Cardiomyocyte-specific expression of CYP2J2 prevents development of cardiac remodelling induced by angiotensin II

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Aims
Cardiac remodelling is one of the key pathological changes that occur with cardiovascular disease. Previous studies have demonstrated the beneficial effects of CYP2J2 expression on cardiac injury. In the present study, we investigated the effects of cardiomyocyte-specific CYP2J2 expression and EET treatment on angiotensin II-induced cardiac remodelling and sought to determine the underlying molecular mechanisms involved in this process.

Methods and results
Eight-week-old mice with cardiomyocyte-specific CYP2J2 expression (αMHC-CYP2J2-Tr) and wild-type (WT) control mice were treated with Ang-II. Ang-II treatment of WT mice induced changes in heart morphology, cardiac hypertrophy and dysfunction, as well as collagen accumulation; however, cardiomyocyte-specific expression of CYP2J2 attenuated these effects. The cardioprotective effects observed in αMHC-CYP2J2-Tr mice were associated with peroxisome proliferator-activated receptor (PPAR)-γ activation, reduced oxidative stress, reduced NF-κB p65 nuclear translocation, and inhibition of TGF-β1/smad pathway. The effects seen with cardiomyocyte-specific expression of CYP2J2 were partially blocked by treatment with PPAR-γ antagonist GW9662. In in vitro studies, 11,12-EET(1 μmol/L) treatment attenuated cardiomyocyte hypertrophy and remodelling-related protein (collagen I, TGF-β1, TIMP1) expression by inhibiting the oxidative stress-mediated NF-κB pathway via PPAR-γ activation. Furthermore, conditioned media from neonatal cardiomyocytes treated with 11,12-EET inhibited activation of cardiac fibroblasts and TGF-β1/smad pathway.

Conclusion
Cardiomyocyte-specific expression of CYP2J2 or treatment with EETs protects against cardiac remodelling by attenuating oxidative stress-mediated NF-κBp65 nuclear translocation via PPAR-γ activation.

Keywords
Epoxyeicosatrienoic acids • Cardiac hypertrophy • CYP2J2 • Cardiac remodelling • Oxidative stress

1. Introduction
Cardiac remodelling is described as changes in the size, shape, and function of the heart resulting from cardiac injury. During this process, structural changes in cardiomyocytes and the myocardial extracellular matrix occur. Evidence from experimental animal models and humans indicates that there is increased oxidative stress in the failing heart that contributes to the pathogenesis of myocardial remodelling. Recent progress towards understanding the mechanisms involved in myocardial remodelling has revealed that reactive oxygen species (ROS) play a central role in regulating the phenotype of cardiomyocytes and fibroblasts, while also acting as a paracrine factor to induce cardiac fibrosis. ROS also acts as a secondary messenger to regulate the expression of cardiac remodelling proteins in various types of cells. All these factors contribute to cardiac hypertrophy and collagen accumulation in the cardiac remodelling process.

CYP2J2 is a cytochrome P450 epoxygenase that is abundantly expressed in the human heart. Our previous studies demonstrated that CYP2J2 overexpression alleviates diabetic cardiomyopathy and TNF-α-induced cardiac injury. Cardiomyocyte-specific expression of CYP2J2 has been shown to attenuate ischaemia-reperfusion injury in isolated perfused mouse hearts and significantly protect against...
doxorubicin-induced cardiac toxicity. CYP2J2 metabolizes arachidonic acid to the four regioisomeric epoxyeicosatrienoic acids (5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET). Previous studies have demonstrated that EETs play important biological roles in cardiovascular homeostasis.

As discussed above, CYP2J2/EETs are protective in cardiovascular system. However, the role of CYP2J2/EETs on cardiac remodelling has not been elucidated. Cardiomyocytes and their paracrine action exert important roles in cardiac remodelling. Therefore, we focused on the role of CYP2J2 and EETs in cardiomyocytes and sought to investigate the mechanisms involved in Ang-II-induced cardiac remodelling. EETs are ligands of peroxisome proliferator-activated receptor (PPAR) and exert antioxidant effects by regulating oxidative stress-related proteins. Oxidative stress and downstream transcription factors such as NF-kB play an essential role in Ang-II-induced cardiac remodelling. Therefore, we hypothesize that CYP2J2 and its EET products may protect against cardiac remodelling by attenuating oxidative stress-mediating NF-kB p65 nuclear translocation through PPAR-γ activation in cardiomyocytes. To test these hypotheses, in vivo experiments were performed with mice exhibiting cardiomyocyte-specific expression of CYP2J2 treated with Ang-II as a means to induce cardiac dysfunction and remodelling. In vitro experiments with cultured cardiomyocytes and cardiac fibroblasts were used to explore the mechanisms of EET actions in these cells.

2. Methods

More details are available in the Supplementary material online.

2.1 Animal experiments

Mice with cardiomyocyte-specific expression of CYP2J2 driven by the α-myosin heavy chain promoter (α-MHC) were generated in Dr Zeldin’s laboratory (NIEHS), and transgenic mice were genotyped as described previously. Eight-week-old male CYP2J2 transgenic mice and age-sex-matched WT controls on a pure C57BL/6 background were used for the experiments. All experimental procedures were approved by the Experimental Animal Research Committee of Tongji Medical College, Huazhong University of Science and Technology, and were in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH.

2.1.1 Animal experiment 1

Animals were divided into experimental groups (n = 10 per group) as follows: CYP2J2 transgenic mice and WT control mice with/without infusion of Ang-II (Sigma-Aldrich, St Louis, MO, USA) at a rate of 1.5 μg/kg/min for 2 weeks by an osmotic mini-pump (Alzet model 1002, Alza) as previously described and with/without PPAR-γ inhibitor (GW9662, Sigma-Aldrich) treatment (0.35 mg/kg per day in drinking water) for 2 weeks as previously described. Control groups of CYP2J2 transgenic mice and WT control mice were treated with saline.

2.1.2 Animal experiment 2

Animals were divided into experimental groups (n = 10 per group) as follows: CYP2J2 transgenic mice and WT control mice with/without infusion of Ang-II at a rate of 1.5 μg/kg/min for 2 weeks by an osmotic mini-pump as previously described. Additionally, WT control mice with infusion of Ang-II were treated with/without various concentrations of hydralazine in drinking water (250, 200, 150, 100, and 50 mg/L).

Systolic blood pressures were measured before 4 days of Ang-II infusion and measured every 2 days during Ang-II infusion. We performed the measurement at room temperature by a photoelectric tail-cuff system (PowerLab; ADInstruments Pty Ltd, New South Wales, Australia) as described previously. Mice were anaesthetized with intraperitoneal injections of xylazine (5 mg/kg) and ketamine (80 mg/kg) mixture before implantation of the osmotic mini-pumps. Anaesthesia was monitored by detecting reflexes, heart rate, and respiratory rate. Two weeks later, mice were euthanized with carbon dioxide after haemodynamic measurements and echocardiography.

2.2 Cell culture

2.2.1 Isolation of neonatal rat cardiomyocyte and cardiac fibroblast

Neonatal rats were anaesthetized with 4–5% isoflurane inhalation anaesthesia. Adequate anaesthesia was assured by the absence of reflexes prior to rapid heart excision. Then cardiac fibroblasts and cardiomyocytes were isolated and cultured as described previously.

2.2.2 Treatments of neonatal cardiomyocytes

Neonatal rat cardiomyocytes (3 × 10^5 cells per well) were serum-starved overnight and then incubated with Ang-II (1 μmol/L) or H2O2 (20 μmol/L) in the presence or absence of 11,12-EET (1 μmol/L), 14,15-EEZE (1 μmol/L), PPAR-γ inhibitor GW9662 (1 μmol/L), PPAR-γ agonist pioglitazone (5 μmol/L), or ROS scavenger NAC (2.5 mM) for 24 or 48 h. Transfections with NF-kBp65siRNA (100 nM, Ribobio, Guangzhou, China) were performed with Lipofectamine 2000 reagent (Invitrogen, Life Technologies Corporation) for 48 h, according to the manufacturer’s instructions, and then cells were incubated with or without Ang-II for 24 or 48 h. The cells incubated with various reagents for 24 and 48 h were used for western blot analysis and cardiomyocytes morphology analysis, respectively.

2.2.3 Neonatal rat cardiomyocytes and cardiac fibroblasts conditioned co-culture

Step 1: Neonatal rat cardiomyocytes (3 × 10^5 cells per well) were incubated with various reagents for 6 h after overnight serum starvation. Then, the medium was discarded, and cells were incubated in fresh medium for another 18 h. Medium of every group was collected, respectively, to cultivate cardiac fibroblasts.

Step 2: Cardiac fibroblasts were plated in 6-well plates. At 75% confluence, cardiac fibroblasts in each well were incubated with corresponding cardiomyocytes conditioned medium, respectively, for 24 h after overnight serum starvation, and then the cells were used for further analysis (western blot analysis and α-SMA staining).

2.3 Haemodynamic measurements and echocardiography

Echocardiographic examination was performed under light (1–2%) isoflurane anaesthesia using a high-resolution imaging system with a 30-MHz high frequency scanhead (VisualSonicsVevo770, VisualSonics Inc., Toronto, Canada) as previously described. Left ventricle haemodynamic measurements were performed under intraperitoneal injection of 0.9% NaCl and 10 mg/kg xylazine using a Millar Catheter System via the left carotid artery as described previously.

2.4 Histological analysis

Heart sections were stained with FITC-conjugated wheat germ agglutinin (Sigma), haematoxylin/eosin, and picrosirius red as previously described.

2.5 DHE staining

Fresh-frozen heart sections were incubated with dihydroethidium (DHE, S0063, Beyotime, Shanghai, China) as previously described.

2.6 Preparation of nuclear and cytosolic extracts

Nuclear and cytosolic extract of LV tissue was isolated as described previously.
2.7 Western blotting analysis
Western blots were performed using various antibodies. Bands were quantified by densitometry using Quantity One software (Bio-Rad, Hercules, CA, USA).

2.8 Detection of intracellular ROS
2′,7′-Dichlorofluoresceindiacetate (DCFH-DA, Sigma) was employed as an ROS-capturing reagent using methods described previously.27 DCF fluorescence was detected by flow cytometry immediately after staining.

2.9 Hydrogen peroxide measurements
Hydrogen peroxide levels were determined using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, A22188) as described.30,31

2.10 Detection of TGF-β1 and TIMP1 secretion
Secretion of TGF-β1 and TIMP1 were detected by an ELISA Kit (Abcam, Cambridge, MA, USA) following the manufacturer’s instructions.

2.11 Measurement of super oxide dismutase 1 and catalase activity
Super oxide dismutase 1 (SOD1) activity and catalase activity were detected using Cu/Zn-SOD Assay Kits (Beyotime) and Catalase Assay Kit (Beyotime) following the manufacturer’s instructions.

2.12 Immunofluorescence
Primary cells were identified by IF staining. Neonatal cardiomyocytes morphology was evaluated by Actin-Tracker Green staining (Beyotime) following the manufacturer’s instructions.

2.13 Detection of EET in plasma and cardiac tissues
EET levels in plasma and cardiac tissues were analysed by both LC/MS/MS and ELISA kit (Detroit R&D Inc., Detroit, MI, USA) as previously described.30,31

2.14 Statistics analysis
All data were expressed as means ± SEM. The in vivo data were analysed using two-way ANOVA. The in vitro data were analysed by one-sample t-test and one-way ANOVA with post hoc analyses performed using the Fisher’s least significant difference test. Statistical significance was defined as P < 0.05.

3. Results
3.1 Expression of CYP2J2 in cardiomyocytes attenuates Ang-II-induced cardiac dysfunction via PPAR-γ activation and lowered blood pressure
Echocardiography and haemodynamic assessments showed that Ang-II induced cardiac dysfunction in WT mice; however, αMHC-CYP2J2-Tr inhibited these changes (Table 1, see Supplementary material online, Table S1). Administration of the PPAR-γ inhibitor, GW9662, partially blocked the effects of CYP2J2 expression (Table 1).

Administration of hydralazine (150 mg/L) mimics the blood pressure-lowering effects seen with CYP2J2 (see Supplementary material online, Figure S2) and exerts weaker effects than CYP2J2 on Ang-II-induced cardiac dysfunction (see Supplementary material online, Table S1).

Table 1: Haemodynamic and echocardiography results

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<th>Control</th>
<th>Ang-II</th>
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<th>CYP2J2-Tr</th>
<th>Ang-II + GW9662</th>
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<td>LVEDP (mmHg)</td>
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<tr>
<td>LVdP/dt max (mmHg/s)</td>
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<td>47 ± 2</td>
<td>47 ± 2</td>
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<td>Ejection fraction (%)</td>
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<td>LVdD/Dp (mmHg)</td>
<td>1.63 ± 0.07</td>
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Ang-II indicates angiotensin II; dP/dt max, maximal slope of systolic pressure increment; dD/dt min, minimal slope of diastolic pressure decrement; LVEDP, left ventricular end-diastolic pressure; LVP, left ventricle; CYP2J2-Tr, transgenic mice.
3.2 Cardiomyocyte-specific expression of CYP2J2 prevents the development of Ang-II-induced cardiac remodelling

We compared cardiomyocyte morphology and heart weights in WT and α-MHC-CYP2J2-Tr mice under different treatment conditions. Heart size, heart weight, and heat weight/body weight ratios were significantly increased in WT mice following Ang-II infusion; however, in the αMHC-CYP2J2 transgenic mice treated with Ang-II, these parameters were normal (Figure 1A–C). Similar findings were observed when paraffin-embedded heart sections were stained with haematoxylin/eosin and Wheat germ agglutinin to evaluate cardiac hypertrophy (Figure 1D–G). Picrosirius red staining demonstrated that Ang-II treatment of WT mice resulted in markedly enhanced collagen accumulation corresponding with increased fibrosis; however, these effects were not observed in αMHC-CYP2J2-Tr mice (Figure 1H and I).

DHE staining for ROS was performed, because changes in ROS are associated with cardiac remodelling. Ang-II treatment of WT mice led to significantly enhanced DHE fluorescent intensity, indicating increased ROS production and oxidative stress. CYP2J2 overexpression completely eliminated these effects (Figure 1J and K). Moreover, the PPAR-γ antagonist GW9662 partially blocked the cardioprotective effects observed in αMHC-CYP2J2-Tr mice. Control studies demonstrated that GW9662 did not induce myocardial remodelling and oxidative stress in the absence of Ang-II treatment (Figure 1). Meanwhile, administration of hydralazine (150 mg/L), which mimics the blood pressure-lowering effects of CYP2J2 (see Supplementary material online, Figure S2), exerts weaker effects than CYP2J2 on Ang-II-induced cardiomyocyte remodelling and oxidative stress (see Supplementary material online, Figure S3).

3.3 αMHC-CYP2J2-Tr mice have reduced expression of cardiac remodelling proteins and NF-κBp65 nuclear translocation, and enhanced activation of PPAR-γ and antioxidant effects

Western blots confirmed that CYP2J2 was expressed in CYP2J2 transgenic mice but not in WT mice (Figure 2A). EETs were significantly increased in plasma and cardiac tissue of αMHC-CYP2J2-Tr mice (see Supplementary material online, Figure S7). To determine whether Ang-II infusion induced up-regulation of cardiac remodelling proteins and activation of the TGF-β1/p-smad3 pathway, western blots were performed to examine TIMP1, α-SMA, TGF-β1, p-smad3, and collagen type I expression. Ang-II induced expression of these proteins in WT mice; however, these changes were not observed in αMHC-CYP2J2-Tr mice (Figure 2A–C). Ang-II infusion stimulated production of H2O2 in the heart (Figure 2D), up-regulation of the NADPH oxidases NOX2 and NOX4 (Figure 2E and F), and inhibited expression and activity of the antioxidant enzymes SOD1 and catalase (Figure 2E, G, and H). These changes were not observed in the αMHC-CYP2J2-Tr mice (Figure 2D–H). In addition, PPAR-γ nuclear translocation was markedly reduced in Ang-II-infused WT mice and p65 nuclear translocation was significantly increased; however, CYP2J2 expression inhibited these effects (Figure 2I and J). Interestingly, the protective effects observed in αMHC-CYP2J2-Tr mice were partially blocked by the PPAR-γ antagonist, GW9662 (Figure 2).

3.4 CYP2J2 overexpression attenuates Ang-II-induced responses in vitro

To further define the role of CYP2J2 overexpression on the local cardiac environment, we compared the effects of neonatal cardiomyocytes from αMHC-CYP2J2-Tr and wild-type mice stimulated with Ang-II. Ang-II induced expression of cardiac remodelling proteins collagen type I, TIMP1, and TGF-β1 in cardiomyocytes from WT mice. These effects were attenuated in cardiomyocytes from αMHC-CYP2J2-Tr mice. 14,15-EEZE blocked the protective effects of CYP2J2 (Figure 3K and L).

3.5 11,12-EET attenuates Ang-II-induced responses in vitro

We further investigated the impact of CYP2J2 metabolites EET on Ang-II-induced remodelling in the neonatal rat cardiomyocytes and H9c2 cell line. Consistent with our in vivo findings, Ang-II stimulated cardiomyocyte hypertrophy and expression of cardiac remodelling proteins collagen type I (COL1), TIMP1, and TGF-β1. Importantly, the addition of 11,12-EET inhibited these effects (Figure 3A–D). Similar results were also observed with intracellular ROS levels and intracellular and extracellular levels of H2O2 (Figure 3F). Ang-II treatment resulted in up-regulation of NADPH oxidases NOX2 and NOX4, while expression and activity of antioxidant enzymes SOD1 and catalase were inhibited. Treatment with 11,12-EET abolished these effects (Figure 3E,G, and H). PPAR-γ nuclear translocation was reduced and p65 nuclear translocation was increased in Ang-II treatment; however, these effects were also inhibited by treatment with 11,12-EET (Figure 3I and J). The protective effects observed with 11,12-EET were blocked by the putative EET receptor antagonist, 14,15-EEZE (Figure 3). In addition, similar effects were seen in the H9c2 cell line (see Supplementary material online, Figure S9).

3.6 Inhibition of Ang-II-induced responses by 11,12-EET is partially mediated by PPAR-γ activation

To determine whether PPAR-γ impacts the activity of the CYP2J2 metabolite 11,12-EET in vitro, we treated neonatal cardiomyocytes with the PPAR-γ activator pioglitazone and PPAR-γ inhibitor GW9662 in conjunction with 11,12-EET. Treatment with the PPAR-γ activator pioglitazone exerted similar effects as EET treatment (Figure 4E,G, and H). The PPAR-γ inhibitor, GW9662, partially blocked the protective effects of EETs (Figure 4). In addition, similar effects were observed in H9c2 cells (see Supplementary material online, Figure S10).

3.7 11,12-EET regulates NF-κB pathway and cardiac remodelling protein expression

We hypothesized that EETs regulate cardiac remodelling through oxidative stress-mediated activation of the NF-κB p65 pathway. We used H2O2, a ROS inducer, and NAC, a ROS scavenger, to investigate the role of oxidative stress in regulating cardiac remodelling and in altering the protective effects of 11,12-EET. Similar to Ang-II treatment, H2O2 enhanced cardiomyocytes hypertrophy and expression of cardiac remodelling proteins, while 11,12-EET treatment attenuated these effects (Figure 5A–D). NAC and EET exert similar and synergistic effects on cardiomyocytes hypertrophy, expression of cardiac remodelling proteins, and p65 nuclear translocation induced by Ang-II (Figure 5E–H, J, and K), but the synergistic effects were not observed in the level of ROS and H2O2 (Figure 5I). In addition, the effects above
Figure 1  Expression of CYP2J2 in cardiomyocytes prevents the development of Ang-II-induced cardiac remodelling. (A–C) Heart size (A), heart weight (B), heart weight: body weight ratio (C) of WT and α-MHC-CYP2J2-Tr mice under various treatment conditions. (D and F) Histological analysis of cardiomyocytes by H&E and Wheat germ agglutinin staining. (E and G) Quantitation of cardiomyocyte size. (H) Heart sections stained with picrosirius red identifying collagen deposition. (I) Quantitation of cardiac fibrosis. (J) DHE staining to evaluate ROS level in heart tissue. (K) Quantification of ROS in cardiac tissue. (n = 10 for each group; *P < 0.05 vs. WT control; **P < 0.05 vs. WT + Ang-II; ***P < 0.05 vs. CYP2J2 + Ang-II).
Figure 2 Cardiomyocyte-specific expression of CYP2J2 inhibited Ang-II-induced changes in cardiac remodelling proteins, oxidative stress-related proteins, NF-κB p65, and PPAR-γ. (A) Representative immunoblots for cardiac remodelling proteins (TGF-β1, p-smad3, smad3, collagen type I, α-SMA, and TIMP) and CYP2J2. (B and C) Quantitation of cardiac remodelling proteins. (D) Detection of H2O2 in heart tissue. (E) Representative immunoblots for oxidative stress-related proteins (NOX2, NOX4, SOD1, catalase). (F and G) Quantitation of NOX2, NOX4, SOD1, and catalase. (H) Detection of SOD1 and catalase activity. (I) Representative immunoblots for NF-κB p65 and PPAR-γ nuclear translocation. (J) Quantitation of NF-κB p65 and PPAR-γ nuclear translocation. (n = 10 for each group; *P < 0.05 vs. WT control; #P < 0.05 vs. WT + Ang-II; &P < 0.05 vs. CYP2J2-Ang-II).
were observed in H9c2 cells (see Supplementary material online, Figure S11A–G).

To assess the role of EETs on the early stage of Ang-II stimulation, we reproduced the rapid Ang-II-induced nuclear translocation of p65. EETs inhibited the p65 nuclear translocation as well as cytosolic IkBα degradation. GW9662 blocked these effects (see Supplementary material online, Figure S4), suggesting that EET attenuates the Ang-II-induced rapid p65 nuclear translocation by inhibiting IkBα degradation via PPAR-γ activation.

We also performed siRNA-mediated knockdown of NF-κB p65 in neonatal cardiomyocytes to determine whether NF-κB p65 participates in Ang-II-mediated cardiac remodelling. Results showed that NF-κB p65-specific siRNA inhibited cardiomyocyte hypertrophy and up-regulation of cardiac remodelling proteins induced by Ang-II (Figure 5L–O). In addition, similar effects were observed in H9c2 cells (see Supplementary material online, Figure S11H and I).

3.8 Cardiomyocyte-specific CYP2J2 overexpression alleviates Ang-II-induced profibrotic effects by acting directly on cardiomyocytes and indirectly on cardiac fibroblasts

Cardiac fibroblasts are an essential component in the development of cardiac fibrosis. Therefore, we hypothesized that CYP2J2 may exhibit indirect effects on cardiac fibroblasts through cardiomyocytes paracrine factors. Conditioned co-culture experiments with neonatal mouse cardiomyocytes and cardiac fibroblasts were performed. Results revealed that Ang-II treatment elevated levels of TIMP1, TGF-β1, and H2O2 in neonatal CYP2J2-Tr mouse cardiomyocytes media, while the effects were attenuated in neonatal αMHC-CYP2J2-Tr mouse cardiomyocytes media. The EET antagonist, 14,15-EEZE, blocked the protective effects of αMHC-CYP2J2-Tr. (Figure 6F). These data indicate that αMHC-CYP2J2-Tr reduced cardiomyocyte secretion of profibrotic factors TIMP1, TGF-β1, and H2O2 via EET production.

Mouse neonatal cardiac fibroblasts were then cultured with neonatal mouse cardiomyocyte conditioned medium to explore the indirect effects of cardiac expression of CYP2J2 on fibroblasts. There was enhanced expression of TIMP1, TGF-β1, collagen I, and α-SMA in cardiac fibroblasts after culture with medium from Ang-II-treated wild-type cardiomyocytes (Figure 6b and j). Importantly, these effects were reversed if the cardiac fibroblast were cultured with medium collected from αMHC-CYP2J2-Tr cardiomyocytes that had been incubated with Ang-II. The effects of CYP2J2 were inhibited with conditioned cardiomyocyte medium incubated with Ang-II and 14,15-EEZE. Similar effects were observed in cardiac fibroblast activation by detecting α-SMA fluorescence (Figure 6G and H).

3.9 11,12-EET alleviates Ang-II-induced profibrotic effects by acting directly on cardiomyocytes and indirectly on cardiac fibroblasts

We performed further in vitro experiments to study the indirect effects of exogenous EETs elevation in cardiomyocytes on cardiac fibroblasts. Results showed that Ang-II treatment elevated levels of TIMP1, TGF-β1, and H2O2 in neonatal cardiomyocytes media and 11,12-EET inhibited the effects. The protective effects of 11,12-EET were blocked with GW9662 (Figure 6A). These data indicate that 11,12-EET inhibited the production of profibrotic factors TIMP1, TGF-β1, and H2O2 in cardiomyocytes via a PPAR-γ-dependent mechanism.

Cardiac fibroblasts were then cultured with neonatal cardiomyocyte conditioned medium. Ang-II-treated cardiomyocytes conditioned medium enhanced phosphorylation of smad3 and up-regulated expression of TIMP1, TGF-β1, collagen I, and α-SMA in cardiac fibroblasts (Figure 6D and E). Importantly, these effects were reversed by conditioned medium collected from cells incubated with Ang-II and 11,12-EET (Figure 6D and E). The effects of 11,12-EET were blocked by conditioned medium incubated with Ang-II, 11,12-EET, and GW9662 (Figure 6D and E). Similar effects were observed in cardiac fibroblast activation by detecting α-SMA fluorescence (Figure 6B and C). Taken together, these data suggest that Ang-II induced production of profibrotic factors TIMP1, TGF-β1, and H2O2 in cardiomyocytes and 11,12-EET decreased the secretion of these factors resulting in indirect inhibitory effects on activation of cardiac fibroblasts. In addition, similar effects were observed in H9c2 cells and cardiac fibroblast conditioned co-culture (see Supplementary material online, Figure S12).

4. Discussion

4.1 Main findings

We used mice with cardiomyocyte-specific expression of CYP2J2, with elevated EETs in cardiomyocytes, to investigate the mechanisms underlying the effects of CYP2J2 on Ang-II-induced cardiac remodelling. We found that cardiomyocyte-specific expression of CYP2J2 inhibited cardiac dysfunction, cardiac fibrosis, cardiac hypertrophy, and elevation of oxidative stress in response to Ang-II. The αMHC-CYP2J2-Tr mice also exhibited a reduction in Ang-II mediated expression of cardiac remodelling proteins (TIMP1, TGF-β1, p-smad3, α-SMA, and collagen I). Furthermore, we elucidated the underlying mechanisms: (i) Our data provide new evidence that cardiomyocyte-specific expression of CYP2J2 attenuates oxidative stress-mediated NF-κB p65 nuclear translocation via PPAR-γ activation in cardiomyocytes after Ang-II treatment. (ii) EETs treatment and cardiac-specific expression of CYP2J2 decreased secretion of profibrotic factors from cardiomyocytes, which results in inhibitory effects on activation of cardiac fibroblasts. (iii) The slight blood pressure-lowering effects and PPAR-γ/IkBα/p65 signaling, to some extent, contribute to the protective effects of CYP2J2/EETs. These main findings were summarized in scheme (Figure 7).

4.2 The role of PPAR-γ in the protective effects of CYP2J2 or EET

PPAR-γ has been shown to act in two different mechanisms. EETs are known ligands of PPAR-γ that exert antioxidant effects by regulating oxidative stress-related proteins.17 A genomic effect was observed in this study when PPAR-γ translocates to the nucleus after activation from EETs resulting in inhibition of Ang-II-induced oxidative stress/ NF-κB pathway. This role of PPAR-γ is supported by various other studies. Some in vivo studies have shown that PPAR-γ ligands attenuate myocardial remodelling by reducing oxidative stress.32 Previous studies also demonstrated that PPAR-γ ligands regulated oxidative stress-related protein activity and expression.33 Secondly, our study indicated that a non-genomic effect was seen when PPAR-γ is activated by EETs, resulting in attenuation of Ang-II-induced IkBα degradation and the subsequent rapid NF-κB activation. EETs may inhibit IKK activity and subsequent IkBα degradation via PPAR-γ activation, because treatment with a PPAR-γ agonist inhibited IKK activity and subsequent IkB
Treatment of neonatal rat cardiomyocytes with 11,12-EET inhibits Ang-II-induced changes in cardiomyocyte size, expression of cardiac remodelling proteins, oxidative stress-related proteins, ROS production, and NF-κBp65/PPAR-γ nuclear translocation. (A and B) Measurement of surface area of cardiomyocytes with various treatments. (C and D) Representative immunoblots and quantitation of cardiac remodelling proteins (TIMP1, TGF-β1, collagen type I). (E) Quantitation of SOD1 and catalase activity. (F) Quantitation of intracellular ROS levels and intracellular and extracellular H2O2 concentration. (G and H) Representative immunoblots and quantitation of oxidative stress-related proteins (NOX2, NOX4, SOD1, and catalase). (I and J) Representative immunoblots and quantitation of NF-κB p65 and PPAR-γ nuclear translocation. (K and L) Representative immunoblots and quantitation of cardiac remodelling proteins of CYP2J2-Tr and WT mouse neonatal cardiomyocytes treated with various reagents. (n = 5 for each experiment; *P < 0.05 vs. control; #P < 0.05 vs. Ang-II; $P < 0.05 vs. Ang-II + 11,12-EET).
Figure 4 PPAR-γ antagonist GW9662 partially blocks the protective effects of 11,12-EET in neonatal rat cardiomyocytes. (A and B) Measurement of surface area of neonatal cardiomyocytes with various treatments. (C–F) Representative immunoblots and quantitation of cardiac remodelling proteins (TIMP1, TGF-β1, collagen type I) of cardiomyocytes with various treatments. (G) Quantitation of SOD1 and catalase activity. (H) Quantitation of intracellular ROS levels and intracellular and extracellular H2O2 concentration. (I and J) Representative immunoblots and quantitation of oxidative stress-related proteins (NOX2, NOX4, SOD1, catalase). (K and L) Representative immunoblots and quantitation of NF-κBp65 and PPAR-γ nuclear translocation. (n = 5 for each experiment; *P < 0.05 vs. control; #P < 0.05 vs. Ang-II; $P < 0.05 vs. Ang-II + 11,12-EET; ¥P < 0.05 vs. Ang-II + pioglitazone).
Figure 5  11,12 EET regulates hypertrophy and cardiac remodelling proteins expression by inhibiting the oxidative stress-mediated NF-κB pathway in neonatal rat cardiomyocytes. (A and B) Measurement of surface area of cardiomyocytes with EET and H$_2$O$_2$ treatment. (C and D) Representative immunoblots and quantitation of cardiac remodelling proteins (TIMP1, TGF-β1, collagen type I) after EET and H$_2$O$_2$ treatment. (n = 5 for each experiment; *P < 0.05 vs. control, **P < 0.05 vs. H$_2$O$_2$, ***P < 0.05 vs. H$_2$O$_2$ + EET) (E and F) Representative immunoblots and quantitation of cardiac remodelling proteins (TIMP1, TGF-β1, collagen type I) after EET and NAC treatment. (G and H) Measurement of surface area of cardiomyocytes with EET and H$_2$O$_2$ treatment. (I) Quantitation of intracellular ROS level and intracellular and extracellular H$_2$O$_2$ concentration. (J and K) Representative immunoblots and quantitation of NF-κBp65 nuclear translocation after 11,12 EET and NAC treatment. (L and M) Representative immunoblots and quantitation of cardiac remodelling protein expression in cardiomyocytes with NF-κB p65 siRNA and Ang-II treatment. (N and O) Measurement of surface area of cardiomyocytes with various treatments. (n = 5 for each experiment; *P < 0.05 vs. control, **P < 0.05 vs. Ang-II, ***P < 0.05 vs. Ang-II + NAC).
Figure 6 CYP2J2 overexpression or 11,12-EET reduces cardiomyocytes profibrotic factor secretion induced by Ang-II and subsequently attenuates cardiac fibroblast activation. (A) Relative fold changes of TGF-β1, TIMP1, and H₂O₂ in neonatal rat cardiomyocytes conditioned medium. (B and C) Representative immunofluorescent images and quantitation of α-SMA positive cells in cultures of neonatal rat cardiac fibroblasts treated with neonatal rat cardiomyocytes conditioned medium. (D and E) Expression of cardiac remodelling proteins (p-smad3, α-SMA, TIMP1, TGF-β1, and collagen I) in neonatal rat cardiac fibroblasts treated with neonatal rat cardiomyocytes conditioned medium. (F and G) Relative fold changes of TGF-β1, TIMP1, and H₂O₂ in neonatal mouse cardiomyocytes conditioned medium. (H and I) Representative immunofluorescent images and quantitation of α-SMA positive cells in cultures of neonatal mouse cardiac fibroblasts treated with neonatal mouse cardiomyocytes conditioned medium. (J and K) Expression of cardiac remodelling proteins in neonatal mouse cardiac fibroblasts treated with neonatal mouse cardiomyocytes conditioned medium. (n = 5 for each experiment; *P < 0.05 vs. control; †P < 0.05 vs. Ang-II; ‡P < 0.05 vs. Ang-II + 11,12-EET).
There are several other potential mechanisms through which EETs may exert a protective effect through PPAR-γ. Recent research demonstrated that IkBα is a PPAR cardiac target gene; therefore, we cannot exclude that EETs may inhibit NF-κB activation by up-regulating expression of IkBα via PPAR-γ activation. Additionally, PPAR-γ may physically interact with NF-κB, resulting in inhibition of NF-κB activation.

Further studies will need to be completed to determine the exact mechanisms involved in this process.

4.3 The antioxidant effects of CYP2J2 or EET

Oxidative stress plays an important role in cardiac remodelling. NADPH oxidase-derived ROS are induced by various factors and are scavenged by antioxidant enzymes. Several studies have demonstrated that NOX2 and NOX4 are the predominant NADPH oxidase isoforms in cardiomyocytes that are essential for Ang-II-induced cardiac remodelling. In the present study, we found that inhibition of oxidative stress is involved in the protective effects of CYP2J2 and EETs on Ang-II-induced cardiac remodelling. Moreover, EET attenuates H2O2-induced response and NAC exerts similar and synergistic effects of EET on cardiomyocyte remodelling. Furthermore, we elucidated that CYP2J2 expression or treatment with EETs attenuates oxidative stress-induced NF-κB p65 nuclear translocation by regulating oxidative stress-related protein expression via PPAR-γ activation. This leads to a reduction in cardiomyocyte hypertrophy, oxidative stress, and expression of collagen I, TIMP1, and TGF-β1. As a result, the release of cardiomyocytes paracrine factors (H2O2, TIMP1, and TGF-β1) is reduced. Subsequently, activation of cardiac fibroblasts and cardiac fibroblast TGF-β1-smad signalling are attenuated leading to a protective effect against Ang-II-induced cardiac remodelling. In addition, CYP2J2 causes a slight reduction in blood pressure and also regulates PPAR-γ/IkBα/p65 signalling that contributes to its protective effects.
proteins induced by Ang-II. This provides evidence that NF-κB is important in the pathogenesis of cardiac hypertrophy and fibrosis; however, the precise mechanism involving Ang-II-induced NF-κB activation is not completely understood. Various studies have demonstrated that Ang-II induces NF-κB activation via canonical (IkB-dependent) mechanisms; however, some studies have demonstrated that non-canonical (IkB-independent) mechanisms play a major role in Ang-II-induced NF-κB activation. Though, the mechanism is complicated and sometimes disreputable, ROS serve as common intracellular agents and exert a pivotal role in Ang-II-induced NF-κB activation. We demonstrated that EETs attenuate Ang-II-induced oxidative stress and subsequently inhibit the sustained p65 nuclear translocation. These results provide a definitive relationship between EETs, oxidative stress, and NF-κB activation. However, it is still not completely understood how the reduction in oxidative stress due to CYP2J2/EETs attenuates NF-κB activation. We speculate that IKK may act downstream of ROS and mediate the protective effects of EETs, because activation of IKK by Ang-II is dependent on ROS, and EETs are reported to reduce IKK activity. Other possible mechanisms may include changes in oxidative stress leading to attenuated MEK1/ERK/RSK signaling and thereby reduced non-canonical NF-κB activation. Previously, Node et al. demonstrated that EETs attenuate rapid p65 nuclear translocation by reducing IkBα degradation. Similar effects of EETs were observed in the Ang-II-induced rapid p65 nuclear translocation (see Supplementary material online, Figure S4); however, the precise mechanism needs to be further elucidated.

In our present study, we provide a long-term genomic mechanism involving inhibition of the sustained p65 nuclear translocation by EET. This mechanism is new and different from the established non-genomic mechanism. Both mechanisms are important in the protective effects of EETs, but the genomic mechanism may expropriate protective effects during the late stage of Ang-II stimulation, while the non-genomic mechanism may exert protective effects during the early, rapid stage of Ang-II stimulation.

4.5 Crosstalk between cardiomyocytes and cardiac fibroblasts

Protective effects of CYP2J2 in cardiomyocytes can be attributed to both direct effects on cardiomyocytes and indirect effects on cardiac fibroblasts. In response to Ang-II, cardiomyocytes and fibroblasts increase TGF-β1 production and convert inactive TGF-β1 to the active form. Ang-II-mediated collagen synthesis is a result of crosstalk between cardiomyocytes and cardiac fibroblast via paracrine actions of TGF-β1 in cardiomyocytes. ROS, an important paracrine factor from cardiomyocytes, triggers phenotypic changes in cardiac fibroblasts resulting in increased TGF-β1 expression followed by collagen expression. Another factor produced by cardiomyocytes known as metalloproteinase inhibitor I (TIMP1) inhibits collagen degradation and has been proposed to promote fibrosis by inducing phenotypic differentiation of fibroblasts into myofibroblasts and subsequent collagen synthesis. Our in vitro experiments demonstrated that cardiomyocytes become hypertrophic and secrete more profibrotic paracrine factors (TIMP1, TGF-β1, H2O2) in response to Ang-II treatment. These profibrotic factors activate cardiac fibroblasts leading to the increased TGF-β1-smad signalling and subsequent remodelling protein up-regulation in cardiac fibroblasts. CYP2J2-specific expression or exogenous EETs treatment in cardiomyocytes reduces cardiomyocyte paracrine factors (TIMP1, TGF-β1, H2O2) resulting in reduced TGF-β1-smad signalling and cardiac remodelling protein expression.

4.6 Summary of the cardiac protective effects and mechanism of CYP2J2/EET

Overall, our data indicate that cardiomyocyte-specific expression of CYP2J2 or EETs attenuates Ang-II-induced cardiac remodelling and dysfuction by reducing oxidative stress leading to diminished NF-κB signalling via PPAR-γ activation. Indirect effects are observed in cardiac fibroblasts including reduced activation of cardiac fibroblast and suppression of the TGF-β1/smad signalling pathway. In addition, a slight reduction in blood pressure and inhibition of the rapid NF-κB activation partially contribute to the protective effects of CYP2J2 and EETs. These results yield significant insights into the beneficial role of CYP2J2 and EETs in cardiomyocytes during cardiac remodelling.

4.7 Limitation

EETs released from cardiomyocytes overexpressing CYP2J2 may also directly affect cardiac fibroblasts; however, this potential action needs to be further explored in future studies.

Supplementary material

Supplementary Material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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References

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