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Original Article

Apocynin attenuates oxidative stress and cardiac fibrosis in angiotensin II-induced cardiac diastolic dysfunction in mice

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Aim: To investigate whether apocynin, a NADPH oxidase inhibitor, produced cardioproteictive effects in Ang II-induced hypertensive mice, and to elucidate the underlying mechanisms.
Methods: C57BL/6 mice were subcutaneously infused Ang II for 4 weeks to mimic cardiac remodeling and fibrosis. Concomitantly the mice were administered apocynin (100 $mg\cdot kg^{-1}\cdot d^{-1}$) or/and the aldosterone receptor blocker eplerenone (200 $mg\cdot kg^{-1}\cdot d^{-1}$) via gavage for 4 weeks. Systolic blood pressure (SBP) and heart rate were measured, and transthoracic echocardiography was performed. For *in vitro* study, cardiac fibroblasts were treated with Ang II (10^{-7} mol/L) in the presence of apocynin (10^{-5} mol/L) or/and eplerenone (10^{-5} mol/L). Immunohistochemistry and Western blotting were used to quantify the expression levels of NADPH oxidase and osteopontin (OPN) proteins in the cells.
Results: Both apocynin and eplerenone significantly decreased SBP, and markedly improved diastolic dysfunction in Ang II-induced hypertensive mice, accompanied with ameliorated oxidative stress and cardiac fibrosis. In the Ang II-treated cardiac fibroblasts, the expression levels of NOX4 and OPN proteins were markedly upregulated. Both Apocynin and eplerenone significantly suppressed the increased expression levels of NOX4 and OPN proteins in the Ang II-treated cells. In all the experiments, apocynin and eplerenone produced comparable effects. Co-administration of the two agents did not produce synergic effects.
Conclusion: Apocynin produces cardioproteictive effects comparable to those of eplerenone. The beneficial effects of apocynin on myocardial oxidative stress and cardiac fibrosis might be mediated partly through a pathway involving NADPH oxidase and OPN.

Keywords: angiotensin; Ang II-induced hypertensive mice; diastolic heart failure; eplerenone; apocynin; osteopontin; NADPH oxidase; NOX4

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Introduction

Nearly half of all patients with chronic heart failure experience diastolic dysfunction[1]. Although great efforts have been devoted to deciphering the pathobiology of diastolic heart failure (DHF), its underlying mechanism and therapeutic strategies remain elusive. In this context, the angiotensin II (Ang II)-induced mouse model of hypertension has been proposed to be an ideal tool to study DHF due to its major features, which include early onset heart failure during the course of hypertension and the preservation of the ejection fraction[2].

Growing evidence implicates reactive oxygen species (ROS) in the pathophysiology of heart failure and Ang II-induced cardiovascular disease[3]. Nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase is a key enzyme that affects ROS production[4]. Osteopontin (OPN), a SIBLING glycoprotein, interacts with various extracellular matrices and is involved in Ang II-induced fibrosis and heart failure[5]. We and others previously found that the progression of diastolic dysfunction could be effectively prevented by the aldosterone receptor blocker eplerenone, and that cardiac hypertrophy and fibrosis were significantly ameliorated through the inhibition of OPN expression[2, 6]. We therefore sought to evaluate the effect of OPN on the progression of reactive fibrosis in the Ang

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II-induced hypertensive mouse heart and to further explore whether apocynin (an NADPH oxidase inhibitor), alone or in conjunction with eplerenone, could regulate OPN expression through the Ang II-aldosterone system.

To achieve this goal, a hypertensive mouse model with reactive fibrosis developed in the interstitial and perivascular regions of the myocardium and without cardiomyocyte necrosis was generated by infusing Ang II. First, we examined whether apocynin had cardioprotective effects that was comparable to those of eplerenone, *eg*, decreasing systolic blood pressure, improving diastolic dysfunction, and ameliorating oxidative stress and cardiac fibrosis. Then, we examined the effects of apocynin and/or eplerenone on the expression of NADPH oxidase and OPN in this model to investigate the possible mechanisms underlying their functions.

Materials and methods

Animals

All animal protocols followed the guidelines of the Shanghai Jiao Tong University Animal Care and Use Committee. Eight-week-old male C57BL/6 mice (*n*=35) were randomized into 5 groups: a sham group, an Ang II-infused group, an apocynin treatment group, an eplerenone treatment group, and an apocynin and eplerenone combination treatment group. Ang II-infused mice were implanted with an osmotic minipump (model 2004, Alza Corp, USA) with an administrating speed of $2\ \mu g\cdot kg^{-1}\cdot min^{-1}$ and raised for 4 weeks. Concomitantly, these mice were treated with or without apocynin and/or eplerenone. Apocynin (Sigma-Aldrich, USA) was administered at a dosage of $100\ mg\cdot kg^{-1}\cdot d^{-1}$ in the drinking water, and eplerenone (Pfizer, USA) was administered by gavage at a dosage of $200\ mg\cdot kg^{-1}\cdot d^{-1}$ continuously for 4 weeks.

Blood pressure and heart rate

The systolic blood pressure (SBP) and heart rate (HR) were measured by using a tail cuff system (BP96A, Softron, Japan). At least 5 preliminary cycles were performed, and then 10 experimental measurements were taken for each mouse.

Echocardiographic analysis

The left ventricular end-diastolic diameter (LVDd), left ventricular end-systolic diameter (LVSd), and left ventricular posterior wall thickness (PW) were measured by transthoracic echocardiography using a Vevo770 instrument (VisualSonics, Toronto, Canada). The percentage of left ventricular fractional shortening (FS) was used to judge the cardiac systolic function; this value was calculated as FS (%)=(LVDd–LVSd)/LVDd*100.

The left lateral position was chosen to obtain optimal Doppler image quality. The left ventricular inflow tract was interrogated from the apical four-chamber view with the sample volume at the tips of the mitral leaflets. The E wave velocity (E/A) ratio and isovolumic relaxation time (IVRT) were measured to estimate cardiac diastolic function.

Histological analysis

The mouse hearts were fixed with 10% formalin, embedded in paraffin, sectioned at 5 μm and then stained with hematoxylin-eosin to evaluate overall morphology and Masson's trichrome to evaluate fibrosis. Myocyte breadth was measured in the hematoxylin-eosin-stained sections, and suitable cross sections were defined as having nearly circular capillary profiles and nuclei. Myocyte breadth was measured in 100 cells (per mouse) from a randomly selected section of the left ventricular lateral-mid free wall. The collagen fraction was calculated as the ratio of the sum of the total interstitial fibrosis area to the sum of the total connective tissue area plus the myocyte area in an entire visual field. The area of perivascular fibrosis was determined as the ratio of the area of fibrosis surrounding the vessel wall to the total vessel area. Approximately 10 arterial cross sections were examined for each mouse heart.

Assay of oxidative stress

Myocardial tissues were homogenized in RIPA lysis buffer. The lipid peroxide content of the left ventricular was determined by estimating its malondialdehyde (MDA) content (Beyotime Institute of Biotechnology, Haimen, China). MDA is an end product of the lipid peroxidation chain reaction and is frequently used as a marker of ROS production[7]. Glutathione peroxidase (GPx) activity was determined by hydrogen peroxide assay (Nanjing Jiancheng, Nanjing, China), and the disappearance rate of NADPH was recorded spectrophotometrically (412 nm) at 37 °C. GPx is an important cellular antioxidant that protects cells from the damaging effects of oxidation products, such as peroxidized lipids.

Immunohistochemistry

Fixed hearts were embedded in paraffin and sectioned at 5 μm. Antigen retrieval was performed in 10 mmol/L citrate buffer antigen, endogenous peroxidase activity was blocked by immersing the sections in 3% H_2O_2, and nonspecific epitope binding was blocked by incubation with 4% bovine serum albumin. The sections were then incubated with NOX4 polyclonal antibody (Santa Cruz Biotechnology, USA) and OPN polyclonal antibody (IBL, Japan) overnight at 4 °C, followed by an incubation for 30 min at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody (1:200), and visualized with diaminobenzidine.

Western blot

Previously frozen heart tissues were lysed in RIPA buffer containing protease inhibitor (Beyotime, Haimen, China), minced with scissors, and sonicated for 10 s on ice. The homogenates were pelleted at 12 000×*g* for 15 min at 4 °C, and the supernatants were collected for Western blotting. Protein concentrations were determined using the Bradford protein assay (Beyotime, Haimen, China). Tissue lysates (30 μg) were separated on 10% Bis-Tris gels and electrotransferred onto polyvinylidene fluoride membranes, which were incubated overnight at 4 °C with antibodies specific to NOX4 (Santa Cruz Biotechnology, USA), OPN (IBL, Japan) and β-actin (Sigma-Aldrich, USA). After incubation with an HRP-conjugated secondary antibody, immune complexes were detected using

the enhanced chemiluminescence method. Protein concentrations were quantified from the band intensities with ImageJ software (NIH, USA).

Cell culture

Cardiac fibroblasts were obtained from the ventricles of C57BL/6 mice and then grown in DMEM with 10% fetal bovine serum as previously described[8], with minor modifications. Experiments were performed on secondary cultures. The cells were plated in MULTI-WELL 6-well plates, grown for 12 h in serum-free DMEM media, and then stimulated with Ang II (10^{-7} mol/L, Sigma-Aldrich, USA) for 4 h in the presence or absence of eplerenone (10^{-5} mol/L, Sigma-Aldrich, USA) and/or apocynin (10^{-5} mol/L, Sigma-Aldrich, USA) for 30 min. The relative NOX4 and OPN levels were determined by Western blotting.

Statistical analysis

The data are presented as the mean±SEM, unless otherwise indicated. Intergroup data were compared using a one-way analysis of variance (ANOVA) with the Tukey correction or unpaired *t*-tests for continuous variables. Two-tailed $P<0.05$ was considered statistically significant.

Results

Blood pressure and heart rate

The measurements of blood pressure and heart rate under drug treatment are summarized in Table 1. Elevated blood pressure induced by Ang II was significantly reduced by apocynin and/or eplerenone. The antihypertensive effect of apocynin was comparable to that of eplerenone, and combination treatment with both drugs failed to further decrease blood pressure. There were no significant differences in heart rate across the five treatment groups.

Cardiac hypertrophy

The Ang II-induced increase in HW/BW (the ratio of heart weight to body weight) was partially inhibited, albeit not significantly, by both apocynin and eplerenone (Table 1). The Ang II-induced increase in LVW/BW (the ratio of left ventricular weight to body weight) was significantly inhibited by apocynin and eplerenone. Cardiomyocyte breadth was measured to confirm the attenuation of ventricular hypertrophy by both apocynin and eplerenone (Figure 1).

Cardiac fibrosis

Representative photomicrographs of the heart and vessels are shown in Figures 2 and 3, respectively. Interstitial fibrosis was significantly increased after Ang II treatment in mice. Importantly, both apocynin and eplerenone almost completely abolished the Ang II-induced interstitial fibrosis. The percentage of perivascular fibrotic areas also increased markedly in Ang II-treated mice (47.1%±3.1%) compared with sham mice (13.2%±0.2%) ($P<0.05$). Compared with Ang II alone, treatment with apocynin, eplerenone or their combination significantly reduced the fibrotic area by 32.4%±1.8%, 29.8%±1.4%, and 29.0%±0.8%, respectively.

To further verify the reduction in fibrosis response by apocynin and/or eplerenone treatment, the protein expression of OPN and the NADPH oxidase subunit in the left ventricle was evaluated by immunohistochemistry. Positively labeled cells were rare in sham mice but abundant in Ang II-infused mice. Both apocynin and eplerenone remarkably blunted the level of Ang II-induced OPN and NOX4 staining (Figure 4).

Cardiac function and remodeling

Left ventricular hypertrophy, as judged by posterior wall thickness, was clearly induced by Ang II treatment (Table 2) and clearly inhibited by apocynin and/or eplerenone. Left ventricular systolic function was measured by percent fractional shortening (%FS) and was similar in all groups. Moreover, the E wave velocity (E/A) ratio was reduced, and isovolumic relaxation time (IVRT) was prolonged in the Ang II group, reflecting the impairment of left ventricular diastolic function. Finally, the left ventricular diastolic dysfunction caused by the administration of Ang II was improved by treatment with apocynin, eplerenone or their combination.

Myocardial oxidative stress

Ang II treatment triggered an increase in MDA levels (Figure 5A) but no change in the GPx activity in the left ventricle (Figure 5B). Treatment with apocynin and/or eplerenone inhibited an increase in the MDA levels in the hearts, but there was no change in GPx activity. The MDA levels and GPx activity observed in cardiac fibroblasts were consistent with those in cardiac tissue (Figure 5C and 5D).

Table 1. Effect of Apo and Eple on BW, BP, HR, HM/BW, and LVW/BW. n=7. Values are expressed as mean±SEM. $^{b}P<0.05$ *vs* Sham group. $^{e}P<0.05$ *vs* the Ang II group.

Parameter	Sham (n=7)	Ang II (n=7)	Ang II+Apo (n=7)	Ang II+Eple (n=7)	Ang II+Apo+Eple (n=7)
BW (g)	26.4±0.9	26.3±1.1	23.9±1.5	23.7±0.6	24.9±0.7
BP (mmHg)	116.4±2.9	183.3±5.1^{b}	153.6±6.4^{be}	147.6±7.6^{be}	136.3±2.8^{e}
HR (bpm)	638.2±6.2	647.5±11.3	638.1±7.1	641.3±7.2	639.2±5.7
HW/BW (mg/g)	4.4±0.1	6.6±0.3^{b}	5.9±0.3^{b}	5.9±0.2^{b}	5.6±0.2
LVW/BW (mg/g)	3.3±0.1	4.8±0.2^{b}	3.7±0.2^{e}	3.6±0.1^{e}	3.5±0.1^{e}

BW, bodyweight; BP, blood pressure; HR, heart rate; HW/BW, radio of heart weight to bodyweight; LVW/BW, radio of left ventricular weight to bodyweight.

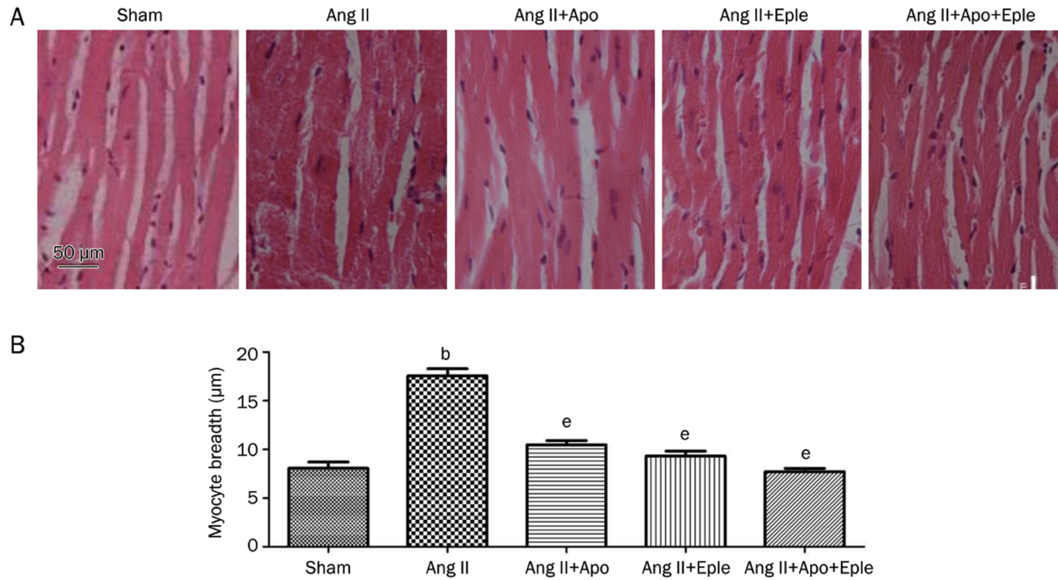

Figure 1. Cardiac myocyte breadths in mice. (A) Representative cross-sections of cardiomyocytes stained with hematoxylin-eosin. (B) Bar graph shows quantitative analysis of cardiac myocyte breadths (μm). Data are expressed as mean±SEM. [b]$P<0.05$ *vs* Sham. [e]$P<0.05$ *vs* Ang II.

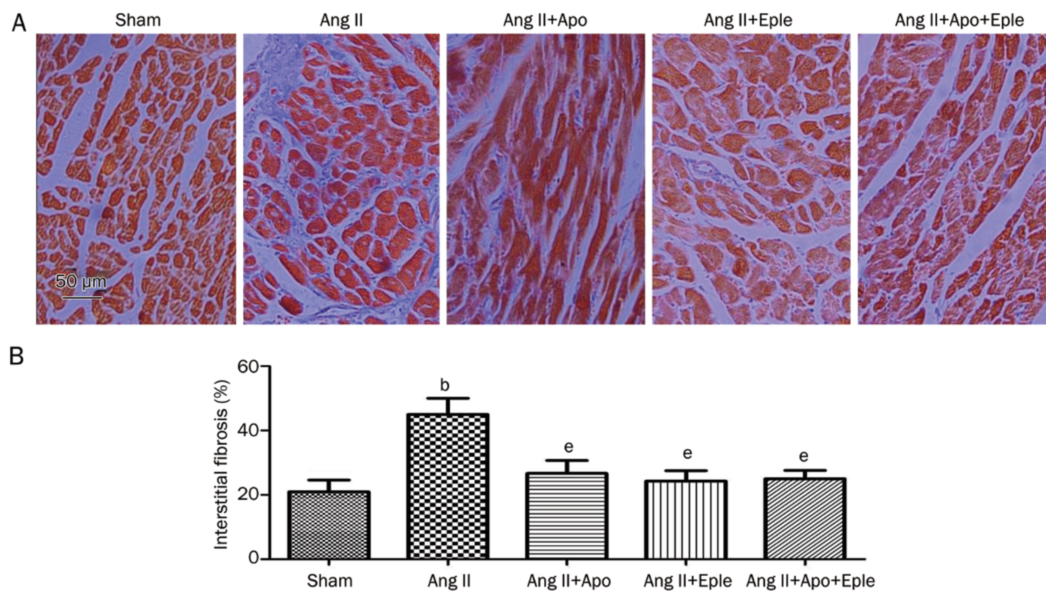

Figure 2. Left ventricular interstitial fibrosis in mice. (A) Representative images of myocardium with interstitial fibrosis stained with Masson trichrome stain. (B) Bar graph shows the quantified interstitial fibrotic areas (%). Data are expressed as mean±SEM. [b]$P<0.05$ *vs* Sham. [e]$P<0.05$ *vs* Ang II.

Effects of Ang II, apocynin, and eplerenone on OPN and NOX4 expression

To investigate whether Ang II treatment stimulated the signaling cascade leading to cardiac fibrosis, we investigated NADPH oxidase and OPN protein levels in cardiac fibroblasts (Figure 6). At baseline, the NOX4 protein was abundantly expressed, whereas NOX1 and NOX2 (gp91phox) were expressed at very low levels (data not shown). Following Ang II treatment (10^{-7} mol/L), NOX4 and OPN protein levels were significantly upregulated. However, NOX4 and OPN protein expression was significantly downregulated when the cells were treated with apocynin (10^{-5} mol/L) and/or eplerenone (10^{-5} mol/L).

Discussion

The most noteworthy finding in this study was that apocynin has cardioprotective effects comparable to that of eplerenone, triggering the reversal of cardiac fibrosis, attenuating oxidative stress, and lowering blood pressure. These effects imply that a pathway involving NADPH oxidase and OPN may regulate

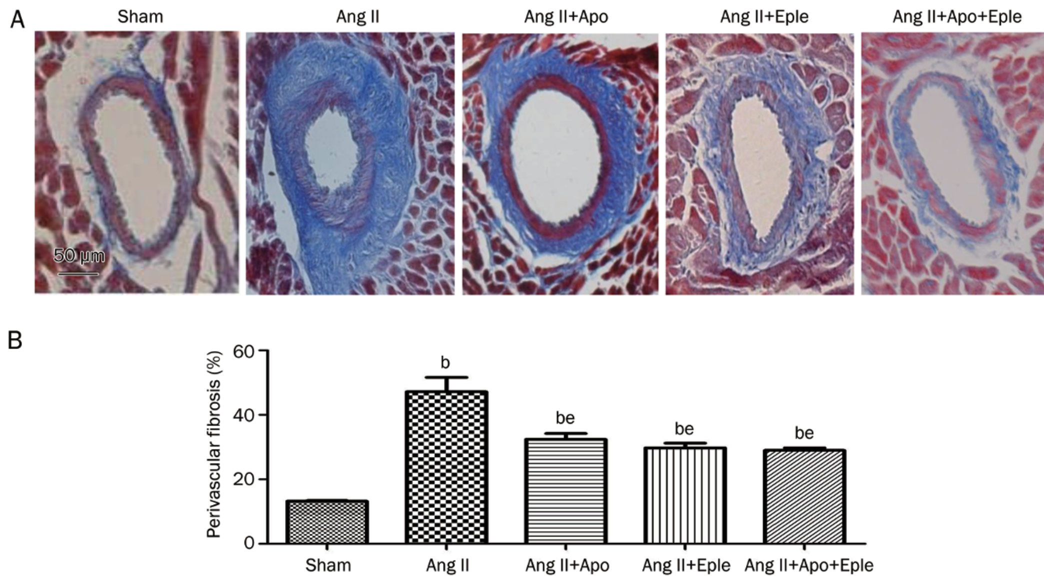

Figure 3. Left ventricular perivascular fibrosis in mice. (A) Representative short-axis images of intramuscular arteries with perivascular fibrosis stained with Masson trichrome stain. (B) Bar graph shows the quantified perivascular fibrotic areas (%). Data are expressed as mean±SEM. [b]*P*<0.05 *vs* sham. [e]*P*<0.05 *vs* Ang II.

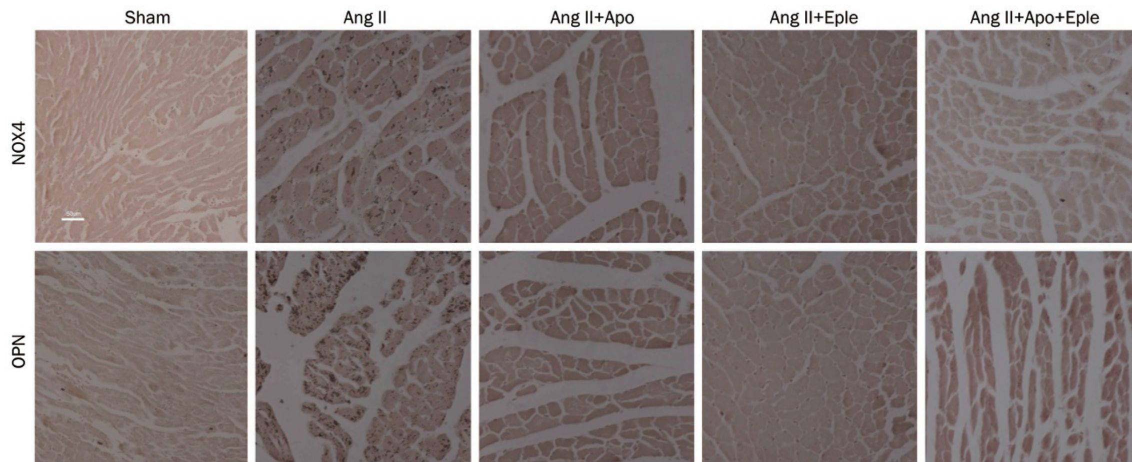

Figure 4. Immunohistochemial analysis of mouse myocardial tissue. Representative photographs of immunostained myocardium and positively stained cells in each group for NOX4 and OPN.

Table 2. Echocardiographic measurements. *n*=5. Values are expressed as mean±SEM. [b]*P*<0.05 *vs* the sham group. [e]*P*<0.05 *vs* Ang II.

Parameter	Sham (*n*=5)	Ang II (*n*=5)	Ang II+Apo (*n*=5)	Ang II+Eple (*n*=5)	Ang II+Apo+Eple (*n*=5)
PW (mm)	0.48±0.02	0.88±0.06[b]	0.69±0.04[be]	0.71±0.05[be]	0.68±0.04[be]
LVDd (mm)	3.35±0.10	3.22±0.14	3.32±0.24	3.25±0.22	3.48±0.26
LVSd (mm)	1.90±0.08	1.81±0.20	1.98±0.37	1.89±0.47	2.04±0.52
FS (%)	43.3±1.69	43.8±2.18	40.4±3.0	41.8±3.4	41.3±2.2
E/A ratio	2.4±0.2	1.5±0.1[b]	2.2±0.3[e]	2.1±0.2[e]	2.3±0.4[e]
IVRT (ms)	12.5±0.4	20.0±1.7[b]	15.0±1.3[e]	15.0±0.8[e]	12.5±1.1[e]

PW, posterior wall thickness; LVDd, left ventricular end-diastolic diameter; LVSd, left ventricular end-systolic diameter; FS, fractional shortening; E/A, ratio of the velocity of E and A waves; IVRT, isovolumic relaxation time.

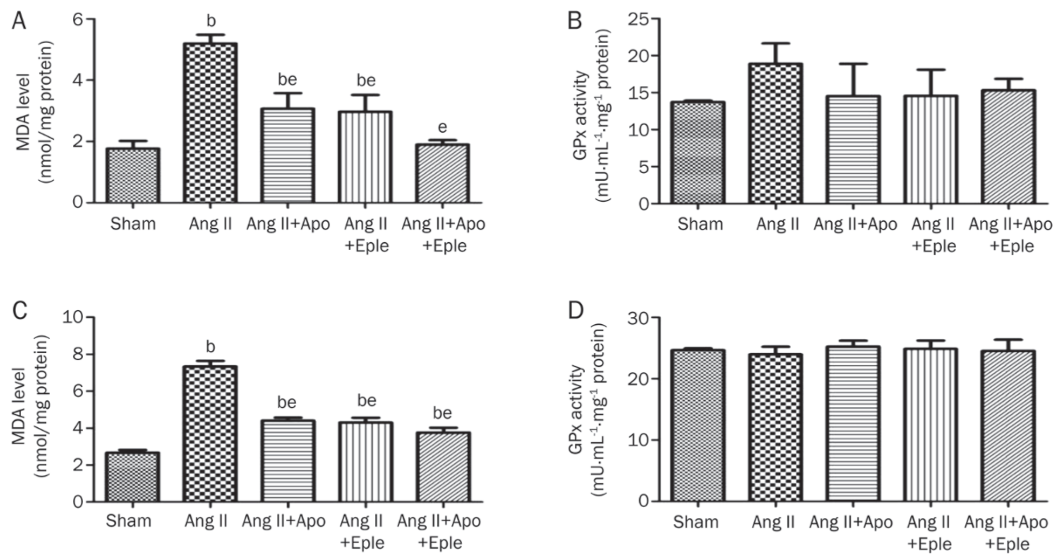

Figure 5. Malondialdehyde (MDA) levels and glutathione peroxidase (GPx) activity in left ventricle and cardiac fibroblasts of mice. (A) MDA levels in the left ventricle of mice. (B) GPx activity in the left ventricle of mice. (C) MDA levels in the cardiac fibroblasts. (D) GPx activity in the cardiac fibroblasts. *n*=5. Data are expressed as mean±SEM. [b]*P*<0.05 *vs* sham. [e]*P*<0.05 *vs* Ang II.

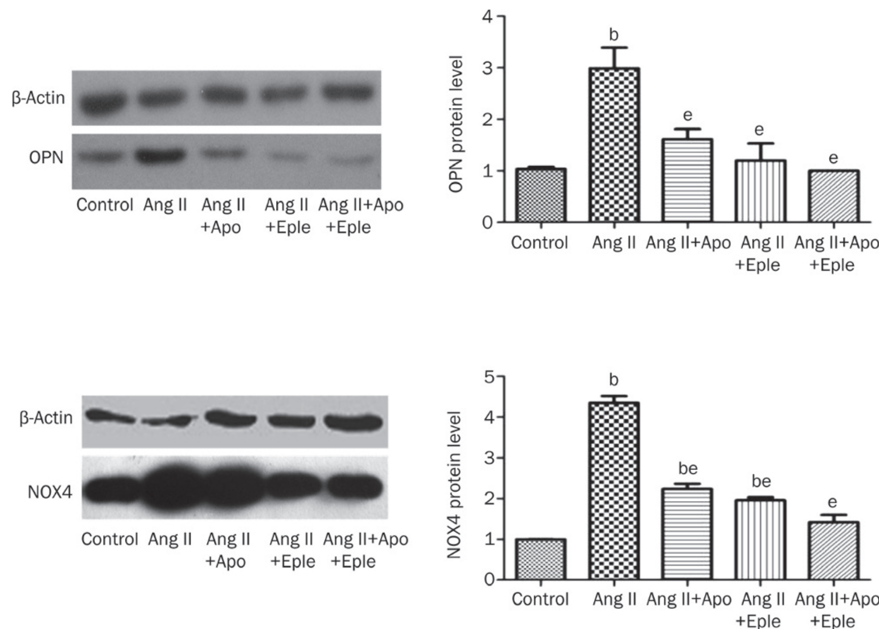

Figure 6. Protein levels of OPN and NOX4 in cardiac fibroblasts determined by Western blot. *n*=3. Data are expressed as mean±SEM. [b]*P*<0.05 *vs* the control. [e]*P*<0.05 *vs* Ang II.

the process of Ang II-induced DHF in mice.

The roles of Ang II in cardiac hypertrophy and remodeling, as well as in the progression of DHF, have been well-established. ROS are believed to be associated with maladaptive responses such as myocardial hypertrophy, apoptosis, and interstitial fibrosis. Experimental data suggest that apocynin acts predominantly as an antioxidant in endothelial cells and vascular smooth muscle cells. However, whether this effect of alleviating excessive oxidation also applies to cardiac oxidative stress in Ang II-induced hypertensive mice with DHF is not yet clear. With this in mind, we observed that the increase in levels of MDA was significantly attenuated by treatment with apocynin in Ang II-induced hypertensive mice with DHF, while myocardial GPx activity remained unaffected, consistent with the findings of Virdis *et al*[9] and Tian *et al*[10].

More importantly, we also noted that treatment with epler-

enone could suppress the formation of ROS. Eplerenone was reported to be superior to apocynin in aldosterone-induced hypertensive rats because eplerenone tends to reduce kidney damage and inhibit NOX expression[11]. Several clinical trials have indicated a remarkable protective impact of eplerenone on cardiac remodeling and heart failure[12, 13]. These observations therefore suggest that lowered blood pressure may suppress ROS formation by modulating the transduction of shear stress-dependent signaling cascades in vascular cells and decrease ROS production accordingly. Further, in light of our observation that eplerenone did not enhance the apocynin-mediated improvement in blood pressure, or *vice versa*, we speculate that the effect of eplerenone on blood pressure that is associated with attenuated cardiac fibrosis and oxidative stress in Ang II-induced hypertensive mice is maximized, and additional treatment with apocynin has no effect.

NADPH oxidase is a transmembrane enzyme that produces superoxide (O_2) by transferring an electron from NADPH to molecular oxygen. A growing body of data suggests that NADPH oxidase is involved in various experimental models of cardiovascular disease and exhibits high activity in human heart failure[14–17]; however, its molecular mechanism remains elusive. As an NADPH oxidase inhibitor, apocynin can prevent and reverse adrenocorticotropic hormone-induced hypertension in rats[16]. In addition, apocynin was also observed to lower SBP, and it was as effective as nifedipine in spontaneously hypertensive rats[15]. However, in Dahl salt-sensitive hypertensive rats with established heart failure, treatment with apocynin failed to reverse left ventricular inflammation and fibrosis[17]. In contrast, in our study, cardiac fibrosis in Ang II-induced hypertensive mice with DHF was significantly ameliorated by apocynin, which lowered the levels of both NADPH oxidase and OPN proteins. One possible reason for this inconsistency is the alleviation of excessive oxidation, as discussed above. Another possibility might be the reduced expression of OPN, which interacts with various extracellular matrices and is involved in the process of Ang II-induced fibrosis and heart failure[5]. Eplerenone treatment stimulated changes similar to those caused by apocynin, suggesting that eplerenone may affect the activation and function of NADPH oxidase and OPN.

Our findings also revealed a role for the NADPH oxidase inhibitor apocynin in further blocking the upregulation of OPN expression in Ang II-induced cardiac fibroblasts. If this observation holds true, we may speculate that the reversal of cardiac remodeling by apocynin might be attributed, at least in part, to the inhibition of NADPH oxidase secondary to the attenuation of OPN expression (known as NADPH oxidase-OPN signaling cascade). However, the possible existence of an OPN-NADPH oxidase signaling cascade cannot be ruled out because both NADPH oxidase and OPN levels can also be lowered by the aldosterone receptor blocker eplerenone.

A literature search did not uncover any evidence supporting either hypothesis. For example, Xie *et al* found that DPI (an inhibitor of the flavoprotein component of NADPH oxidase) inhibited Ang II-stimulated OPN expression in the ventricular myocytes and cardiac microvascular endothelial cells of adult rats, suggesting that NADPH oxidase may be a link between Ang II-stimulated ROS production and OPN expression[18]. Moreover, Umekawa *et al* observed that the CaOx monohydrate-induced production of OPN was significantly reduced after treatment with DPI[19]. In contrast, Lai *et al* proposed that an OPN-NADPH oxidase signaling cascade promotes pro-matrix metalloproteinase-9 activation in aortic mesenchymal cells[20]. Given these contradictory claims, it remains possible that NADPH oxidase and OPN might mutually promote each other's expression during the reversal of cardiac remodeling.

In summary, our findings provide evidence that apocynin had cardioprotective effects comparable to those of eplerenone in restoring DHF by triggering the reversal of cardiac fibrosis, attenuating oxidative stress, and lowering blood pressure, which collectively implicated a potential pathway involving NADPH oxidase and OPN in regulating Ang II-induced DHF in mice. Although further studies to elucidate these complex, multistep molecular pathways are warranted, the reduction of OPN in hypertensive heart might be a key step in perivascular fibrosis, and thus a prime target for clinical therapy.

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Author contribution

Nan JIA and Wen-quan NIU designed the research; Yu-qiong LI and Xiao-bo LI performed the research; Shu-jie GUO, Shao-li CHU, Ping-jin GAO, and Ding-liang ZHU contributed new analytical tools and reagents; Yu-qiong LI and Wen-quan NIU analyzed the data; and Yu-qiong LI, Nan JIA, and Wen-quan NIU wrote and revised the paper.

Abbreviations

Ang II, angiotensin II; OPN, osteopontin; NADPH, nicotinamide adenine dinucleotide phosphate; DHF, diastolic heart failure; ROS, reactive oxygen species; LVDd, left ventricular end-diastolic diameter; LVSd, left ventricular end-systolic diameter; PW, posterior wall thickness; IVRT, isovolumic relaxation time; MDA, malondialdehyde; GPx, glutathione peroxidase; Ab, antibody; ANOVA, one-way analysis of variance; LVW/BW, the ratio of the left ventricular weight to body weight.

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