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SUPPLEMENTAL MATERIAL

Nigro et al., http://www.jem.org/cgi/content/full/jem.20101174/DC1

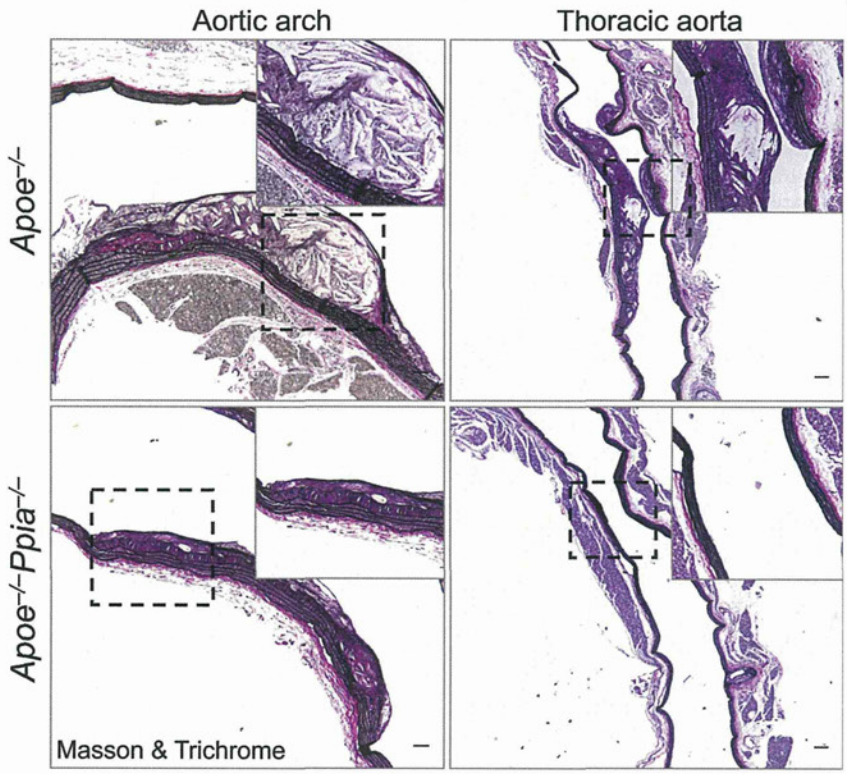

Figure S1. CyPA deficiency prevents atherosclerosis formation. Representative examples of aortic longitudinal cross sections from the aortic arch and thoracic aorta stained with Masson and Trichrome. *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice are protected in the elastin degradation in both the aortic arch and thoracic aorta. Insets show higher magnification images of areas in the dashed boxes. Data are representative of two separate experiments with similar results (n = 4 each group). Bars: (left) 25 µm; (right) 200 µm.

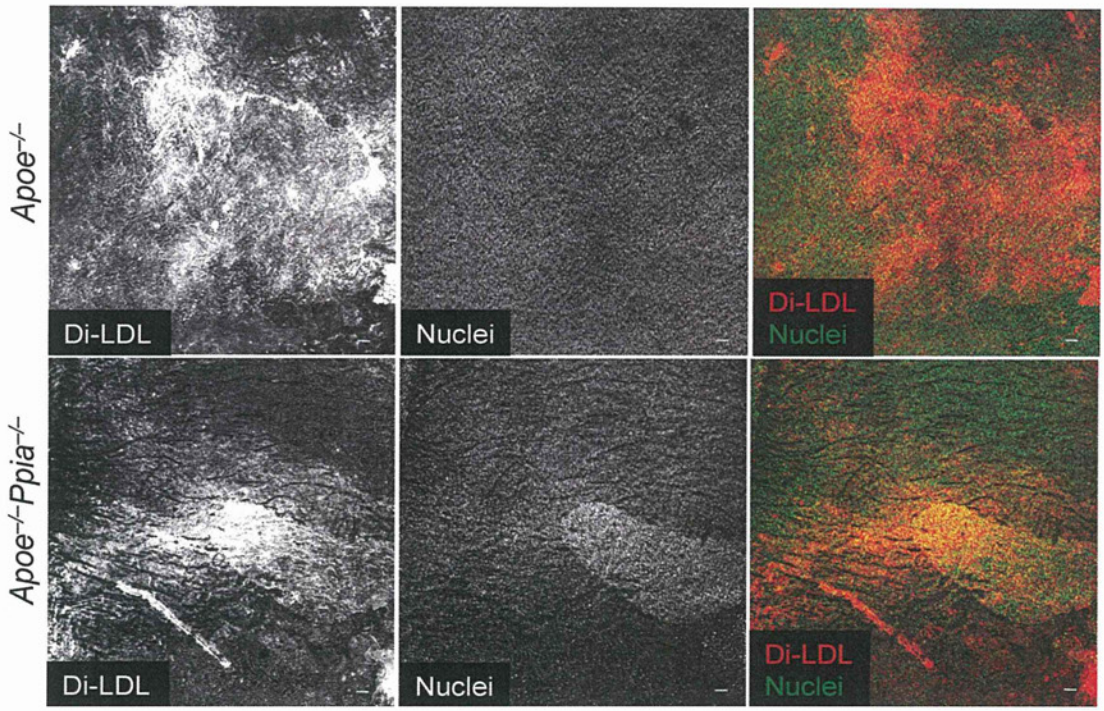

Figure S2. CyPA deficiency decreases LDL uptake in the lesser curvature of the mouse aorta. Aortas from *Apoe*$^{-/-}$ ($n = 4$) and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ ($n = 4$) mice were harvested for qualitative analysis of DiI-labeled LDL uptake in the athero-prone areas of the aortic arch. Data are representative of two separate experiments with similar results. Bars, 50 µm.

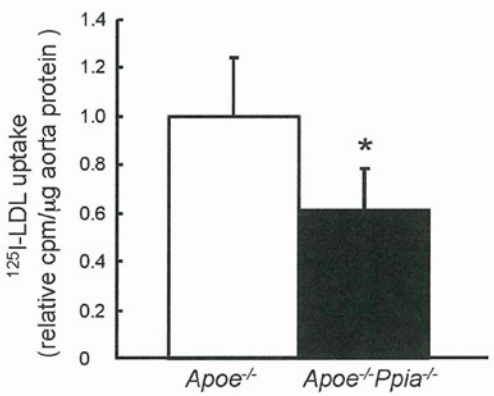

Figure S3. CyPA regulates LDL uptake. Aortas from *Apoe*$^{-/-}$ ($n = 4$) and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ ($n = 4$) mice were incubated with [^{125}I]LDL uptake for 3 h. Measurement of cell-associated label was performed in a gamma counter. Results are mean ± SD; *, $P < 0.01$ compared with *Apoe*$^{-/-}$ mice. Results show pooled data from two experiments.

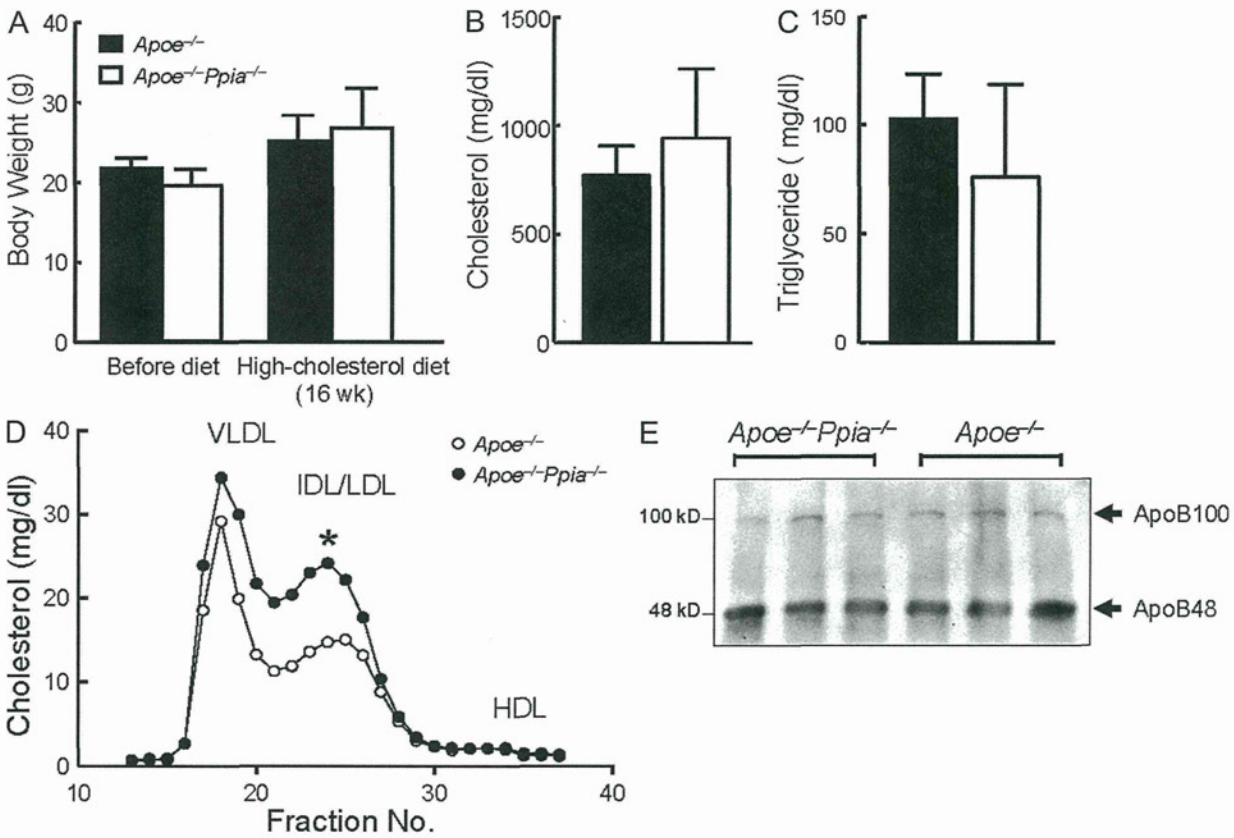

Figure S4. Metabolic parameters in the absence of CyPA. (A) Body weight of *Apoe*$^{-/-}$ ($n = 8$) and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ ($n = 10$) mice before and after 16 wk on a high-cholesterol diet. (B and C) Total cholesterol (B) and triglyceride (C) levels of *Apoe*$^{-/-}$ ($n = 18$) and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ ($n = 18$) mice after 16 wk on a high-cholesterol diet. (D) Lipoprotein profiles from *Apoe*$^{-/-}$ ($n = 9$) and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ ($n = 9$) mice after 16 wk of a high-cholesterol diet. (E) Levels of apoB-100 and apoB-48, the main protein markers of LDL, were determined by Western blot analysis. Plasma apoB levels were similar in animals fed a high-cholesterol diet. Results with three representative animals are shown for each genotype. Equal volumes of plasma were loaded in each lane. Results in A–D are mean ± SD; *, $P < 0.01$ compared with *Apoe*$^{-/-}$ mice. Results in A–D show pooled data from two experiments.

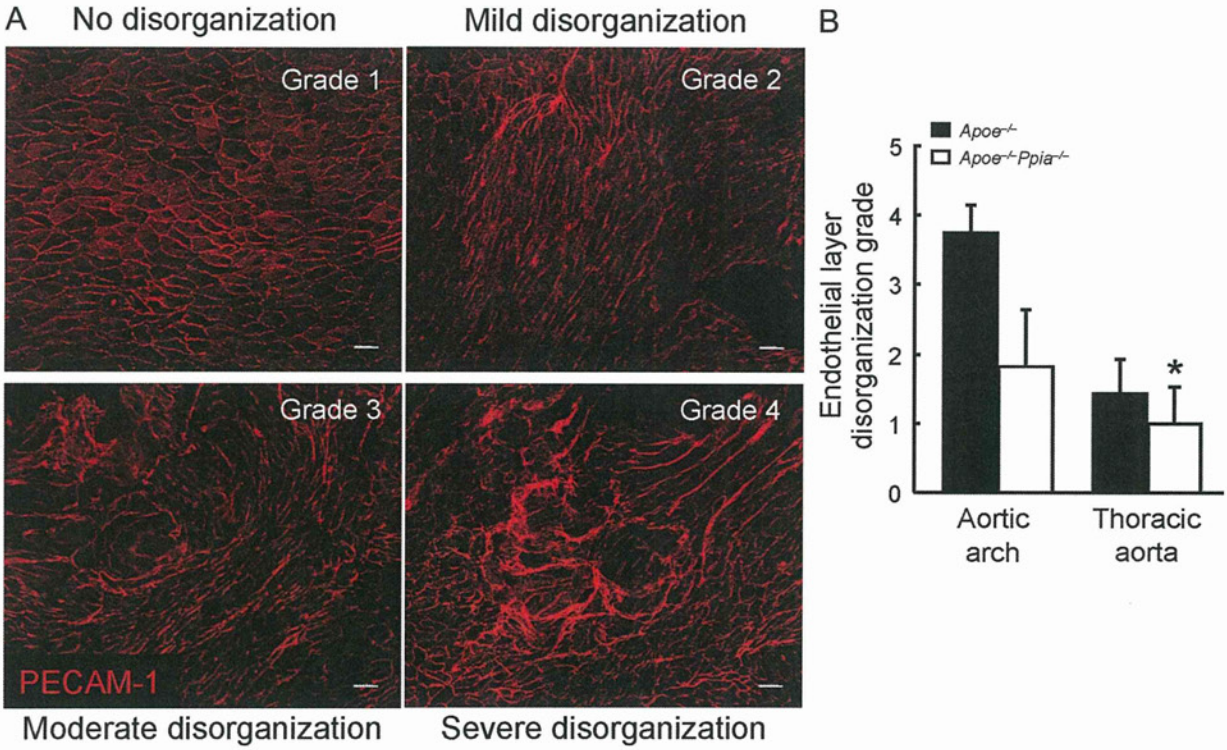

Figure S5. CyPA promotes EC damage and disorganization. (A) EC disorganization-grading (four grades) keys by using PECAM-1 en face aortic staining. Bars, 10 µm. (B) Statistical analysis based on the EC layer disorganization grade shows a significant decrease in EC damage in *Apoe*$^{-/-}$ *Ppia*$^{-/-}$ mice compared with *Apoe*$^{-/-}$ mice ($n = 4$ each group). Results are mean ± SD; *, $P < 0.01$ compared with *Apoe*$^{-/-}$ mice. Results in B show pooled data from two experiments.

Arteriosclerosis, Thrombosis, and Vascular Biology

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Cyclophilin A Promotes Cardiac Hypertrophy in Apolipoprotein E–Deficient Mice

Kimio Satoh, Patrizia Nigro, Asad Zeidan, Nwe Nwe Soe, Fabrice Jaffré, Masayoshi Oikawa, Michael R. O'Dell, Zhaoqiang Cui, Prashanthi Menon, Yan Lu, Amy Mohan, Chen Yan, Burns C. Blaxall and Bradford C. Berk

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Objective—Cyclophilin A (CyPA, encoded by *Ppia*) is a proinflammatory protein secreted in response to oxidative stress in mice and humans. We recently demonstrated that CyPA increased angiotensin II (Ang II)–induced reactive oxygen species (ROS) production in the aortas of apolipoprotein E ($Apoe^{-/-}$) mice. In this study, we sought to evaluate the role of CyPA in Ang II–induced cardiac hypertrophy.

Methods and Results—Cardiac hypertrophy was not significantly different between $Ppia^{+/+}$ and $Ppia^{-/-}$ mice infused with Ang II (1000 ng/min per kg for 4 weeks). Therefore, we investigated the effect of CyPA under conditions of high ROS and inflammation using the $Apoe^{-/-}$ mice. In contrast to $Apoe^{-/-}$ mice, $Apoe^{-/-}Ppia^{-/-}$ mice exhibited significantly less Ang II–induced cardiac hypertrophy. Bone marrow cell transplantation showed that CyPA in cells intrinsic to the heart plays an important role in the cardiac hypertrophic response. Ang II–induced ROS production, cardiac fibroblast proliferation, and cardiac fibroblast migration were markedly decreased in $Apoe^{-/-}Ppia^{-/-}$ cardiac fibroblasts. Furthermore, CyPA directly induced the hypertrophy of cultured neonatal cardiac myocytes.

Conclusion—CyPA is required for Ang II–mediated cardiac hypertrophy by directly potentiating ROS production, stimulating the proliferation and migration of cardiac fibroblasts, and promoting cardiac myocyte hypertrophy. ***(Arterioscler Thromb Vasc Biol.* 2011;31:1116-1123.)**

Key Words: cardiac hypertrophy ■ cardiac remodeling ■ oxidative stress

Cardiac hypertrophy is a fundamental response of cardiac cells to common clinical disorders, such as arterial hypertension, valvular heart disease, myocardial infarction, cardiomyopathy, and congenital heart disease.[1] Emerging data reveal that the communication between cardiac fibroblasts and myocytes plays a critical role in cell-cell signaling in the heart, and it is implicated in the process of cardiac remodeling and overall heart function during development and cardiopathology.[2–5] There are numerous lines of evidence indicating that cardiac fibroblasts and myocytes release into their local microenvironment proteins that regulate neighboring cells via paracrine mechanisms.[2–5] Although multiple factors have been implicated in this intercellular crosstalk, the discovery of new hypertrophic players and a better understanding of the underlying mechanisms hold the key to successful therapy of hypertrophic heart disease.

Cyclophilin A (CyPA, encoded by *Ppia*) was originally found as a binding partner of cyclosporine A.[6] Intracellular CyPA is a chaperone protein that has several functions, including peptidyl-prolyl isomerase activity and protein trafficking, such as nuclear translocation of ERK1/2[7] and apoptosis-inducing factor.[8] We and others have provided evidence that CyPA is secreted in response to reactive oxygen species (ROS) from vascular smooth muscle cells[9] and rat neonatal cardiac myocytes.[10] Moreover, we have shown the involvement of CyPA in angiotensin II (Ang II)–induced aortic aneurysms and oxidative stress.[11,12]

Ang II plays a key role in many physiological and pathological processes in cardiac cells, including cardiac hypertrophy.[13] Therefore, understanding the molecular mechanisms responsible for Ang II–mediated myocardial pathophysiology will be critical to developing new therapies for cardiac dysfunction.[14] One important mechanism now recognized to be involved in Ang II–induced cardiac hypertrophy is ROS production,[15,16] but the precise mechanism by which ROS cause hypertrophy remains unknown. Our recent study provides strong mechanistic evidence of synergy between CyPA and Ang II to increase ROS generation.[11]

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From the Aab Cardiovascular Research Institute (K.S., P.N., A.Z., N.N.S., F.J., M.O., M.R.O., Z.C., P.M., Y.L., A.M., C.Y., B.C. Blaxall, B.C. Berk) and Department of Medicine (K.S., P.N., A.Z., N.N.S., F.J., M.O., M.R.O., Z.C., P.M., Y.L., A.M., C.Y., B.C. Blaxall, B.C. Berk), University of Rochester School of Medicine and Dentistry, Rochester, NY; Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Aoba-ku, Sendai, Japan (K.S.).

Drs Satoh and Nigro contributed equally to this work.

Correspondence to Bradford C. Berk, MD, PhD, Aab Cardiovascular Research Institute, University of Rochester, Box CVRI, 601 Elmwood Ave, Rochester, NY 14642. E-mail bradford_berk@urmc.rochester.edu

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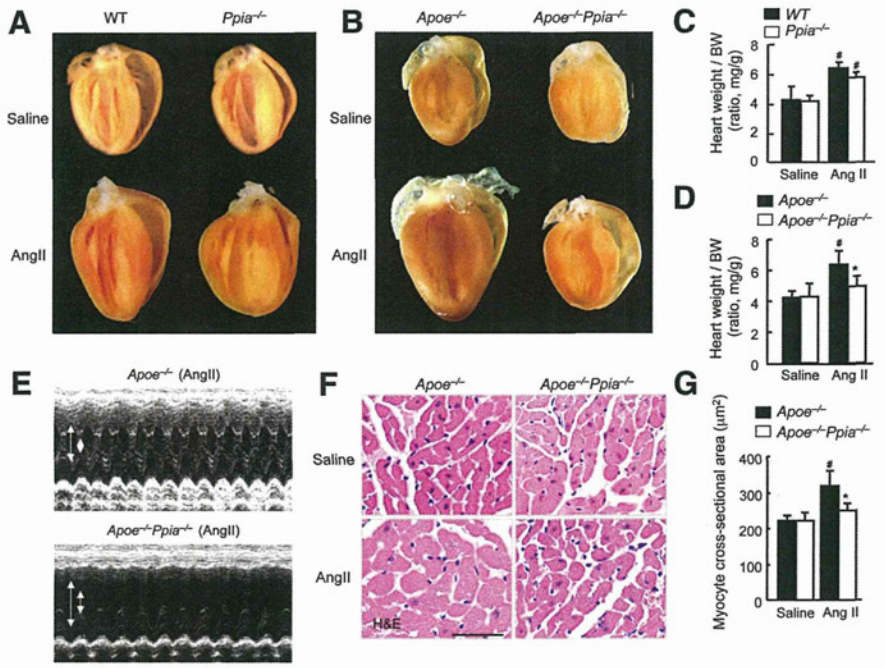

Figure 1. CyPA deficiency prevents Ang II–induced cardiac hypertrophy in *Apoe*$^{-/-}$ mice. A, Representative photographs showing macroscopic features of cardiac hypertrophy induced by Ang II infusion in *Ppia*$^{-/-}$ mice vs WT mice. Ang II–induced cardiac hypertrophy was not prevented in *Ppia*$^{-/-}$ mice compared with WT mice. B, Representative photographs of the hearts of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice infused with Ang II or saline for 4 weeks. Ang II–induced cardiac hypertrophy was prevented in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice compared with *Apoe*$^{-/-}$ mice. C, Ang II–induced cardiac hypertrophy (heart weight/BW ratio) was not significantly decreased in *Ppia*$^{-/-}$ mice (n=5) compared with WT (n=6). #P<0.05 in saline vs Ang II; *P<0.05 in *WT* vs *Ppia*$^{-/-}$ mice. D, Ang II–induced cardiac hypertrophy (heart weight/BW ratio) was significantly reduced in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (n=11) compared with *Apoe*$^{-/-}$ mice (n=15). No significant differences were found in the control groups (saline infusion) of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (n=4, respectively). E, Representative M-mode images of cardiac hypertrophy assessed by echocardiography after 4 weeks of Ang II infusion. Arrows indicate the diastolic LV cavity and systolic LV cavity. F, H&E staining of representative cross-sections of cardiac myocytes of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice 4 weeks after Ang II infusion. G, Myocyte cross-sectional area was significantly reduced in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (n=7) compared with *Apoe*$^{-/-}$ mice (n=9). Results are mean±SD. #P<0.05 in saline vs Ang II; *P<0.05 in *Apoe*$^{-/-}$ vs *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice.

Because ROS stimulate myocardial hypertrophy, matrix remodeling, and cellular dysfunction,[17] we tested the hypothesis that CyPA enhances Ang II–induced cardiac ROS production and therefore cardiac hypertrophy.

Methods

An expanded Supplemental Methods section is available online at http://atvb.ahajournals.org.

Analysis of Cardiac Hypertrophy

Ang II–infused models were used to assess the effect of CyPA deficiency on cardiac hypertrophy.[18] Five- to 6-week-old male mice on a normal chow diet were infused with 1000 ng/min per kg Ang II (MP Biomedicals, Solon, OH) or saline vehicle for 4 weeks. Ang II was dissolved in sterile saline and infused using Alzet osmotic pumps (model 2004, Durect Corp, Cupertino, CA). Mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). Pumps were placed into the subcutaneous space of anesthetized mice through a small incision in the back of the neck that was closed with suture. After 4 weeks of Ang II infusion, the animals were again anesthetized with ketamine (80 mg/kg) and xylazine (5 mg/kg). The heart tissue was perfused with calcium- and magnesium-free phosphate-buffered saline (PBS) and then fixed with phosphate-buffered 10% formalin solution and subsequently prepared for histological analysis as previously described.[19] Heart weight was measured, and the ratio of heart weight to body weight (BW) was calculated to determine an index of cardiac hypertrophy. Five sections were obtained from each heart and mounted on slides and stained with Masson's trichrome or hematoxylin/eosin (H&E). To evaluate the perivascular fibrosis, short-axis images of coronary arteries were scanned at ×200 magnification. The area of perivascular fibrosis (the ratio of the fibrosis area surrounding the vessel to the total vessel area) was calculated. To evaluate the extent of cardiac myocyte hypertrophy, cross-sectional images of cardiac myocytes were scanned at ×400 magnification. Approximately 10 cross-sections of cardiac myocytes were analyzed in each heart. Average values for each heart were used for analysis.

Bone Marrow Transplantation

Bone marrow transplantation was performed as described.[20] Briefly, recipient mice were lethally irradiated and received an intravenous injection of 5×10^6 donor bone marrow cells suspended in 100 μL of PBS with 2% fetal bovine serum. After transplantation, the mice were placed on a regular chow diet for 6 weeks followed by infusion of 1000 ng/kg per minute Ang II for 4 weeks. Transgenic mice ubiquitously expressing green fluorescent protein (GFP) were obtained from the Jackson Laboratory (Bar Harbor, ME). The chimeric rate assessed by GFP$^+$ cells in the peripheral blood was more than 99% by fluorescence-activated cell sorter analysis (FACSCantoII, Becton Dickinson, San Jose, CA).

ROS Analysis

The evaluation of ROS production in response to Ang II was performed as described before.[21] After treatment with Ang II (1 μmol/L), cardiac fibroblasts were washed with PBS and loaded with 2,7-dichlorofluoresceine diacetate (5 μmol/L; Molecular Probes) for 30 minutes. Hearts were perfused with PBS (pH 7.4) at 100 mm Hg for 5 minutes at 4°C. Heart tissue was harvested and embedded in OCT (Tissue-Tek, Miles Inc., Elkhart, IL) and snap-frozen. Dihydroethidine hydrochloride (5 μmol/L, Molecular Probes) was topically applied to the freshly cut frozen heart sections (10 μm) for 30 minutes at 37°C. Dichlorofluorescein (DCF) and dihydroethidium fluorescence was revealed by confocal microscopy (Olympus, Fluoview).[22]

A lucigenin assay was performed as previously described, with some modifications.[23] Briefly, control cells or cells treated with Ang II for 4 hours were harvested and pelleted by centrifugation (1200 rpm, 4°C, 5 minutes). To start the assay, cells were resuspended in Hanks' balanced salt solution (Cellegro) containing lucigenin (final concentration, 500 μmol/L). Photon emission was measured every 15 seconds for 20 minutes in a luminometer (Wallac Plate Reader, model 1420). A buffer blank (<5% of the cell signal) was subtracted from each reading before transformation of the data.

[^{3}H]Leucine Incorporation Study

The effect of conditioned medium (CM) from Ang II–stimulated cardiac fibroblasts for protein synthesis in cardiac myocytes was

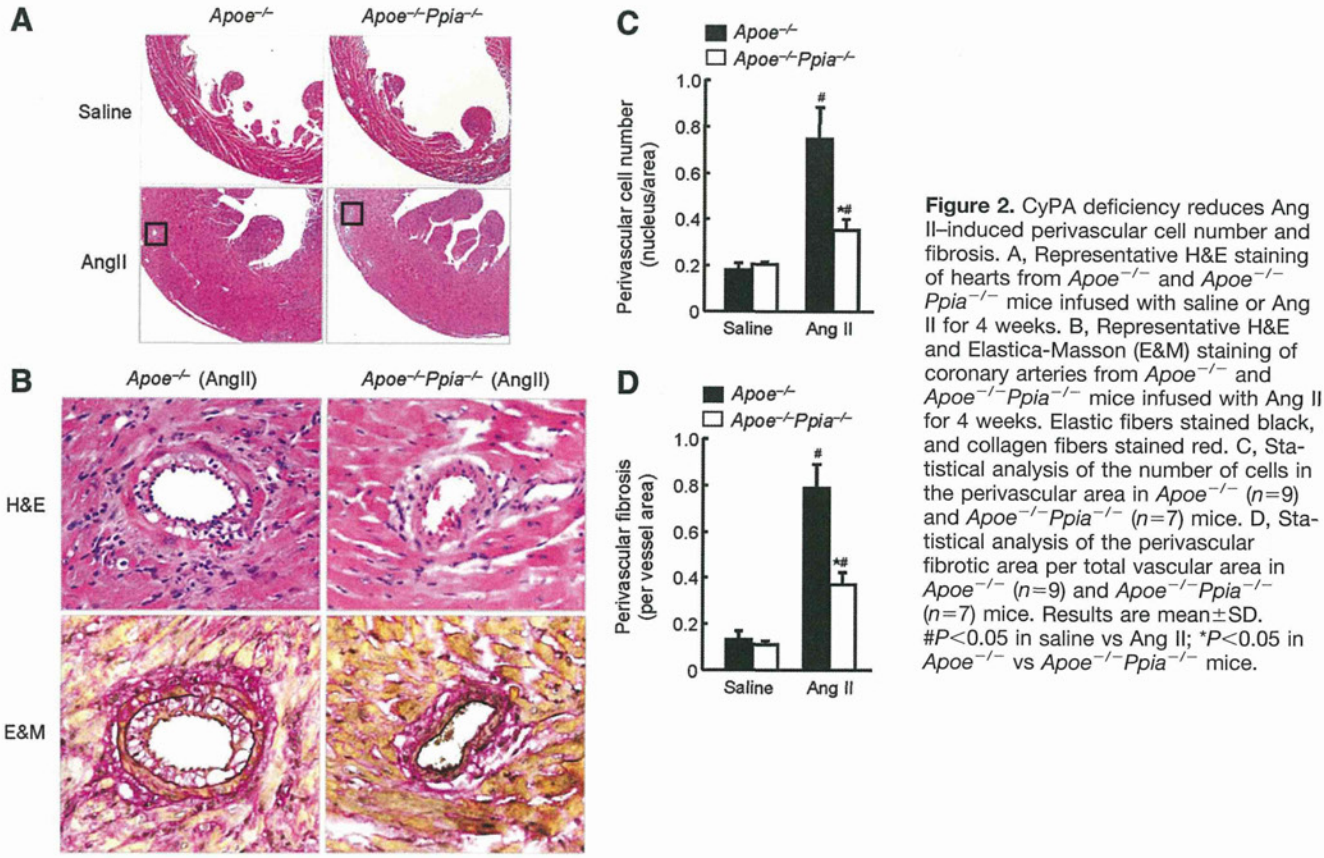

Figure 2. CyPA deficiency reduces Ang II–induced perivascular cell number and fibrosis. A, Representative H&E staining of hearts from $Apoe^{-/-}$ and $Apoe^{-/-}Ppia^{-/-}$ mice infused with saline or Ang II for 4 weeks. B, Representative H&E and Elastica-Masson (E&M) staining of coronary arteries from $Apoe^{-/-}$ and $Apoe^{-/-}Ppia^{-/-}$ mice infused with Ang II for 4 weeks. Elastic fibers stained black, and collagen fibers stained red. C, Statistical analysis of the number of cells in the perivascular area in $Apoe^{-/-}$ (n=9) and $Apoe^{-/-}Ppia^{-/-}$ (n=7) mice. D, Statistical analysis of the perivascular fibrotic area per total vascular area in $Apoe^{-/-}$ (n=9) and $Apoe^{-/-}Ppia^{-/-}$ (n=7) mice. Results are mean±SD. #$P<0.05$ in saline vs Ang II; *$P<0.05$ in $Apoe^{-/-}$ vs $Apoe^{-/-}Ppia^{-/-}$ mice.

determined by [^{3}H]leucine incorporation as previously described.[24] Briefly, neonatal rat cardiac myocytes were plated at a density of 250,000 cells/well in 12-well plates and maintained in Dulbecco's modified Eagle's medium supplemented with insulin, transferrin, and selenium, 5% horse serum, 5% fetal calf serum, 50 U/mL penicillin, and 50 μg/mL streptomycin for 24 hours. The cells were then starved in serum-free Dulbecco's modified Eagle's medium for 48 hours. Cardiac myocytes were stimulated with cardiac fibroblast-derived CM for 24 hours following incubation with [^{3}H]leucine (2 μCi/mL) for 24 hours. To precipitate proteins, ice-cold 10% trichloroacetic acid was added to the wells. After 30 minutes of incubation on ice, the myocytes were lysed with 0.5 N NaOH and incubated for 10 minutes on ice. All samples were mixed with scintillation cocktail (Biosafe-II) before counting.

Statistical Analysis

Quantitative results are expressed as mean±SD. Comparisons of parameters among 2 groups were made by the unpaired Student t test. Comparisons of parameters among >2 groups were made by 1-way ANOVA, and comparisons of different parameters between the 2 genotypes were made by 2-way analysis of variance (ANOVA), followed by a post hoc analysis using the Bonferroni test. Statistical significance was evaluated with StatView (StatView 5.0, SAS Institute Inc, Cary, NC). A value of $P<0.05$ was considered to be statistically significant.

Results

CyPA Augments Ang II–Induced Cardiac Hypertrophy In Vivo

To define the role of CyPA in cardiac hypertrophy, we studied the $Ppia^{-/-}$ (knockout) and wild-type (WT) mice following Ang II infusion for 4 weeks. Ang II significantly increased heart weight/BW in both $Ppia^{-/-}$ and WT mice (Figure 1A and 1C). However, there were no significant differences in heart weight/BW (Figure 1A and 1C), systolic blood pressure, or interventricular septum thickness (Supplemental Table I) between $Ppia^{-/-}$ and WT mice before or after Ang II treatment. Although there was an apparent increase in echo-estimated left ventricle (LV) mass in the WT compared with $Ppia^{-/-}$, when gravimetric heart was normalized to the BW, the relative increase in heart weight/BW did not differ significantly (1.25-fold versus 1.20-fold).

Because CyPA is a proinflammatory cytokine secreted in response to oxidative stress, we hypothesized that the role of CyPA in cardiac hypertrophy would require a situation in which there was increased ROS generation or inflammation. Previously, it was demonstrated that the hearts of apolipoprotein E ($Apoe$)$^{-/-}$ mice exhibit increased ROS production.[11] Because we showed that ROS generation stimulates secretion of CyPA from vascular smooth muscle cells, we compared the secretion of CyPA from WT and $Apoe^{-/-}$ cardiac fibroblasts in response to Ang II. Secretion of CyPA was barely detected in conditioned media (CM) from WT fibroblasts (Supplemental Figure I). In contrast, there was abundant CyPA secretion from Ang II–treated $Apoe^{-/-}$ cardiac fibroblasts. We attempted similar experiments in cultured adult cardiac myocytes. However, substantial cell death ensued on culture in the required serum-free medium.

The above data suggest that $Apoe^{-/-}$ mice would provide an ideal model by which to study the role of CyPA in Ang II–induced cardiac hypertrophy. Consistent with previous findings,[18] we showed that Ang II infusion for 4 weeks significantly increased cardiac hypertrophy in $Apoe^{-/-}$ mice (Figure 1B and 1D). Despite equal increases in blood pressure (Supplemental

Figure IIA), *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice had significantly smaller increases in heart weight after treatment with Ang II compared with *Apoe*$^{-/-}$ mice (Figure 1B and 1D). Echocardiography showed no significant difference in LV mass and interventricular septum thickness between *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice before Ang II treatment (Supplemental Table II). However, after Ang II infusion, *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice had significantly smaller increases in LV mass and interventricular septum thickness compared with *Apoe*$^{-/-}$ mice (Figure 1E, Supplemental Figure IIB and IIC). Moreover, in *Apoe*$^{-/-}$ mice, Ang II significantly increased cardiac myocyte size compared with control (saline-infused mice) (Figure 1F and 1G). In contrast, in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice, Ang II–induced cardiac myocyte hypertrophy was significantly reduced (Figure 1F and 1G). These results suggest that CyPA is required for cardiac hypertrophy induced by Ang II in *Apoe*$^{-/-}$ mice.

Morphologically, the hearts of saline-infused *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice did not differ from the hearts of *Apoe*$^{-/-}$ mice, as shown by H&E staining (Figure 2A). In response to Ang II, LV wall thickness significantly increased in *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice, although to a lesser extent in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (Figure 2A). Most impressively, there was an obvious reduction in perivascular cell number, suggesting decreased proliferation or migration of cells to the perivascular area in the *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (Figure 2B and 2C). There was also a decrease in collagen content in the perivascular area, as shown by Elastica-Masson staining (Figure 2B), in the *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice after Ang II treatment. Perivascular fibrosis area was markedly decreased in the coronary arteries of *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (Figure 2D). These data suggest that CyPA contributes to cardiac hypertrophy and perivascular fibrosis in *Apoe*$^{-/-}$ mice.

CyPA Deficiency Prevents Ang II–Induced ROS Production in Cardiac Tissue

Because ROS are key mediators of Ang II action, we next investigated whether CyPA altered the redox state of the heart after Ang II treatment. Heart sections were incubated with dihydroethidium, which in the presence of superoxide anions is transformed to fluorescent oxyethidium. In the saline-infused heart, ROS production (red fluorescence) was low in both *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (Figure 3A, 3B, and 3E). After Ang II treatment, in the whole heart, oxyethidium fluorescence was ≈2-fold greater in *Apoe*$^{-/-}$ mice compared with *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice (Figure 3C, 3D, and 3E). In the perivascular area of *Apoe*$^{-/-}$ mice, ROS production was increased by 4-fold after Ang II–treatment (Figure 3C and 3F). In contrast, in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice, the perivascular increase in ROS was markedly reduced (Figure 3D and 3F). These data suggest that CyPA is a key determinant for Ang II–mediated ROS production.

Cardiac-Derived CyPA Promotes Recruitment of Bone Marrow–Derived Cells

We have shown that CyPA has direct chemotactic effects on bone marrow–derived cells and promotes vascular cell proliferation and remodeling.[19] To determine whether cardiac CyPA promotes recruitment of bone marrow–derived cells and mediates Ang II–induced cardiac hypertrophy, GFP^{+}

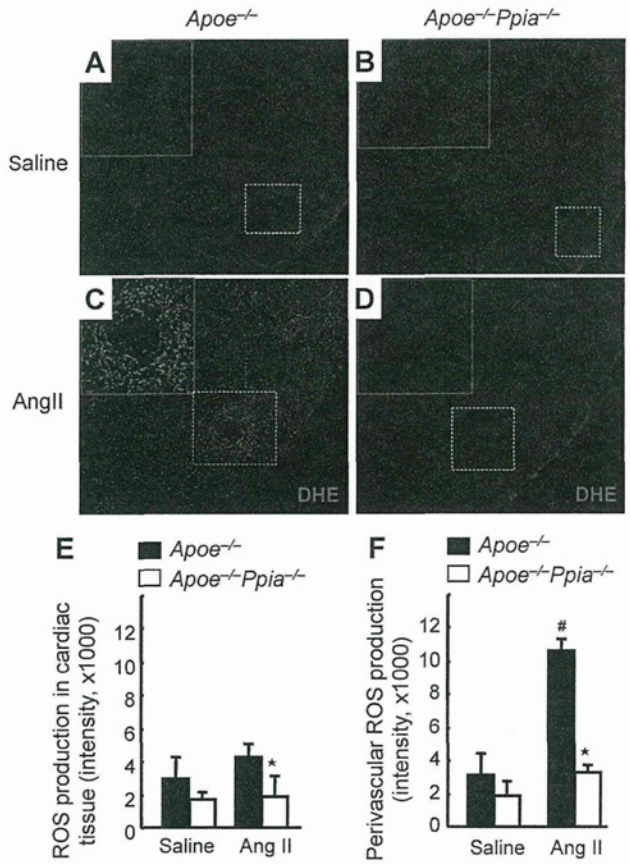

Figure 3. CyPA is crucial for cardiac ROS formation. A to D, Representative in situ dihydroethidium (DHE) staining of hearts. The hearts from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice infused with saline or Ang II for 7 days were used for analysis. Images were obtained using the same magnification (×100) and shutter speed. E and F, Densitometric analysis of oxyethidium (red fluorescence) in the cardiac tissue (E) and the perivascular area (F). Results are mean±SD (n=4 in each group). #P<0.05 in saline vs Ang II; *P<0.05 in *Apoe*$^{-/-}$ vs *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice.

bone marrow cells (*Ppia*$^{+/+}$) were transplanted into irradiated *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice. After 42 days, these chimeric mice were treated with Ang II for 4 weeks. There was no significant difference in the blood pressure between chimeric mice before and after Ang II infusion (data not shown). Ang II dramatically increased the number of bone marrow–derived cells (GFP^{+} cells) present in the cardiac tissue in the *Apoe*$^{-/-}$ recipient mice (Figure 4C and 4E). Most significant was the accumulation of bone marrow–derived cells in the perivascular area of *Apoe*$^{-/-}$ recipient mice (Figure 4C and 4F), which was significantly reduced in the perivascular area of *Apoe*$^{-/-}$*Ppia*$^{-/-}$ recipient mice (Figure 4D and 4F). Because many of these bone marrow–derived cells are inflammatory cells, and inflammation is associated with increased ROS, it is possible that they will contribute to the elevated ROS observed in the perivascular area.

After transplantation of *Ppia*$^{+/+}$ bone marrow cells to the *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice, cardiac hypertrophy was still significantly lower in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ recipient mice compared with *Apoe*$^{-/-}$ recipient mice (Figure 4G). These data suggest that *Ppia*$^{+/+}$ inflammatory cells are not important in Ang II–induced cardiac hypertrophy. We next prepared chimeric

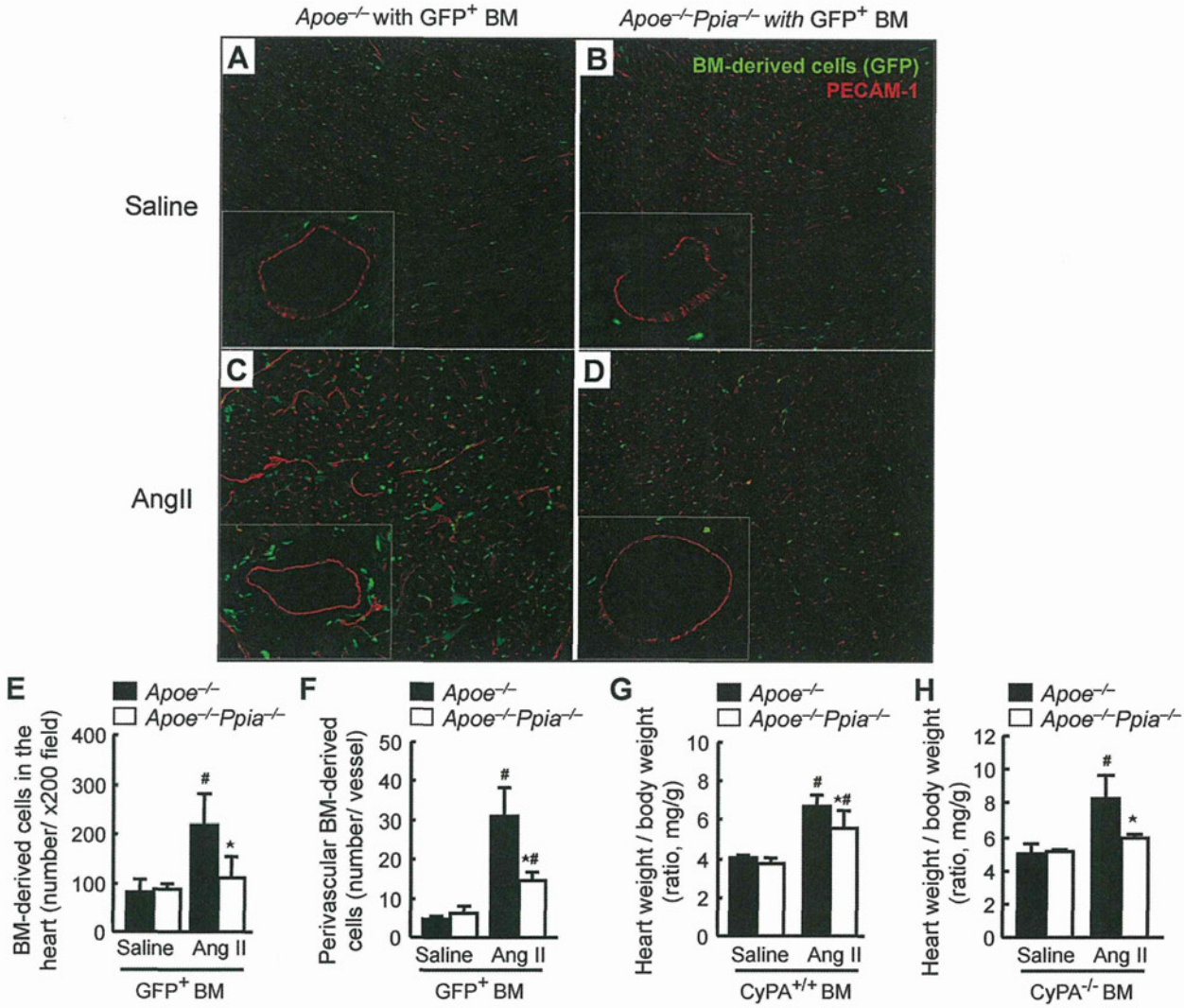

Figure 4. Bone marrow reconstitution shows a strong effect of cardiac CyPA for recruitment of bone marrow–derived cells in response to Ang II and development of cardiac hypertrophy. GFP$^+$ bone marrow cells (*Ppia*$^{+/+}$) were transplanted into irradiated *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice. After 6 weeks, these chimeric mice with GFP$^+$ bone marrow were infused with saline (A and B) or Ang II for 4 weeks (C and D). A to D, Representative platelet and endothelial cell adhesion molecule-1 staining (red) of hearts from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ recipient mice with GFP$^+$ bone marrow (green). E and F, Statistical analysis of the number of migrating GFP$^+$ bone marrow cells in the cardiac tissue (E) or perivascular area (F) in the hearts of *Apoe*$^{-/-}$ (n=9) and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ (n=8) mice. G, Ang II–induced cardiac hypertrophy (heart weight/BW ratio) was significantly lower in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ recipient mice (n=9) compared with *Apoe*$^{-/-}$ recipient mice (n=8) with *Ppia*$^{+/+}$ bone marrow. H, Heart weight/BW ratio after Ang II infusion for 4 weeks was significantly lower in *ApoE*$^{-/-}$*Ppia*$^{-/-}$ (n=6) compared with *ApoE*$^{-/-}$ (n=6) chimeric mice with *Ppia*$^{-/-}$ bone marrow. Results are mean±SD. #P<0.05 in saline vs Ang II; *P<0.05 in *Apoe*$^{-/-}$ vs *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice.

mice transplanted with *Ppia*$^{-/-}$ bone marrow to investigate the role of recipient environment. There was no significant difference in the heart weight/BW of saline-infused groups. However, after Ang II infusion for 4 weeks, there was still a significantly greater heart weight/BW in the *ApoE*$^{-/-}$ compared with the *ApoE*$^{-/-}$*Ppia*$^{-/-}$ chimeric mice, even after transplantation with *Ppia*$^{-/-}$ bone marrow (Figure 4H). These data further demonstrate that cardiac-derived CyPA, not bone marrow–derived CyPA, is most important in Ang II–induced cardiac hypertrophy.

CyPA Augments Ang II–Induced ROS Production and Proliferation in Cultured Cardiac Fibroblasts

On the basis of the above results, we next evaluated the effects of CyPA on cells present in cardiac tissue. We first studied cardiac fibroblasts because of the increase in perivascular collagen (Figure 2B) and the large increase in CyPA secretion (Supplemental Figure I) in response to Ang II. We isolated cardiac fibroblasts from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice and measured ROS by DCF staining. In response to 1 μmol/L Ang II, there was a 2-fold increase in ROS production in *Apoe*$^{-/-}$ (Figure 5A and 5B). There was a dramatic reduction in Ang II–induced ROS production in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ fibroblasts (Figure 5A and 5B), suggesting that CyPA plays an autocrine role in cardiac fibroblast ROS generation, similar to our findings in vascular smooth muscle cells.[11] To verify the results with DCF, we also measured ROS by lucigenin chemiluminescence. As shown in Figure 5C, Ang II–induced ROS production was significantly decreased by ≈50% in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ fibro-

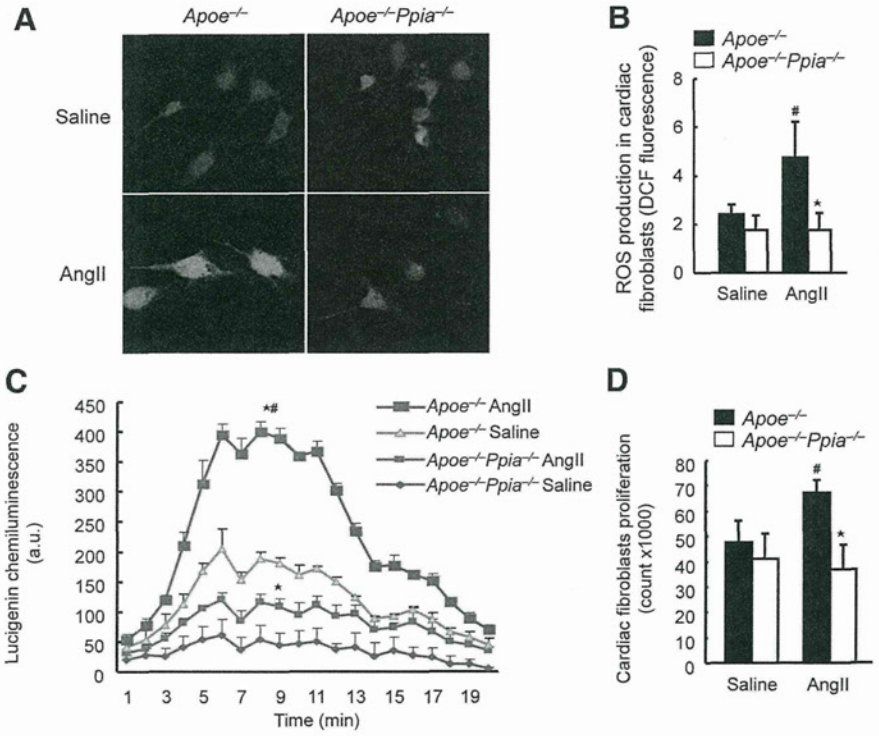

Figure 5. CyPA activates cardiac fibroblasts by enhancing ROS production. A, Representative DCF staining of mouse cardiac fibroblasts. Ang II–induced ROS generation after 4 hours was decreased in CyPA-deficient cardiac fibroblasts. B, Densitometric analysis of DCF fluorescence in response to Ang II shows significant reduction in $Ppia^{-/-}$ cardiac fibroblasts at 4 hours (n=8 in each group). C, Superoxide production in cardiac fibroblasts exposed to lucigenin for 4 hours. Results are mean±SD of 3 independent experiments performed in triplicate. #P<0.05 in saline vs Ang II; *P<0.05 in $Apoe^{-/-}$ vs $Apoe^{-/-}Ppia^{-/-}$ mice. D, Proliferation of cardiac fibroblasts. $Apoe^{-/-}$ and $Apoe^{-/-}Ppia^{-/-}$ fibroblasts were treated with saline or Ang II. After 48 hours of incubation, cells were counted (n=3 in each group). Results are mean±SD. #P<0.05 in saline vs Ang II; *P<0.05 in $Apoe^{-/-}$ vs $Apoe^{-/-}Ppia^{-/-}$ cardiac fibroblasts.

blasts compared with $Apoe^{-/-}$ fibroblasts, similar to results with DCF.

Excessive fibroblast proliferation induces myocardial stiffening, an important component of pathological cardiac hypertrophy.[25,26] To evaluate whether CyPA is important in fibroblast proliferation, cell number in response to Ang II was assessed. Proliferation was significantly augmented by Ang II in $Apoe^{-/-}$ cardiac fibroblasts, whereas there was no change in $Apoe^{-/-}Ppia^{-/-}$ fibroblasts (Figure 5D). These results suggest that CyPA-mediated cardiac fibroblast proliferation contributes to the perivascular cellular response.

Extracellular CyPA Promotes Proliferation and Migration of Cultured Cardiac Fibroblasts

We next evaluated the effect of extracellular CyPA on fibroblast proliferation. Interestingly, there was a small but significantly greater growth rate of $Apoe^{-/-}$ fibroblasts compared with $Apoe^{-/-}Ppia^{-/-}$ fibroblasts treated with vehicle measured by cell number (Figure 6A). Addition of recombinant CyPA (100 nmol/L) significantly increased proliferation of $Apoe^{-/-}$ fibroblasts (Figure 6A) and compared to $Apoe^{-/-}Ppia^{-/-}$ cardiac fibroblasts (Figure 6A).

We next studied the effect of CyPA on migration of fibroblasts. There was a concentration-dependent increase in migration, which was significant at 100 nmol/L CyPA (Figure 6B). These findings highlight the importance of extracellular and intracellular CyPA in cardiac fibroblast proliferation and migration.

Extracellular CyPA Has a Direct Effect on Cardiac Myocyte Hypertrophy

To evaluate whether CyPA could be a direct hypertrophic factor, we investigated the effect of extracellular CyPA on protein synthesis of isolated rat neonatal cardiac myocytes. Treatment with recombinant CyPA significantly increased [^{3}H]leucine incorporation in cardiac myocytes, suggesting the critical role of extracellular CyPA for the hypertrophic response (Figure 6C). To confirm further our data, we measured atrial natriuretic peptide and brain natriuretic peptide mRNA expression, 2 accepted markers of cardiac hypertrophy. We found that CyPA upregulated atrial natriuretic peptide and brain natriuretic peptide in neonatal cardiac myocytes (Figure 6D).

Cardiac fibroblasts isolated from $Apoe^{-/-}$ mice secreted substantial amounts of CyPA in response to Ang II (Supplemental Figure I). Therefore, we investigated the role of fibroblast-derived secreted CyPA in mediating Ang II–induced hypertrophic response in cardiac myocytes. We treated rat neonatal cardiac myocytes with CM prepared from Ang II–stimulated cardiac fibroblasts. Protein synthesis, as measured by [^{3}H]leucine incorporation in myocytes, was used as a parameter of hypertrophy. CM prepared from Ang II–stimulated $Apoe^{-/-}$ fibroblasts significantly augmented [^{3}H]leucine incorporation in cardiac myocytes (Figure 6E). In contrast, there was no effect when myocytes were stimulated with CM prepared from the $Apoe^{-/-}Ppia^{-/-}$ fibroblasts (Figure 6E). These results suggest that CyPA secreted from cardiac fibroblasts can act as a hypertrophic factor on cardiac myocytes. However, on the basis of these data, we cannot exclude the possibility that CyPA is involved in the secretion of another factor, either from fibroblasts or myocytes, which in turn induces cardiac hypertrophy. Together, these results indicate that both intracellular CyPA and extracellular CyPA play critical roles in the mechanisms that contribute to cardiac hypertrophy.

Discussion

The major finding of the present study is that CyPA is a novel mediator of cardiac hypertrophy. We characterized 4 important pathological mechanisms by which CyPA promotes

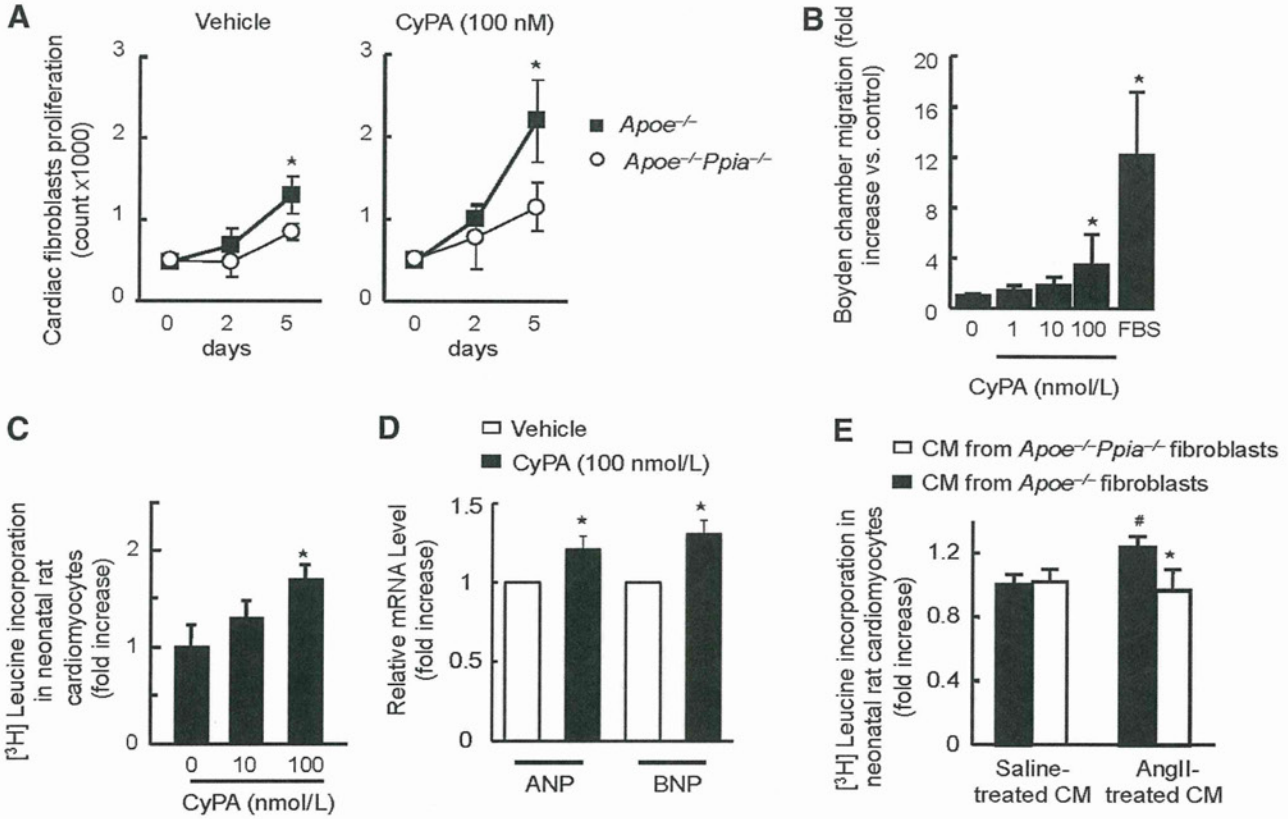

Figure 6. CyPA promotes proliferation and migration of cardiac fibroblasts and hypertrophy of cardiac myocytes. A, After starvation for 24 hours, cardiac fibroblasts from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice were stimulated with 100 nmol/L CyPA or vehicle for 5 days. Medium was changed at day 2, and cells were counted at days 2 and 5. Data are mean±SD. **P*<0.01. *n*=4 in each group. B, Recombinant CyPA promotes cardiac fibroblasts migration in a dose-dependent manner. Data are mean±SD. **P*<0.01 compared with control. *n*=6 in each group. C and D, Neonatal rat cardiac myocytes were treated with recombinant CyPA (0, 10, 100 nmol/L) for 24 hours. Hypertrophy was assessed by the [^{3}H]leucine incorporation method (C) and by measuring atrial natriuretic peptide and brain natriuretic peptide mRNA levels (D). Results are mean±SD. **P*<0.05. E, Cardiac myocytes were stimulated with CM prepared from cardiac fibroblasts that were treated with saline or Ang II for 12 hours. Hypertrophy of neonatal rat cardiac myocytes was determined by means of [^{3}H]leucine incorporation. Data were normalized to myocytes stimulated by CM prepared from saline-treated *Apoe*$^{-/-}$ fibroblasts. *n*=9 in each group. Results are mean±SD. #*P*<0.05 in saline treated CM vs Ang II; **P*<0.05 in *Apoe*$^{-/-}$ vs *Apoe*$^{-/-}$*Ppia*$^{-/-}$ CM.

cardiac hypertrophy (Supplemental Figure III): (1) CyPA is a key determinant for ROS generation, (2) secretion of CyPA in the *Apoe*$^{-/-}$ background is characterized by increased oxidative stress and inflammation, (3) CyPA has a direct hypertrophic effect on cardiac myocytes, and (4) CyPA stimulates proliferation of cardiac fibroblasts that also secrete CyPA, which indirectly stimulates cardiac myocyte hypertrophy. Together, these direct and indirect effects of CyPA contribute to cardiac hypertrophy in *Apoe*$^{-/-}$ mice in response to Ang II.

To examine the involvement of CyPA in the process of the cardiac hypertrophy, we used the Ang II–infusion approach, a well-established mouse model by which to study cardiac hypertrophy. Here, we observed no significant differences in the magnitude of the hypertrophy induced by Ang II between WT and *Ppia*$^{-/-}$ mice on a C57Bl/6 background. Therefore, we investigated the effect of CyPA in *Apoe*$^{-/-}$ mice because they are characterized by high ROS and inflammation.[27] We have recently shown that extracellular CyPA promotes vascular ROS production, which will further induce CyPA secretion and enhance ROS generation.[11] The fact that the CyPA effect was most apparent in the setting of *Apoe* deficiency supports our concept that CyPA plays a key role under conditions in which ROS production contributes to cardiac hypertrophy. However, it is also conceivable that the hyperlipidemia or heightened inflammation characteristic of this model could have contributed to the observed effects.

We anticipated that the *Apoe*$^{-/-}$ background would greatly exacerbate the CyPA-mediated hypertrophic response because Ang II–induced CyPA secretion is significantly elevated in these mice.[11] Consistent with this concept, we found that CyPA secretion from cardiac fibroblasts isolated from WT mice was dramatically lower compared with *Apoe*$^{-/-}$ fibroblasts when stimulated with Ang II.

CyPA has important roles in the immune system and is a well-described regulator of T-lymphocyte functions.[28] It is relevant to note that the primary sources of CyPA responsible for cardiac hypertrophy are likely cells in the heart and not inflammatory cells, because transplantation with *Ppia*$^{+/+}$ bone marrow cells still resulted in less cardiac hypertrophy in *Apoe*$^{-/-}$ *Ppia*$^{-/-}$ compared with *Apoe*$^{-/-}$ mice (Figure 4). In addition, transplantation of *Apoe*$^{-/-}$*Ppia*$^{-/-}$ bone marrow into *Apoe*$^{-/-}$ did not prevent cardiac hypertrophy in response to Ang II (Figure 4H). These data suggest the importance of cardiac-derived CyPA for recruitment of bone marrow–derived cells to perivascular tissues to create an environment that is prohypertrophic.

The interaction between cardiac myocytes and cardiac fibroblasts is a key event during Ang II–induced cardiac hypertrophy.[2,5,24,29,30] In particular, several studies have shown that cardiac myocyte hypertrophy was stimulated by growth factors and cytokines secreted from cardiac fibroblasts.[2–4,31,32] To prove that CyPA could be one of these factors that in a paracrine fashion ultimately induces cardiac myocyte hypertrophy, we first showed that CyPA is released from cardiac fibroblasts after Ang II treatment, and then we proved that extracellular CyPA stimulates cardiac hypertrophy. However, we cannot exclude the involvement of CyPA produced by vascular smooth muscle cells or cardiac myocytes in the enhancement of hypertrophy and fibrosis.

The precise mechanism by which CyPA directly enhances cardiac hypertrophy remains to be elucidated, in part because the CyPA receptor has not been identified. Nonetheless, the present study suggests that inhibition of CyPA may be a useful therapeutic strategy to attenuate cardiac hypertrophy in patients who experience high oxidative stresses, such as smoking, hypertension, and hyperlipidemia.

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Disclosures

None.

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Supplemental Material

Cyclophilin A promotes cardiac hypertrophy in apolipoprotein E-deficient mice

Kimio Satoh,[1,2,3] Patrizia Nigro,[1,3] Asad Zeidan,[1] Nwe Nwe Soe,[1] Fabrice Jaffré,[1] Masayoshi Oikawa,[1] Michael R. O'Dell,[1] Zhaoqiang Cui,[1] Prashanthi Menon,[1] Yan Lu,[1] Amy Mohan,[1] Chen Yan,[1] Burns C. Blaxall,[1] and Bradford C. Berk[1]

[1]Aab Cardiovascular Research Institute and Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, NY, 14642, USA. [2]Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, 980-8574, Japan.

[3] These authors equally contributed to this work.

Supplementary methods

Generation of mice

All animal experiments were conducted in accordance with the experimental protocols that were approved by the Institutional Animal Care and Use Committee at the University of Rochester. *Ppia*$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and were backcrossed to C57BL/6J mice for 10 generations. The *Apoe*$^{-/-}$ mice on a C57BL/6J background were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). Double knockout *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice were generated by crossing *Ppia*$^{-/-}$ mice with *Apoe*$^{-/-}$ mice. The F1 generation was backcrossed with *Apoe*$^{-/-}$ mice to fix the *Apoe*$^{-/-}$ genotype, and littermates were crossed. All mice were genotyped by PCR on tail clip samples, and all experiments were performed with generations F4–F6 using littermate *Apoe*$^{-/-}$*Ppia*$^{+/+}$ as wild-type controls. Animals were housed under a 12-hour light and 12-hour dark regimen and placed on a normal chow diet.

Blood pressure measurement and echocardiography

Blood pressures were obtained from the mice using a noninvasive tail-cuff system (BP-2000 Blood Pressure Analysis System; Visitech Systems, Apex, North Carolina, USA) as described previously.[1] Unanesthetized mice were restrained on a temperature-controlled mouse board, and echocardiography was performed on mice using a Vevo 770 ultrasound system (Visual Sonics, Toronto, Canada). An echocardiographer blind to animal genotype captured 2-dimensional long axis views of the left ventricle. An M-mode cursor was positioned perpendicular to the interventricular septum and the posterior wall of the left

ventricle at the level of the papillary muscles. The following measurements were obtained for systole and diastole using 5 cardiac cycles: interventricular septal thickness, left ventricular posterior wall thickness, left ventricular internal diameter, heart rate, and left ventricular mass.

Histological Analysis

After hemodynamic measurements, animals were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). For morphological analysis, aortas were perfused with normal saline and fixed with 10% phosphate-buffered formalin at physiological pressure for 5 minutes. The whole hearts were harvested, fixed for 24 hours, embedded in paraffin, and cross-sections (5 μm) were prepared. Paraffin sections were stained with Elastica-Masson staining or used for immunostaining.

Immunohistochemistry

Formaldehyde-fixed paraffin sections were incubated with primary antibody overnight at 4ºC. The primary antibody used was PECAM-1 (1:100 dilution; BD Pharmingen). As a negative control, species- and isotype-matched IgG were used in place of the primary antibody. Slides were viewed with a microscope (BX41, Olympus) and with digital camera (Spot Insight 2, Diagnostic Instruments, Inc.).

Preparation of conditioned medium

Conditioned medium from AngII-stimulated cardiac fibroblasts or control medium from

DMEM-incubated cells was collected and filtered to remove cell debris. The medium was concentrated 100-fold with a Centricon Plus-20 filter (Millipore Corporation, Bedford, MA) to yield concentrated conditioned medium [1].

RNA isolation and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RNA was isolated from rat neonatal cardiac myocytes using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Semi-quantitative RT-PCR was performed with a C1000^{TM} Thermal Cycler (Bio-Rad) by first synthesizing cDNA using oligo(dT) primers with the Reverse Transcription PCR kit (Promega) followed by standard PCR using Go-Taq (Promega) according to the manufacturer's instructions. Rat atrial natriuretic peptide (ANP), brain nariuretic peptide (BNP), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were measured using the following primer pairs: ANP: 5'-ATCTGATGGATTTCAAGAACC-3' (Forward) and 5'-CTCTGAGACGGGTTGACTTC-3' (Reverse);
BNP: 5'-ACAATCCACGATGCAGAAGCT-3'
(Forward) and 5'-GGGCCTTGGTCCTTTGAGA-3' (Reverse);
GAPDH: 5'- GACATGCCGCCTGGAGAAAC-3' (Forward) and 5'-AGCCCAGGATGCCCTTTAGT-3' (Reverse).

Expression and Purification of His-tagged CyPA from High Five^{TM} cells

The recombinant baculovirus encoding N-terminal 6 x His tagged rat CyPA protein was

constructed using the Invitrogen Bac-to-Bac® Baculovirus expression system (Invitrogen, Carlsbad, CA, USA) as described below. Briefly, a 508bp cDNA fragment containing the full-length CDS of rat *PPIA* was obtained by excising from BamHI/EcoRI sites of plasmid pGEX-2TK-CyPA. After gel purification, it was inserted in the *Bam*HI/*Eco*RI sites of pFastBac™HT B vector. The resulting plasmid was termed pFastBac His-CyPA. After transformation and amplification in *E. coli*, purified pFastBac His-CyPA was sequenced and was verified that CyPA sequence was fused at its N-terminal to 6 x His tag. This plasmid was further transformed into DH10Bac™ *E.coli* for transposition into the bacmid. After package, amplification, purification from Sf9 cells, the resulting baculovirus was titred and was further used to infect High Five™ cells. Finally, His-CyPA was purified from High Five™ cells by using Ni-NTA Agarose (Qiagen, Germandtown, MD, USA) according to the manufacturer's instructions.

Mouse adult ventricular cardiac fibroblasts Isolation

Wild-type C57B6/j mice aged 12-16 weeks were anesthetized with 0.5 ml heparin (100 U/ml) and 0.5 ml of a ketamine/midazolam in saline combination via intraperitoneal injection. Once anesthetized, the heart was removed, immediately suspended on a Langerdorf apparatus by cannulation of the aortic root and perfused at constant rate of 4 ml/min at 37˚C starting with 4 min of perfusion buffer (5 mM $NaHCO_3$, 30 mM taurine, 10 mM BDM, 5mM Glucose, pH 7.4). Subsequently, enzymatic digestion was achieved by the infusion of Calcium-free digestion buffer (120mg Collagenase type II in 50 ml perfusion buffer) for 3 min followed by 10 minutes of perfusion with calcium containing digestion