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大動脈瘤治療薬開発を目指した基礎的・臨床的基盤研究

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I． 総括研究報告

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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

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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たし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、血漿サイクロフィリン 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。

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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20kDa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。

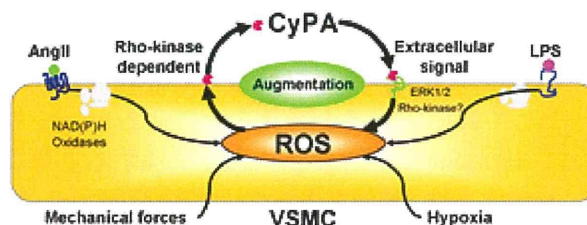
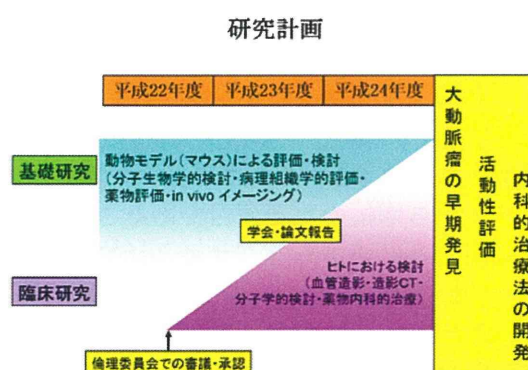


図1. 血管平滑筋への酸化ストレス刺激と細胞外サイクロフィリンAによる増幅回路の形成 (Satoh K, et al. *Circ J* 2010)。

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漿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。これらの血漿サイクロフィリンA濃度をELISA法で測定し、疾患ごとに評価した。これまで、66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。

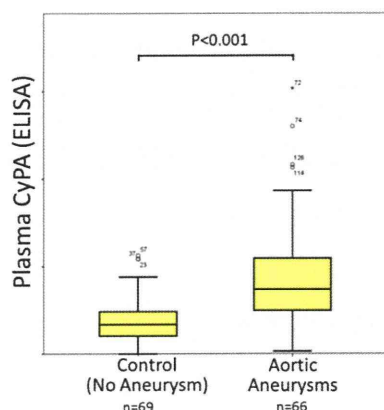


図3. 腹部大動脈瘤患者の末梢血における血漿サイクロフィリンA濃度

初期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、血漿サイクロフィリンA濃度の腹部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 Exp Med.* 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漿サイクロフィリン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漿サイクロフィリンA濃度測定を行った。冠動脈造影検査を施行した連続320症例において検討を行った結果、冠動脈狭窄を有する症例では著しい上昇を確認した。

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)年次集会以て発表した (*Circulation* 2011, Orlando)。

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、血漿サイクロフィリン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. 研究助成期間の研究発表

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- 3) 佐藤公雄 新規酸化ストレス増幅蛋白サイクロフィリン A による心血管病促進機構 (第 52 回日本脈管学会総会 Japanese College of Angiology Award (JCAA) 受賞講演 2011 年 10 月 20 日、岐阜)
- 4) 佐藤公雄 Cyclophilin A Is a Novel Biomarker for Oxidative Stress and Atherosclerosis (第 28 回国際心臓研究学会 (ISHR) 日本部会総会・シンポジウム 2011 年 12 月 2 日、東京)
- 5) 佐藤公雄 Plasma Cyclophilin A Is a Novel Predictor of Coronary Artery Disease (日本血管生物医学会 2011 年 12 月 10 日、東京)
- 6) 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)
- 7) 佐藤公雄 新規酸化ストレスバイオマーカー・サイクロフィリン A による心血管病促進機構 (第 64 回日本酸化ストレス学会学術集会 2011 年 7 月 2 日、北海道)
- 8) 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)
- 9) 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)
- 10) 佐藤公雄 Cyclophilin A Is a Novel Biomarker for Cardiovascular Diseases. Nature Medicine Vascular Medicine Award (日本血管生物医学会・受賞講演、2010 年 12 月 2 日、大阪)

- 11) 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)
- 12) 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 Plays an Important Role in the Pathogenesis of Pulmonary Arterial Hypertension in Humans- Involvement of Rho-kinase Pathway - (アメリカ心臓協会・年次総会、2011年11月15日、米国オーランド)
- 2) Cyclophilin A Mediates Pulmonary Vascular Remodeling by Rho-kinase Activation in Patients with Pulmonary Hypertension 佐藤公雄 Cyclophilin A Plays an Important Role in the Pathogenesis of Pulmonary Arterial Hypertension in Humans- Involvement of Rho-kinase Pathway - (アメリカ心臓協会・年次総会、2010年11月9日、米国シカゴ)
- 3) 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)
- 4) 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)
- 5) 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)
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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

Ⅱ．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
佐藤公雄, 下川宏明	血管内皮機能検査		『血圧』	先端医学社	東京	2011	1-5

雑誌

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Satoh K, et al.	Statin ameliorates hypoxia-induced pulmonary hypertension associated with down-regulated stromal cell-derived factor-1.	<i>Cardiovasc Res.</i>	81	226-234	2009

Ⅲ. 研究成果の刊行・別冊

Cyclophilin A is an inflammatory mediator that promotes atherosclerosis in apolipoprotein E-deficient mice

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Cyclophilin A (CyPA; encoded by *Ppia*) is a ubiquitously expressed protein secreted in response to inflammatory stimuli. CyPA stimulates vascular smooth muscle cell migration and proliferation, endothelial cell adhesion molecule expression, and inflammatory cell chemotaxis. Given these activities, we hypothesized that CyPA would promote atherosclerosis. Apolipoprotein E-deficient (*ApoE*^{-/-}) mice fed a high-cholesterol diet for 16 wk developed more severe atherosclerosis compared with *ApoE*^{-/-}*Ppia*^{-/-} mice. Moreover, CyPA deficiency was associated with decreased low-density lipoprotein uptake, VCAM-1 (vascular cell adhesion molecule 1) expression, apoptosis, and increased eNOS (endothelial nitric oxide synthase) expression. To understand the vascular role of CyPA in atherosclerosis development, bone marrow (BM) cell transplantation was performed. Atherosclerosis was greater in *ApoE*^{-/-} mice compared with *ApoE*^{-/-}*Ppia*^{-/-} mice after reconstitution with CyPA^{+/+} BM cells, indicating that vascular-derived CyPA plays a crucial role in the progression of atherosclerosis. These data define a role for CyPA in atherosclerosis and suggest CyPA as a target for cardiovascular therapies.

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Abbreviations used: BAEC, bovine aortic EC; CHX, cycloheximide; CsA, cyclosporine A; CyPA, cyclophilin A; DCF, dichlorofluorescein; DHE, dihydroethidium; EC, endothelial cell; eNOS, endothelial NO synthase; HUVEC, human umbilical vein EC; LDL, low-density lipoprotein; mRNA, messenger RNA; NO, nitric oxide; PI, propidium iodide; ROS, reactive oxygen species; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; VSMC, vascular smooth muscle cell.

Atherosclerosis is a disease of the vasculature that is characterized by chronic inflammation of the arterial wall (Hansson and Libby, 2006). The development of atherosclerosis is initiated by the activation of endothelial cells (ECs) leading to expression of adhesion molecules for inflammatory cells (Berk, 2008). In addition, these activated ECs facilitate the passage of lipid components in the plasma, such as low-density lipoproteins (LDLs; Hansson, 2005). A critical element in the progression of atherosclerosis is the development of an oxidizing environment caused by the activation of macrophages that become loaded with oxidized LDL and other lipids. These macrophages produce reactive oxygen species (ROS) and secrete cytokines and growth factors that contribute to the progression of atherosclerotic plaques and promote vulnerable lesions (Weber et al., 2008).

Cyclophilin A (CyPA) is a ubiquitously distributed protein belonging to the immunophilin family recognized as the intracellular receptor for the potent immunosuppressive drug cyclosporine A (CsA; Handschumacher et al., 1984). CyPA possesses peptidyl-prolyl isomerase activity and plays an important role in protein folding and trafficking (e.g., nuclear translocation of ERK1/2 [Pan et al., 2008] and AIF [apoptosis-inducing factor; Zhu et al., 2007]). Interestingly, it has been shown that CyPA is a part of a cytosolic trafficking complex consisting of caveolin, heat-shock protein 56, cyclophilin 40, CyPA, and cholesterol (Uittenbogaard et al., 1998). Although CyPA was initially believed to function primarily as an intracellular protein, recent studies have revealed that it can be secreted by cells in

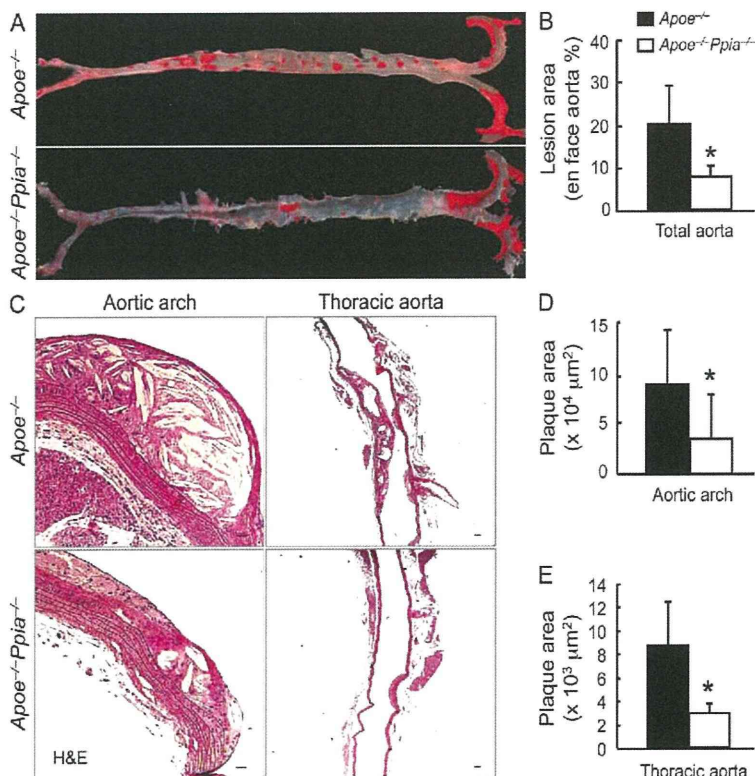
P. Nigro and K. Satoh contributed equally to this paper.

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Supplemental Material can be found at:
<http://jem.rupress.org/content/suppl/2010/12/17/jem.20101174.DC1.html>

response to inflammatory stimuli, especially ROS (Jin et al., 2000; Suzuki et al., 2006; Satoh et al., 2009). Extracellular CyPA is a potent leukocyte chemoattractant for human monocytes, neutrophils, eosinophils, and T cells (Sherry et al., 1992; Xu et al., 1992; Allain et al., 2002; Yurchenko et al., 2002; Arora et al., 2005; Damsker et al., 2007; Pan et al., 2008), and it stimulates inflammatory responses when injected in vivo (Sherry et al., 1992). Most importantly, plasma CyPA is significantly increased in patients with inflammatory diseases such as rheumatoid arthritis (Kim et al., 2005) and sepsis (Tegeder et al., 1997). We have shown that ROS promote secretion of CyPA from vascular smooth muscle cells (VSMCs; Jin et al., 2000; Liao et al., 2000) and that extracellular CyPA stimulates EC adhesion molecule expression in vitro (Jin et al., 2004; Suzuki et al., 2006). Furthermore, we found that CyPA mediates vascular remodeling by promoting inflammation and VSMC proliferation (Satoh et al., 2008), and it is indispensable for the development of angiotensin II-induced aortic aneurysms (Satoh et al., 2009; Weintraub, 2009).

Given these functions of CyPA, we hypothesized that CyPA would contribute to the development of atherosclerosis. In this study, we report that CyPA is atherogenic by enhancing LDL uptake, adhesion molecule expression, and inflammatory cell migration. Our data suggest that CyPA and its signaling pathways are novel targets for atherosclerosis therapy.



RESULTS

Atherosclerosis development is dependent on CyPA

To study the functional role of CyPA in atherogenesis, we used the *Apoe*^{-/-} mouse, a well-known model of atherosclerosis (Nakashima et al., 1994). We generated *Apoe*^{-/-}*Ppia*^{-/-} (double knockout) mice, and fed them a high-cholesterol diet for 16 wk. To visualize lipid-rich atherosclerotic plaques, aortas were stained with Oil red O. As shown in Fig. 1 A, *Apoe*^{-/-}*Ppia*^{-/-} mice compared with *Apoe*^{-/-} mice exhibited significantly less atherosclerosis: aortic coverage of 7.5 ± 2% in *Apoe*^{-/-}*Ppia*^{-/-} versus 19.3 ± 8.2% in *Apoe*^{-/-} (Fig. 1 B).

In another cohort of mice, we quantified plaque area in hematoxylin- and eosin (H&E)-stained cross sections of the aortic arch and thoracic aorta. Lesion area was significantly decreased in both the aortic arch (Fig. 1, C and D) and thoracic aorta (Fig. 1, C and E) of *Apoe*^{-/-}*Ppia*^{-/-} mice compared with *Apoe*^{-/-} mice. As expected, the elastic lamina was frequently degraded with large regions exhibiting disruption in *Apoe*^{-/-} mice compared with *Apoe*^{-/-}*Ppia*^{-/-} mice (Fig. S1). These results demonstrate a remarkable reduction in atherosclerosis in CyPA-deficient mice and strongly support our hypothesis that CyPA contributes to atherosclerosis.

The absence of CyPA decreases the lesion area and the migration of macrophages in the aortic sinus

The aortic sinus is particularly prone to intimal lesion development, and the cusps of the valves provide a useful positional cue in comparative experiments of sectioned tissues (Tangirala et al., 1995). As shown in Fig. 2 (A and B), *Apoe*^{-/-}*Ppia*^{-/-} mice compared with *Apoe*^{-/-} mice exhibited significantly less atherosclerosis measured by plaque area (H&E staining). There was also significantly less lipid deposition and cholesterol clefts in *Apoe*^{-/-}*Ppia*^{-/-} mice (Fig. 2 A, Masson and Trichrome and Oil red O). Both H&E and Masson and Trichrome showed a significant reduction in intima formation in *Apoe*^{-/-}*Ppia*^{-/-} mice (Fig. 2 A).

A crucial step in atherogenesis is the infiltration of monocytes into the subendothelial space of large arteries where they differentiate

Figure 1. CyPA deficiency limits atherosclerosis formation.

(A) Representative photographs showing Oil red O staining of aortas from *Apoe*^{-/-} and *Apoe*^{-/-}*Ppia*^{-/-} mice fed a high-cholesterol diet for 16 wk. (B) Lesion area was significantly decreased in *Apoe*^{-/-}*Ppia*^{-/-} mice (*n* = 15) compared with *Apoe*^{-/-} mice (*n* = 21). (C) Longitudinal cross sections from the aortic arch and thoracic aorta stained with H&E. Bars: (left) 25 μm; (right) 200 μm. (D and E) Quantification of plaque area showed that *Apoe*^{-/-}*Ppia*^{-/-} mice (*n* = 7) exhibited decreased atherosclerosis compared with *Apoe*^{-/-} mice (*n* = 9) in both the aortic arch (D) and thoracic aorta (E). (B, D, and E) Results are mean ± SD; *, *P* < 0.01 compared with *Apoe*^{-/-} mice. Results in A–E show pooled data from two experiments.

into macrophages and become functionally active (Galkina and Ley, 2009). As shown in Fig. 2 (A and D), the *Apoe*^{-/-}*Ppia*^{-/-} mice fed high-cholesterol diet for 16 wk showed significantly fewer Mac3-positive macrophages compared with the *Apoe*^{-/-} mice. All these data suggest that CyPA is a key protein involved in the atherosclerosis progression and migration of inflammatory cells in the lesion area.

CyPA regulates LDL infiltration and the expression of scavenger receptors

Because it is well established that the transport of LDL cholesterol into the artery wall is the initiating event that triggers atherosclerosis (Glass and Witztum, 2001), we examined the role of CyPA in this process. To measure LDL uptake into vessels of *Apoe*^{-/-} and *Apoe*^{-/-}*Ppia*^{-/-} mice, we performed ex vivo incubation with fluorescent DiI-labeled LDLs and en face imaging of the aorta from the EC surface to a depth of 50 μ m into the intima. CyPA deficiency caused a 43% decrease in DiI-LDL uptake in the lesser curvature of the aortic arch (atherosclerosis-prone area; Fig. 3, A and B; and Fig. S2). To strengthen this important finding, we confirmed these data with an independent method. *Apoe*^{-/-} and *Apoe*^{-/-}*Ppia*^{-/-} aortas were incubated with [¹²⁵I]LDL for a subsequent determination of incorporated

radioactivity as measured by a gamma counter. As shown in Fig. S3, LDL uptake was significantly reduced in aortas from *Apoe*^{-/-}*Ppia*^{-/-} compared with *Apoe*^{-/-} mice. To gain insight into how CyPA regulates lipid uptake, we examined expression of several scavenger receptors in aortas after 12 wk of high-cholesterol diet. The expression of lectin-like oxidized LDL receptor (LOX-1) and CD36 were significantly decreased in *Apoe*^{-/-}*Ppia*^{-/-} mice compared with *Apoe*^{-/-} mice, whereas SR-BI was decreased by an equivalent 40% (Fig. 3, C and D). The expression of SR-A did not differ significantly between *Apoe*^{-/-} and *Apoe*^{-/-}*Ppia*^{-/-} mice (Fig. 3, C and D). Collectively, the data demonstrate that CyPA influences LDL uptake by regulating the expression of scavenger receptors on the vessel wall.

CyPA deficiency induces the development of a more proatherogenic lipoprotein profile in *Apoe*^{-/-} mice

Next, we performed phenotypic characterization of *Apoe*^{-/-} and *Apoe*^{-/-}*Ppia*^{-/-} mice fed a high-cholesterol diet for 16 wk. Body weights were similar in all the groups of mice before and after a high-cholesterol diet (Fig. S4 A). There were no significant differences in plasma cholesterol and triglyceride levels (Fig. S4, B and C). We next examined lipoprotein profiles by gel filtration chromatography (Fig. S4 D).

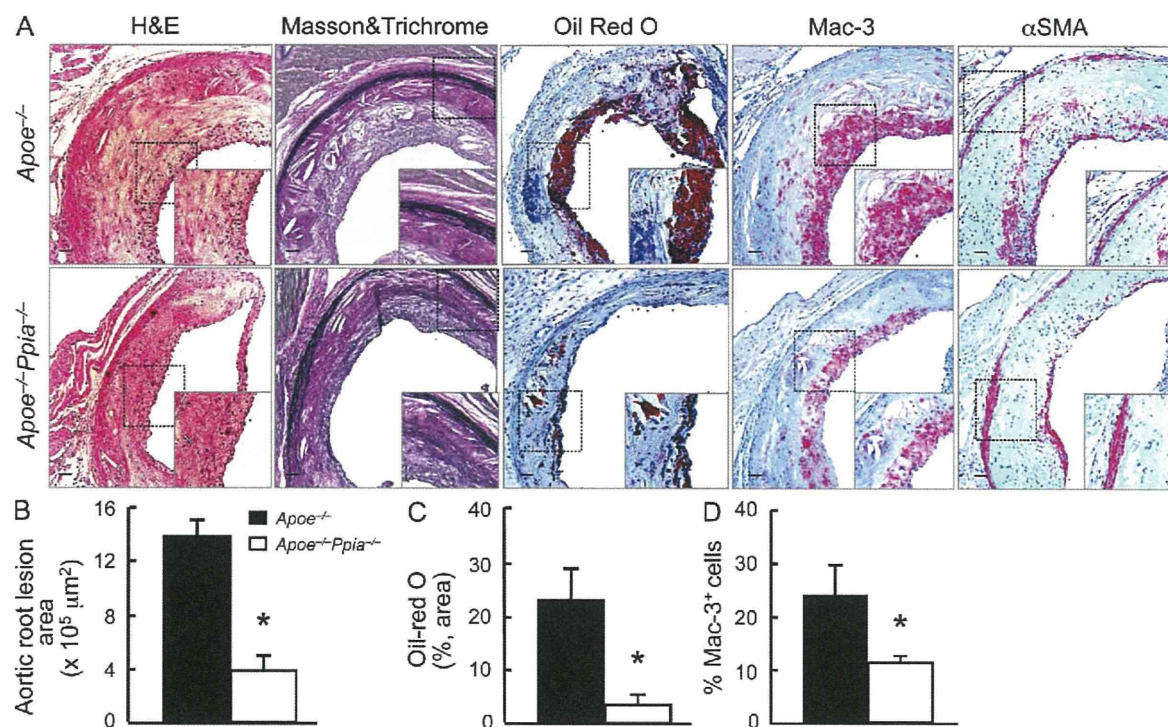


Figure 2. CyPA deficiency reduces lesion size and inflammatory cell accumulation in the aortic sinus. (A) Representative histological analysis of the aortic sinus stained with H&E, Masson and Trichrome, Oil red O, Mac3 (a macrophage marker), and α -smooth muscle cell actin (α -SMA). Insets are higher magnification images of the areas in the dashed boxes. Bars, 100 μ m. (B–D) Quantification of the plaque area (B), lipid accumulation (C), and macrophage accumulation (D) shows a significant reduction in *Apoe*^{-/-}*Ppia*^{-/-} mice ($n = 7$) compared with *Apoe*^{-/-} ($n = 9$) mice. Results are mean \pm SD; *, $P < 0.01$ compared with *Apoe*^{-/-} mice. Results in A–D show pooled data from two experiments.

Although there were no differences observed in cholesterol and triglyceride levels, *Apoe*^{-/-}*Ppia*^{-/-} mice demonstrated increases in the VLDL (very low-density lipoprotein)- and IDL (intermediate-density lipoprotein)/LDL-sized lipoproteins compared with *Apoe*^{-/-} mice. No differences were observed in the HDL (high-density lipoprotein) fraction or in the levels of plasma apoB-100 and apoB-48 (Fig. S4 E). Collectively, these results indicate that loss of CyPA, in the context of the *Apoe*^{-/-} genetic background, leads to the appearance of a more proatherogenic lipoprotein profile.

BM-derived CyPA does not play a significant role in atherosclerosis formation

CyPA has important roles in the immune system and is a well-described regulator of T lymphocyte functions (Colgan et al., 2004). Extracellular CyPA is a potent chemoattractant for inflammatory cells (Xu et al., 1992; Allain et al., 2002; Yurchenko et al., 2002). CyPA has also been reported to stimulate migration of BM-derived cells in vitro (Khromykh et al., 2007).

To determine the role of BM-derived CyPA in atherosclerosis, BM transplantation experiments were performed.

BM cells from *Apoe*^{-/-} and *Apoe*^{-/-}*Ppia*^{-/-} mice were transplanted into 6-wk-old lethally irradiated *Apoe*^{-/-} mice. After 4 wk of reconstitution, mice were fed with a high-cholesterol diet for 12 wk. Surprisingly, there were no differences in atherosclerosis measured by lesion area in the entire aorta (Fig. 4, A and B; Oil red O) or aortic root (Fig. 4, C and D; H&E). These data demonstrate that atherosclerosis in *Apoe*^{-/-}*Ppia*^{-/-} mice was not altered by specific CyPA deficiency in BM-derived cells. Note that the atherosclerosis lesion area was reduced by ~50% in *Apoe*^{-/-} mice after irradiation (compare Fig. 4 B with Fig. 1 B).

We next assessed the development of high-cholesterol-induced atherosclerosis in *Apoe*^{-/-}*Ppia*^{-/-} chimeric mice that were transplanted with *Apoe*^{-/-} BM or *Apoe*^{-/-}*Ppia*^{-/-} BM. There was no significant difference in atherosclerosis lesions between the chimeric mice with *Apoe*^{-/-} BM versus *Apoe*^{-/-}*Ppia*^{-/-} BM (unpublished data).

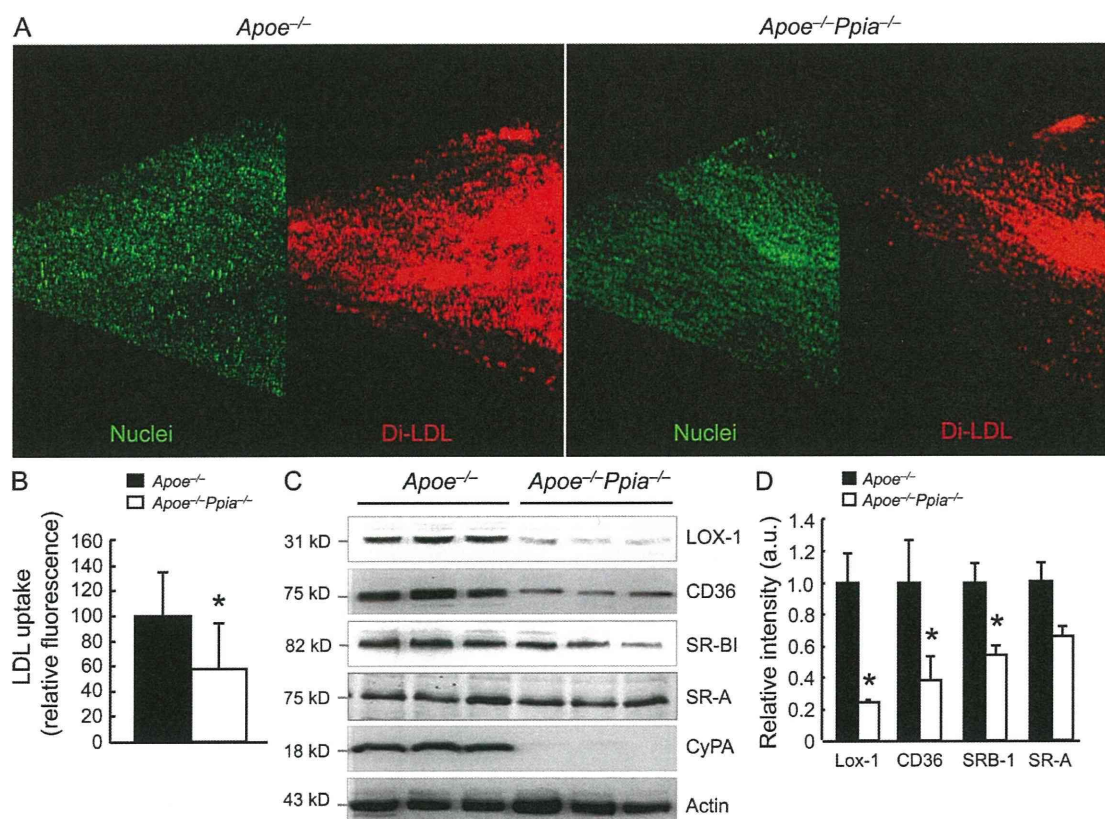


Figure 3. CyPA regulates LDL entry into the aortic wall and the expression of the scavenger receptors. (A) En face fluorescence images of aortic arches from 6-wk-old *Apoe*^{-/-} and *Apoe*^{-/-}*Ppia*^{-/-} mice ($n = 3$ each group) after incubation with DiI-labeled LDL (red) and SYTOX green nucleic acid stain (green). (B) Quantification of DiI-LDL fluorescence intensity from the en face images. The data are quantified as fluorescence-positive area. (C) Western blot analyses of aortic extracts from *Apoe*^{-/-} and *Apoe*^{-/-}*Ppia*^{-/-} mice fed a high-cholesterol diet for 12 wk. Results for three representative mice are shown for each genotype. (D) Densitometric analyses of the blots in C show significant decreases in LOX-1, CD36, and SR-BI scavenger receptors in *Apoe*^{-/-}*Ppia*^{-/-} mice versus *Apoe*^{-/-} mice. (B and D) Results are mean \pm SD; *, $P < 0.01$ compared with *Apoe*^{-/-} mice. Results in A–D show pooled data from two experiments.

To investigate the chemoattractive function of CyPA in vivo, we studied the trafficking of BM-derived cells into atherosclerotic lesions. To analyze BM cells that migrated into lesions, we used donor BM cells harvested from mice constitutively expressing the GFP protein (GFP⁺ BM which are *Ppia*^{+/+}) and performed BM transplantation in lethally irradiated *Apoe*^{-/-} and *Apoe*^{-/-}*Ppia*^{-/-} mice. There was no significant difference in the reconstitution ratio (percentage of GFP⁺ cells in the peripheral blood) in GFP⁺ BM-transplanted *Apoe*^{-/-}*Ppia*^{-/-} mice compared with GFP⁺ BM-transplanted *Apoe*^{-/-} mice. However, there was still a significant difference in the atherosclerosis lesion area in the *Apoe*^{-/-} GFP⁺ BM mice compared with *Apoe*^{-/-}*Ppia*^{-/-} GFP⁺ BM mice ($9.6 \pm 1.3\%$ vs. $2.3 \pm 0.3\%$), and migration of GFP⁺ BM cells into the lesser curvature region was

much greater in the *Apoe*^{-/-} recipient mice (Fig. 4 E, left) compared with *Apoe*^{-/-}*Ppia*^{-/-} recipient mice (Fig. 4 E, right) after high-cholesterol diet. High-magnification images (Fig. 4 G, top) showed the presence of enlarged foam cells in the *Apoe*^{-/-} mice with GFP⁺ BM (arrows), which is consistent with the concept that BM-derived cells differentiate into foam cells upon exposure to hyperlipidemia. Direct demonstration that the BM-derived GFP⁺ cells were localized beneath the ECs (and not just adherent to the luminal surface) was obtained by examination of cross-sectional images of z-series stacks (Fig. 4 G, bottom). These results suggested that CyPA present in BM-derived cells is less important for recruitment to the sites of atherosclerosis lesions than CyPA present in vessels and other non-BM cells.

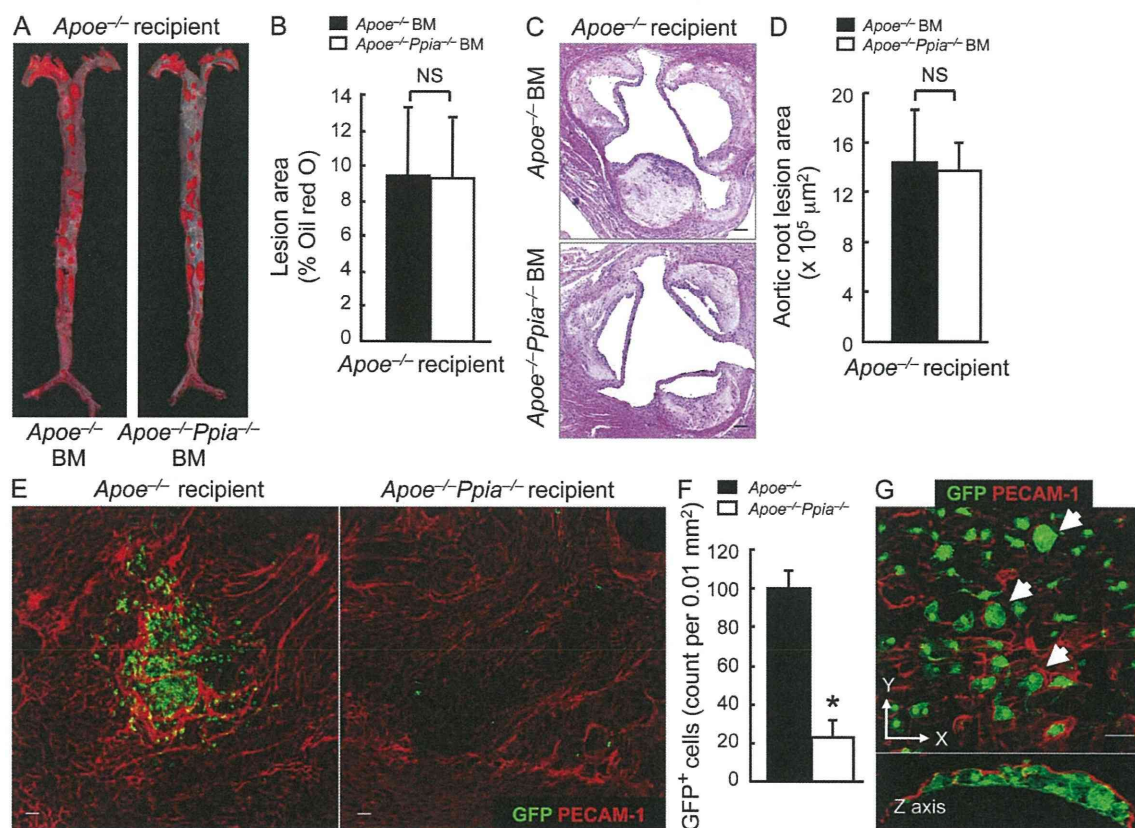


Figure 4. BM-derived CyPA is not crucial in atherosclerosis. (A) Representative Oil red O staining of aortas from *Apoe*^{-/-} mice transplanted with *Apoe*^{-/-} BM ($n = 7$) or *Apoe*^{-/-}*Ppia*^{-/-} BM ($n = 8$) and fed a high-cholesterol diet for 12 wk. (B) Quantification of the lesion area shows that BM transplant of *Apoe*^{-/-}*Ppia*^{-/-} cells does not decrease lesion development in *Apoe*^{-/-} mice. (C and D) Representative histological analysis of aortic sinus stained with H&E (C) and quantification of the aortic root lesion area (D). (B and D) Results are mean \pm SD; $P > 0.01$. (E) *Ppia*^{+/+} BM cells (GFP⁺) were transplanted into irradiated *Apoe*^{-/-} ($n = 4$) or *Apoe*^{-/-}*Ppia*^{-/-} ($n = 4$) mice as described in Materials and methods. Representative PECAM-1 en face staining (Alexa Fluor 546; red) and migration of the GFP⁺ cells in aortic arch from *Apoe*^{-/-} and *Apoe*^{-/-}*Ppia*^{-/-} mice with *Ppia*^{+/+} BM under high-cholesterol diet for 12 wk. (F) Number of migrating GFP⁺ cells was dramatically higher in the aortic wall of *Apoe*^{-/-} ($n = 4$) compared with *Apoe*^{-/-}*Ppia*^{-/-} mice ($n = 4$). Results are mean \pm SD; *, $P < 0.01$ compared with *Apoe*^{-/-} mice. (G) En face confocal microscopy of aortic arch from *Apoe*^{-/-} mice transplanted with GFP⁺ cells. Top panel shows foam cells, which were identified by large size and diffuse GFP pattern (arrows). Bottom panel shows the reconstruction in z axis of the images shown in the top panel. GFP⁺ cells are clearly present in the subendothelial space defined by the PECAM-1 positive fluorescence above them. Results in A–G show pooled data from two experiments. Bars: (C) 100 μ m; (E and G) 10 μ m.

CyPA regulates endothelial nitric oxide (NO) synthase (eNOS) expression

The aforementioned results suggest that the athero protection observed in the *Apoe*^{-/-}*Ppia*^{-/-} was caused by decreased inflammation mediated by the absence of CyPA. The vascular endothelium by virtue of its strategic location between the plasma and the underlying tissue is endowed with a large array of functions that are vital for the initiation of atherosclerosis. Therefore, we performed an extensive examination of the endothelium of aortic arch and thoracic aorta by using PECAM-1 en face staining to visualize ECs. *Apoe*^{-/-}*Ppia*^{-/-} mice showed a decreased EC disorganization in both the aortic segments when compared with *Apoe*^{-/-} mice (Fig. S5, A and B).

To evaluate further the mechanisms by which CyPA promotes inflammation, we measured VCAM-1 (vascular cell adhesion molecule 1) expression, which is highly expressed in activated ECs and promotes atherosclerosis (Cybulsky and Gimbrone, 1991; Nakashima et al., 1998; Cybulsky et al., 2001). En face staining (Fig. 5, A and B) of aortic tissues showed that VCAM-1 expression was significantly reduced in mice lacking CyPA.

eNOS function is critical for vascular homeostasis via generation of NO, and its loss is proatherogenic (Knowles et al., 2000; Chen et al., 2001; Kuhlencordt et al., 2001; Kawashima and Yokoyama, 2004). Furthermore, the progression of atherosclerosis is associated with decreases in both eNOS expression (Oemar et al., 1998; Handa et al., 2008) and NO production (Li and Förstermann, 2009). Therefore, we compared eNOS expression in aortas from *Apoe*^{-/-} and *Apoe*^{-/-}*Ppia*^{-/-} mice by en face staining. As shown in Fig. 6 A, eNOS protein expression was significantly higher in the *Apoe*^{-/-}*Ppia*^{-/-} mice compared with *Apoe*^{-/-} mice. In addition, the location of eNOS differed, being predominantly perinuclear in the *Apoe*^{-/-} mice and diffuse (especially membrane associated) in the *Apoe*^{-/-}*Ppia*^{-/-} mice. To define the mechanisms responsible for decreased eNOS expression, we studied the effect of altering CyPA levels in cultured human umbilical vein ECs (HUVECs). To increase eNOS expression and stimulate cellular responses that are atheroprotective, cells were placed in a cone and plate viscometer, and steady laminar s-flow at physiological shear stress (12 dyn/cm²; termed s-flow here) was applied. As shown in Fig. 6 (B and C), s-flow-mediated induction of eNOS was significantly increased by

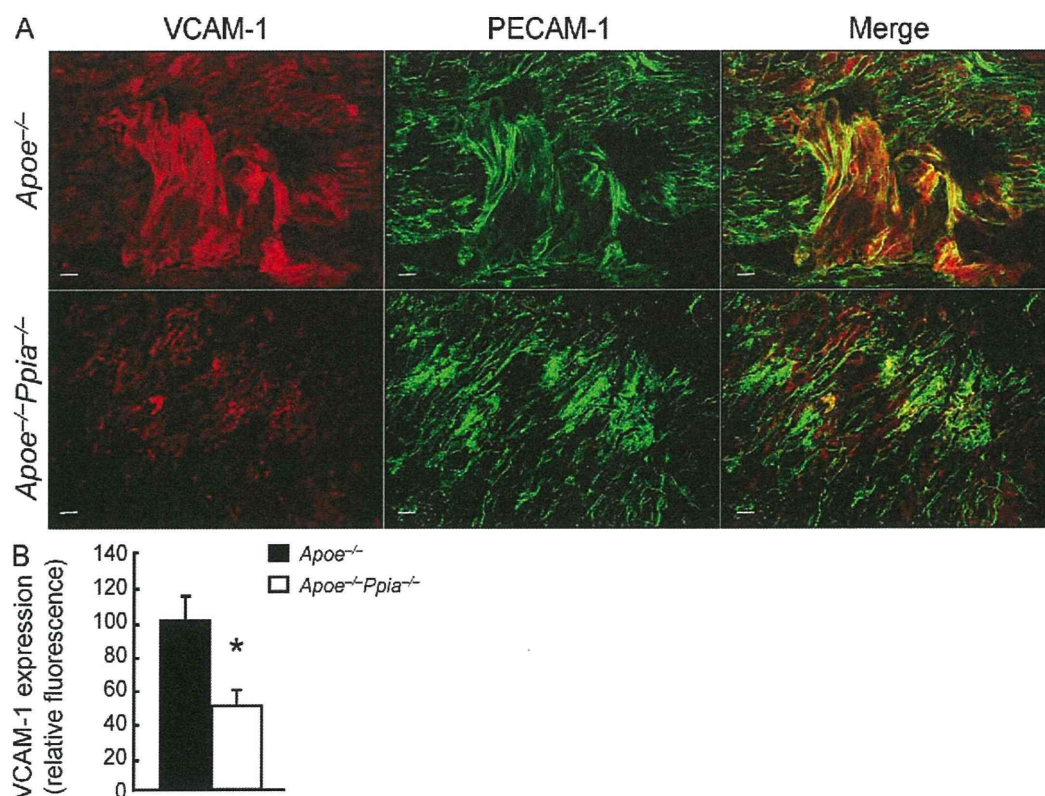


Figure 5. VCAM-1 expression is significantly down-regulated in *Apoe*^{-/-}*Ppia*^{-/-} mice compared with *Apoe*^{-/-} mice. (A) Representative en face staining for VCAM-1 expression (Alexa Fluor 546; red) in the aortic arch. EC morphology was changed in the atherosclerosis regions where ECs are stretched and may have lost PECAM-1 staining (Alexa Fluor 488; green) at some cell junctions. Bars, 10 μ m. (B) Densitometric analysis of the en face staining also shows a significant reduction in VCAM-1 expression in *Apoe*^{-/-}*Ppia*^{-/-} mice compared with *Apoe*^{-/-} mice ($n = 4$ each group). Results are mean \pm SD; *, $P < 0.01$ compared with *Apoe*^{-/-} mice. Results in A and B show pooled data from two experiments.