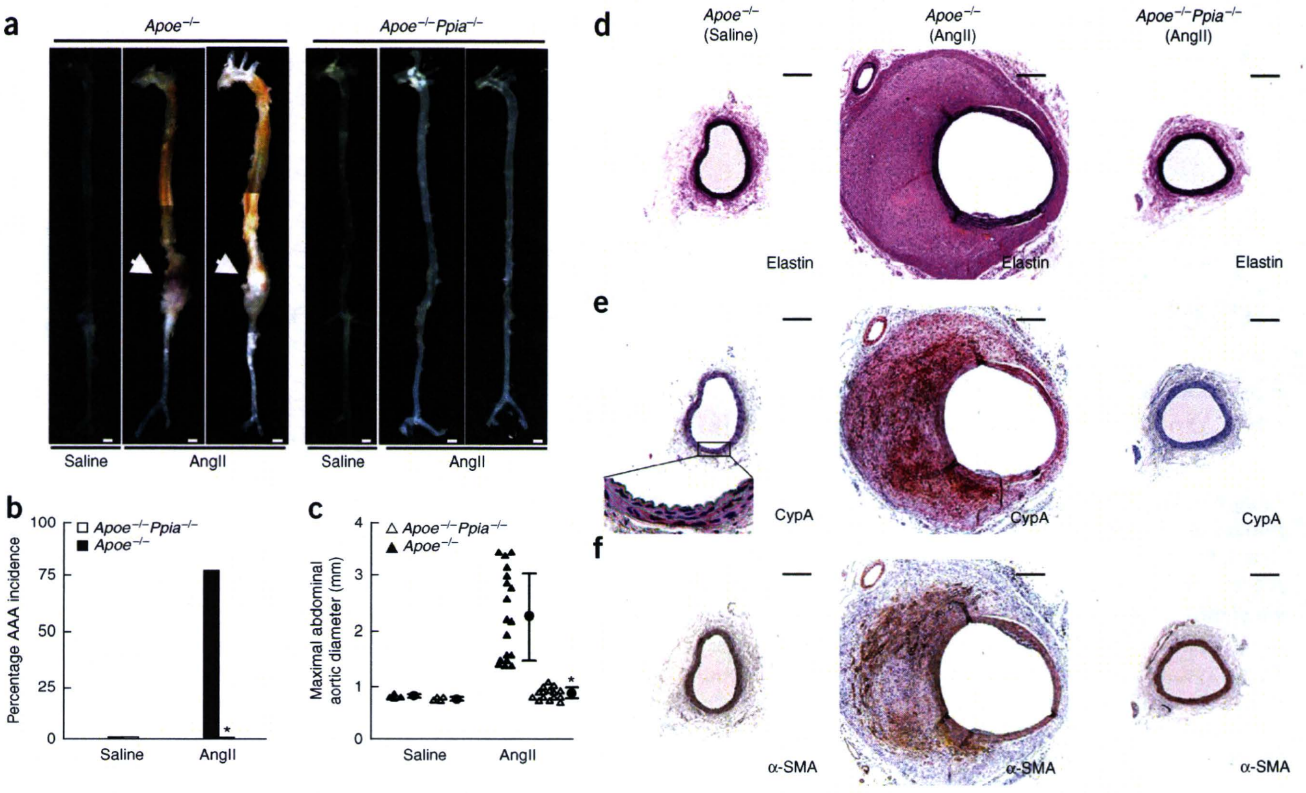

Figure 1 CypA deficiency prevents AngII-induced AAA formation. *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice were infused with AngII or saline for 4 weeks. (**a**) Representative photographs showing macroscopic features of aneurysms induced by AngII. The arrows indicate typical AAAs in *Apoe*$^{-/-}$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

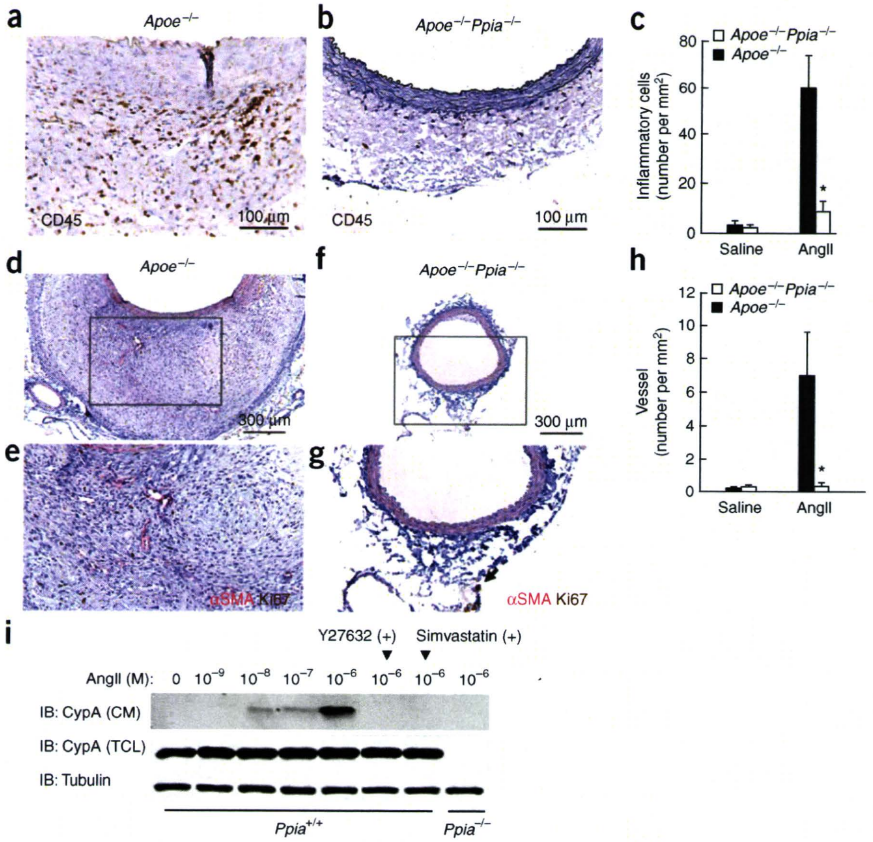

Figure 2 CypA deficiency reduces AngII-induced inflammatory cell accumulation and microvessel formation. (**a**,**b**) Representative CD45 staining of suprarenal aortas from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice infused with AngII for 4 weeks. (**c**) Number of migrating CD45^{+} cells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

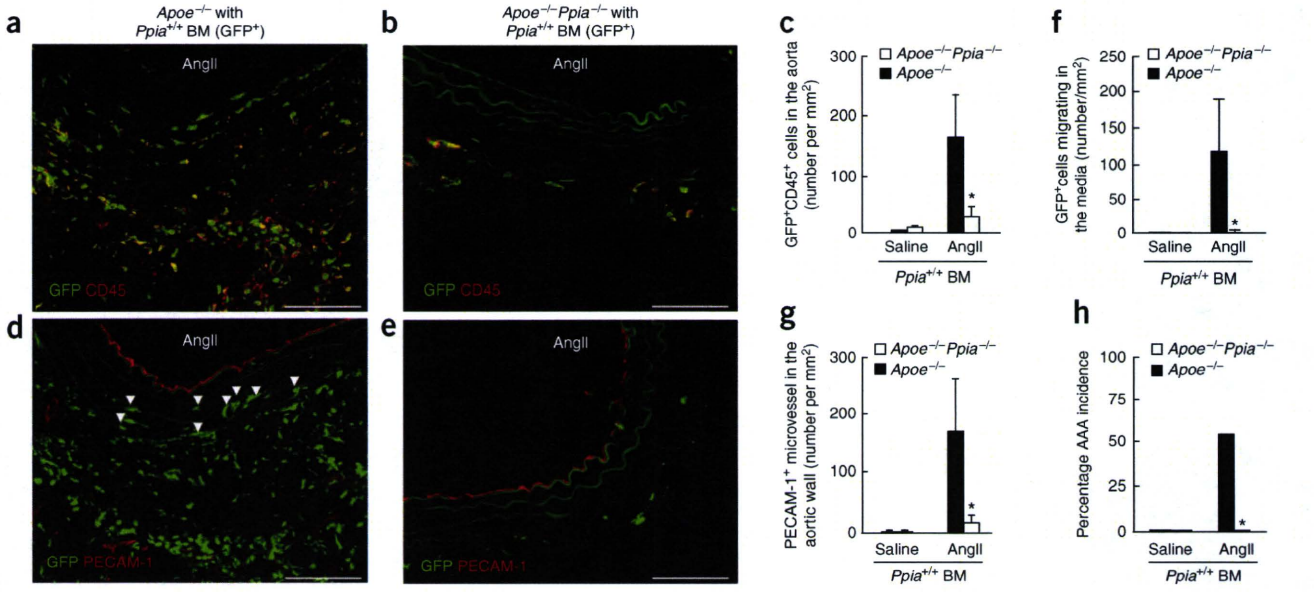

Figure 3 Bone marrow (BM) reconstitution shows a key role for vascular-derived CypA in AAA formation. *Ppia*$^{+/+}$ BM cells (GFP$^+$) were transplanted into irradiated *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice as described in the **Supplementary Methods** online. (**a**,**b**) Representative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

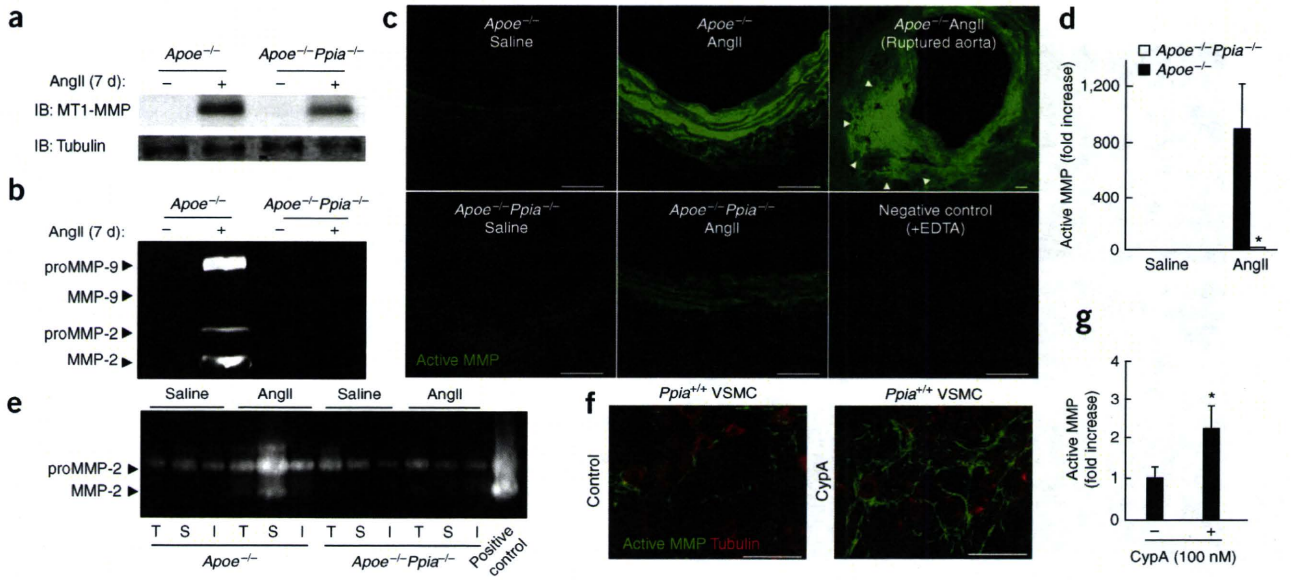

Figure 4 CypA is crucial for secretion and activation of MMPs. (**a**) Representative western blot of MT1-MMP expression in aortas of mice of the indicated genotypes after infusion of AngII for 7 d. $n = 3$ per group. (**b**) Gelatin zymography of conditioned medium from whole-aorta organ culture. Aortas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

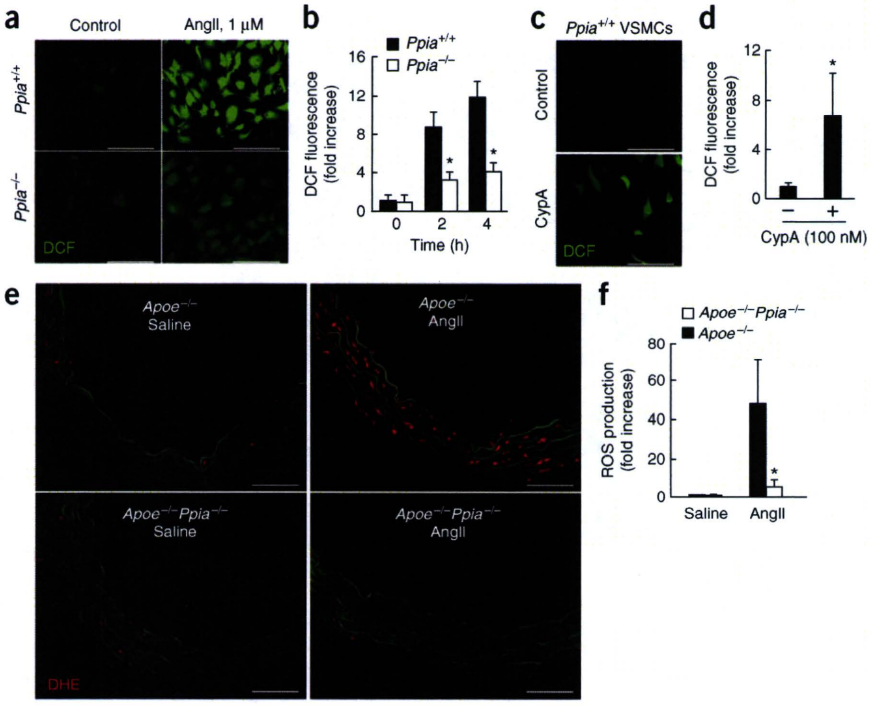

Figure 5 AngII-induced ROS formation in VSMCs requires CypA. (**a**) Representative dichlorofluorescein (DCF) staining of aortic VSMCs. Control, saline. Scale bars, 50 μm. (**b**) Densitometric analysis of DCF fluorescence in response to AngII. Results are means ± s.d. of five independent experiments.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

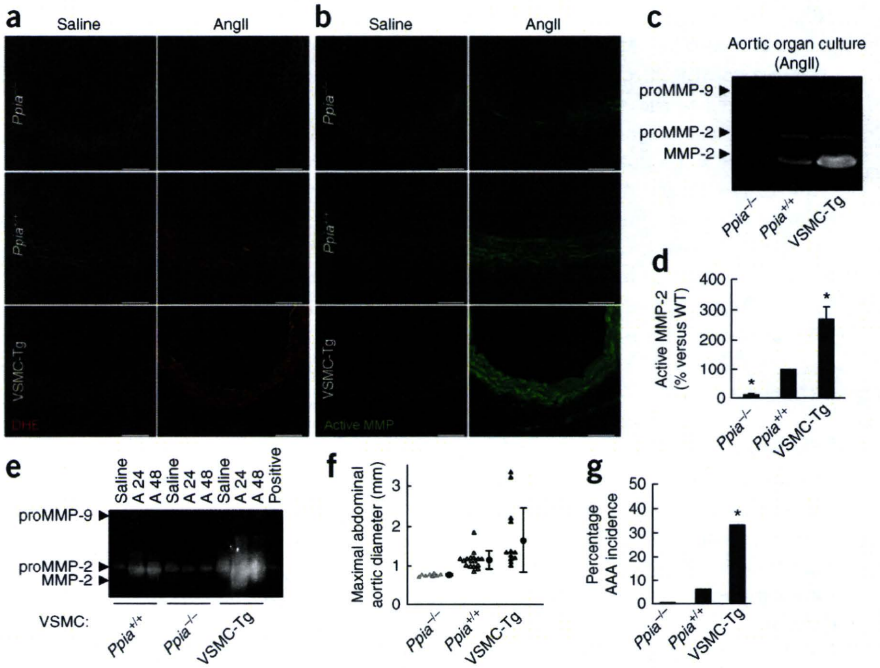

Figure 6 VSMC-derived CypA has a crucial role in aortic ROS production, MMP-2 activation and AAA formation. (**a**,**b**) DHE staining (**a**) and *in situ* zymography (**b**) of suprarenal aortas from mice of the indicated genotypes after treatment with saline or AngII for 7 d. All sections are shown 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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1. Kunieda, T. *et al.* Angiotensin II induces premature senescence of vascular smooth muscle cells and accelerates the development of atherosclerosis via a p21-dependent pathway. *Circulation* **114**, 953–960 (2006).
2. Griendling, K.K. & FitzGerald, G.A. Oxidative stress and cardiovascular injury: Part II: animal and human studies. *Circulation* **108**, 2034–2040 (2003).
3. Taniyama, Y. & Griendling, K.K. Reactive oxygen species in the vasculature: molecular and cellular mechanisms. *Hypertension* **42**, 1075–1081 (2003).
4. Bruemmer, D. *et al.* Angiotensin II–accelerated atherosclerosis and aneurysm formation is attenuated in osteopontin-deficient mice. *J. Clin. Invest.* **112**, 1318–1331 (2003).
5. Sun, J. *et al.* Mast cells promote atherosclerosis by releasing proinflammatory cytokines. *Nat. Med.* **13**, 719–724 (2007).
6. Yoshimura, K. *et al.* Regression of abdominal aortic aneurysm by inhibition of c-Jun N-terminal kinase. *Nat. Med.* **11**, 1330–1338 (2005).
7. Thomas, M. *et al.* Deletion of p47phox attenuates angiotensin II–induced abdominal aortic aneurysm formation in apolipoprotein E–deficient mice. *Circulation* **114**, 404–413 (2006).
8. Gavazzi, G. *et al.* NOX1 deficiency protects from aortic dissection in response to angiotensin II. *Hypertension* **50**, 189–196 (2007).

9. Thompson, R.W. & Baxter, B.T. MMP inhibition in abdominal aortic aneurysms. *Ann. NY Acad. Sci.* **878**, 159–178 (1999).
10. Manning, M.W., Cassis, L.A. & Daugherty, A. Differential effects of doxycycline, a broad-spectrum matrix metalloproteinase inhibitor, on angiotensin II–induced atherosclerosis and abdominal aortic aneurysms. *Arterioscler. Thromb. Vasc. Biol.* **23**, 483–488 (2003).
11. Valentin, F., Bueb, J.L., Kieffer, P., Tschirhart, E. & Atkinson, J. Oxidative stress activates MMP-2 in cultured human coronary smooth muscle cells. *Fundam. Clin. Pharmacol.* **19**, 661–667 (2005).
12. Clempus, R.E. & Griendling, K.K. Reactive oxygen species signaling in vascular smooth muscle cells. *Cardiovasc. Res.* **71**, 216–225 (2006).
13. McCormick, M.L., Gavrila, D. & Weintraub, N.L. Role of oxidative stress in the pathogenesis of abdominal aortic aneurysms. *Arterioscler. Thromb. Vasc. Biol.* **27**, 461–469 (2007).
14. Rajagopalan, S. *et al.* Angiotensin II–mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J. Clin. Invest.* **97**, 1916–1923 (1996).
15. Browatzki, M. *et al.* Angiotensin II stimulates matrix metalloproteinase secretion in human vascular smooth muscle cells via nuclear factor-κB and activator protein 1 in a redox-sensitive manner. *J. Vasc. Res.* **42**, 415–423 (2005).
16. Daugherty, A., Manning, M.W. & Cassis, L.A. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E–deficient mice. *J. Clin. Invest.* **105**, 1605–1612 (2000).
17. Daugherty, A. & Cassis, L.A. Mouse models of abdominal aortic aneurysms. *Arterioscler. Thromb. Vasc. Biol.* **24**, 429–434 (2004).
18. Handschumacher, R.E., Harding, M.W., Rice, J., Drugge, R.J. & Speicher, D.W. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science* **226**, 544–547 (1984).
19. Jin, Z.G. *et al.* Cyclophilin A is a secreted growth factor induced by oxidative stress. *Circ. Res.* **87**, 789–796 (2000).
20. Liao, D.F. *et al.* Purification and identification of secreted oxidative stress–induced factors from vascular smooth muscle cells. *J. Biol. Chem.* **275**, 189–196 (2000).
21. Suzuki, J., Jin, Z.G., Meoli, D.F., Matoba, T. & Berk, B.C. Cyclophilin A is secreted by a vesicular pathway in vascular smooth muscle cells. *Circ. Res.* **98**, 811–817 (2006).
22. Khromykh, L.M. *et al.* Cyclophilin A produced by thymocytes regulates the migration of murine bone marrow cells. *Cell. Immunol.* **249**, 46–53 (2007).
23. Golledge, J., Muller, J., Daugherty, A. & Norman, P. Abdominal aortic aneurysm: pathogenesis and implications for management. *Arterioscler. Thromb. Vasc. Biol.* **26**, 2605–2613 (2006).
24. Saraff, K., Babamusta, F., Cassis, L.A. & Daugherty, A. Aortic dissection precedes formation of aneurysms and atherosclerosis in angiotensin II-infused, apolipoprotein E–deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **23**, 1621–1626 (2003).
25. Gavrila, D. *et al.* Vitamin E inhibits abdominal aortic aneurysm formation in angiotensin II–infused apolipoprotein E–deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **25**, 1671–1677 (2005).
26. Chen, X.L., Tummala, P.E., Olbrych, M.T., Alexander, R.W. & Medford, R.M. Angiotensin II induces monocyte chemoattractant protein-1 gene expression in rat vascular smooth muscle cells. *Circ. Res.* **83**, 952–959 (1998).
27. Bharadwaj, U. *et al.* Effects of cyclophilin A on myeloblastic cell line KG-1 derived dendritic like cells (DLC) through p38 MAP kinase activation(1,2). *J. Surg. Res.* **127**, 29–38 (2005).
28. Satoh, K. *et al.* Important role of endogenous erythropoietin system in recruitment of endothelial progenitor cells in hypoxia-induced pulmonary hypertension in mice. *Circulation* **113**, 1442–1450 (2006).
29. Pyo, R. *et al.* Targeted gene disruption of matrix metalloproteinase-9 (gelatinase B) suppresses development of experimental abdominal aortic aneurysms. *J. Clin. Invest.* **105**, 1641–1649 (2000).
30. Longo, G.M. *et al.* Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms. *J. Clin. Invest.* **110**, 625–632 (2002).
31. Visse, R. & Nagase, H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ. Res.* **92**, 827–839 (2003).
32. Yurchenko, V. *et al.* Active site residues of cyclophilin A are crucial for its signaling activity via CD147. *J. Biol. Chem.* **277**, 22959–22965 (2002).
33. Rajagopalan, S., Meng, X.P., Ramasamy, S., Harrison, D.G. & Galis, Z.S. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases *in vitro*. Implications for atherosclerotic plaque stability. *J. Clin. Invest.* **98**, 2572–2579 (1996).
34. Castier, Y., Brandes, R.P., Leseche, G., Tedgui, A. & Lehoux, S. p47phox-dependent NADPH oxidase regulates flow-induced vascular remodeling. *Circ. Res.* **97**, 533–540 (2005).
35. Satoh, K. *et al.* Cyclophilin A mediates vascular remodeling by promoting inflammation and vascular smooth muscle cell proliferation. *Circulation* **117**, 3088–3098 (2008).
36. Mehta, P.K. & Griendling, K.K. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am. J. Physiol. Cell Physiol.* **292**, C82–C97 (2007).
37. Luchtefeld, M. *et al.* Angiotensin II induces MMP-2 in a p47phox-dependent manner. *Biochem. Biophys. Res. Commun.* **328**, 183–188 (2005).
38. Eagleton, M.J. *et al.* Early increased MT1-MMP expression and late MMP-2 and MMP-9 activity during angiotensin II induced aneurysm formation. *J. Surg. Res.* **135**, 345–351 (2006).
39. Shimokawa, H. & Takeshita, A. Rho-kinase is an important therapeutic target in cardiovascular medicine. *Arterioscler. Thromb. Vasc. Biol.* **25**, 1767–1775 (2005).
40. Wang, Y.X. *et al.* Fasudil, a Rho-kinase inhibitor, attenuates angiotensin II–induced abdominal aortic aneurysm in apolipoprotein E–deficient mice by inhibiting apoptosis and proteolysis. *Circulation* **111**, 2219–2226 (2005).
41. Habashi, J.P. *et al.* Losartan, an AT1 antagonist, prevents aortic aneurysm in a mouse model of Marfan syndrome. *Science* **312**, 117–121 (2006).
42. Cassis, L.A., Rateri, D.L., Lu, H. & Daugherty, A. Bone marrow transplantation reveals that recipient AT1a receptors are required to initiate angiotensin II–induced atherosclerosis and aneurysms. *Arterioscler. Thromb. Vasc. Biol.* **27**, 380–386 (2007).
43. Ejiri, J. *et al.* Oxidative stress in the pathogenesis of thoracic aortic aneurysm: protective role of statin and angiotensin II type 1 receptor blocker. *Cardiovasc. Res.* **59**, 988–996 (2003).
44. Guo, H. *et al.* Characterization of the gene for human EMMPRIN, a tumor cell surface inducer of matrix metalloproteinases. *Gene* **220**, 99–108 (1998).
45. Haug, C., Lenz, C., Diaz, F. & Bachem, M.G. Oxidized low-density lipoproteins stimulate extracellular matrix metalloproteinase inducer (EMMPRIN) release by coronary smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* **24**, 1823–1829 (2004).
46. Chen, X.F. *et al.* Extracellular matrix metalloproteinase inducer (EMMPRIN) is present in smooth muscle cells of human aneurysmal aorta and is induced by angiotensin II *in vitro*. *Clin. Sci. (Lond.)* published online, doi:10.1042/CS20080235 (2008).

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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