

# Melatonin attenuates angiotensin II-induced cardiomyocyte hypertrophy through the CyPA/CD147 signaling pathway

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Received: 18 May 2016 / Accepted: 29 August 2016 / Published online: 2 September 2016  
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**Abstract** Melatonin is well known for its cardioprotective effects; however, whether melatonin exerts therapeutic effects on cardiomyocyte hypertrophy remains to be investigated, as do the mechanisms underlying these effects, if they exist. Cyclophilin A (CyPA) and its corresponding receptor, CD147, which exists in a variety of cells, play crucial roles in modulating reactive oxygen species (ROS) production. In this study, we explored the role of the CyPA/CD147 signaling pathway in angiotensin II (Ang II)-induced cardiomyocyte hypertrophy and the protective effects exerted by melatonin against Ang II-induced injury in cultured H9C2 cells. Cyclosporine A, a specific CyPA/CD147 signaling pathway inhibitor, was used to manipulate CyPA/CD147 activity. H9C2 cells were then subjected to Ang II or CyPA treatment in either the absence or presence of melatonin. Our results indicate that Ang II induces cardiomyocyte hypertrophy through the CyPA/CD147 signaling pathway and promotes ROS production, which can be blocked by melatonin pretreatment in a concentration-dependent manner, in cultured H9C2 cells and that CyPA/CD147 signaling pathway inhibition protects against Ang II-induced cardiomyocyte hypertrophy. The protective effects of melatonin against Ang II-

induced cardiomyocyte hypertrophy depend at least partially on CyPA/CD147 inhibition.

**Keywords** Melatonin · Hypertrophy · Reactive oxygen species · CyPA · CD147 · CsA

## Introduction

Cardiac hypertrophy is an adaptive response to high pressure, volume overload, and neurohormonal activation, and is associated with many heart diseases. Ventricular hypertrophy is closely related to an increased risk of heart failure. Therefore, alleviating hypertrophy is an important step for the prevention of heart failure [1]. It is generally accepted that angiotensin II (Ang II), a crucial molecular component of the renin–Ang–aldosterone system, plays a significant role in cardiovascular homeostasis [2–4]. Additionally, multiple studies have demonstrated that Ang II-induced cardiac hypertrophy depends primarily on reactive oxygen species (ROS) and inflammatory factor overproduction [5, 6]. Excessive ROS levels can cause cardiomyocyte apoptosis, extracellular matrix remodeling, cardiac hypertrophy, and, ultimately, impaired cardiac contractile function [7].

Cyclophilin A (CyPA) is a pro-inflammatory cytokine secreted by several cell types, including monocytes/macrophages, vascular smooth muscle cells, activated platelets, and endothelial cells [8, 9]. Under pathological conditions, these cells secrete CyPA into the extracellular space [10, 11]. CD147, an extracellular matrix metalloproteinase inducer, is thought to be the main cell surface receptor mediating CyPA signal transduction [12, 13]. Cyclosporine A (CsA), an immunosuppressant, inhibits CyPA peptidyl-prolyl isomerase (PPIase) activity

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[14]. It was recently reported that CyPA plays a crucial role in cardiac hypertrophy pathophysiology and may be a key determinant of ROS production in cardiomyocytes [15].

Melatonin is an important chronobiological regulatory molecule that is produced primarily in the pineal gland at night [16]. Studies have revealed that melatonin helps prevent many diseases, including inflammatory diseases, ischemia/reperfusion injury, hypertension, and various cardiovascular diseases [17–23]. Moreover, recent studies indicate that the role of melatonin in cardiovascular disease is dependent on its activity as a powerful antioxidant [24–26]. However, the effects of melatonin on cardiomyocyte hypertrophy and the underlying mechanism of its actions remain to be explored. In this study, we explored the role and mechanism of action of melatonin in Ang II-induced cardiomyocyte hypertrophy.

## Methods

### Reagents

Melatonin, CyPA, CsA, and Ang II were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-CyPA polyclonal antibody and rabbit anti-CD147 monoclonal antibody were purchased from Abcam (Cambridge, MA, USA). Mouse anti-GAPDH monoclonal antibody and mouse anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) monoclonal antibody were purchased from Cell Signaling Technology (Danvers, MA, USA), and rabbit anti-NOx2 polyclonal antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

### Cell culture and treatments

H9C2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing 4.5 g/L D-glucose, 10 % fetal bovine serum (HyClone, Thermo Fisher Scientific, Waltham, MA, USA), penicillin (100 units/mL), and streptomycin (100  $\mu$ g/mL) in a 95 % O<sub>2</sub>–5 % CO<sub>2</sub> humidified incubator at 37 °C until the desired cell density was reached.

Ang II (Sigma-Aldrich) was dissolved in sterile deionized water, and melatonin, CyPA, and CsA (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) for better dissolution. Both solutions were stored at –20 °C. The cells were treated according to three experimental designs, and 0.01 % DMSO was used alone as a sham control. First, the

cells were treated with Ang II (100 nmol/L, Sigma-Aldrich) for 24 h, with or without 60 min of melatonin pretreatment at different concentrations (125, 250, or 500  $\mu$ mol/L). The melatonin concentrations employed in our experiment were chosen based on the results of previous studies that demonstrated the cardioprotective effects of physiological and pharmacological doses of melatonin, as well as the role of endogenous melatonin secretion in cardioprotection [27–32]. Second, the CsA groups were pretreated with CsA (100 nmol/L, Sigma-Aldrich) for 60 min [33–35], and then the Ang II and CsA groups were treated with Ang II (100 nmol/L, Sigma-Aldrich) for 24 h. Third, H9C2 cells were pretreated with different melatonin doses for 60 min before being treated with CyPA (100 nmol/L, Sigma-Aldrich) for 24 h, as described above [14]. The cells were harvested for further analysis after these treatments.

### [<sup>3</sup>H] leucine incorporation

[<sup>3</sup>H] leucine incorporation was measured as described previously [36]. H9C2 cells were incubated with [<sup>3</sup>H] leucine (2  $\mu$ Ci/mL) for 24 h before being treated with ice-cold 10 % trichloroacetic acid for 1 h at 4 °C. The precipitates were washed twice with ice-cold water and dissolved in 1 mL of NaOH (100 mmol/L), before being counted with a scintillation counter.

### Immunofluorescence analysis and cell surface area measurements

Cultured H9C2 cells on coverslips were fixed in 4 % paraformaldehyde for 15 min before being permeabilized in 0.5 % Triton X-100 for 20 min. Then, these cells were blocked in 1 % bovine serum albumin (BSA) or 1 % horse serum for 30 min at room temperature before being incubated sequentially overnight at 4 °C with primary antibodies to  $\alpha$ -SMA (1:50, diluted in 1 % BSA), and CyPA and CD147 (1:200, diluted in 1 % BSA). After being washed three times with PBS, the cells were incubated with the corresponding secondary antibodies for 1 h. Finally, the samples were stained with 4',6-diamino-2-phenylindole (Sigma-Aldrich, USA) for 5 min, washed with PBS, and wet-mounted using a fluorescence quenching solution (Sigma-Aldrich, USA). All images were detected using a confocal laser scanning microscope (Zeiss LSM 710, Germany) and analyzed with Image Pro Plus 6.0 software (Media Cybernetics Company, USA). The sizes of the positively stained H9C2 cells were analyzed simultaneously using ImageJ software (National Institutes of Health, Bethesda, MD, USA), and the surface area of at least 50 randomly chosen cells was analyzed on each coverslip.

## Western blot analysis

The cells were harvested and washed with PBS, and protein was extracted using a cell lysis buffer (Beyotime Institute of Biotechnology, China). Equal amounts of protein (50  $\mu$ g) from each group were separated via 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore, Billerica, MA) using a wet transfer system (Bio-Rad, Hercules, CA). After being blocked with 5 % nonfat milk for 2 h, the membranes were incubated overnight at 4 °C with primary antibodies against GAPDH, CyPA, CD147, NOx2, and  $\alpha$ -SMA (1:1000 dilution), followed by three washes in TBST. The membranes were subsequently incubated with secondary antibodies labeled with horseradish peroxidase [37] and visualized via enhanced chemiluminescence (Millipore). Protein levels were detected using an ImageQuant LAS 4000 chemiluminescence reader (GE) and analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

## Intracellular ROS level evaluation

Intracellular ROS levels in H9C2 cells were detected with the fluorescent indicator 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich), as described previously [38]. Once the cardiomyocytes had grown to the desired cell density and had been exposed to the appropriate treatment, they were washed twice with PBS (pH 7.4) and then incubated with 10  $\mu$ M DCFH-DA for 30 min at 37 °C in the dark. The incubated cells were washed three times with PBS, and then, the DCFH fluorescence in each well was detected at an emission wavelength of 520 nm and an excitation wavelength of 488 nm using a confocal laser scanning microscope (Zeiss LSM 710, Germany).

## Real-time PCR analysis

Total RNA was isolated from cultured cardiomyocytes using Trizol (Invitrogen, Carlsbad, CA, USA). First-strand cDNAs were synthesized from 2  $\mu$ g of total RNAs in a 20-mL reaction mixture using a PrimeScript<sup>TM</sup> RT reagent kit (TaKaRa Biotechnology, Dalian, China). PCR product amplification was performed using an Eppendorf Mastercycler ep realplex detection system (Eppendorf, Hamburg, Germany) with FastStart Universal SYBR Green Master mix (Roche, Germany), according to the manufacturer's protocol. The final real-time PCR analysis results were presented as the ratio of the mRNA of interest to that of GAPDH, which was used as an internal control. The relative mRNA levels were calculated using the  $2^{-\Delta\Delta C_t}$  method.

The primers used for the PCR analysis were as follows: atrial natriuretic peptide (ANP), forward 5'-TAA GCC

CTT GTG GTG TGT CA-3' and reverse 5'-GCA AGA CCC CAC TAG ACC AC-3',  $\beta$ -myosin heavy chain ( $\beta$ -MHC), forward 5'-AAG GGC CTG AAT GAG GAG TA-3' and reverse 5'-AAA GGC TCC AGG TCT GAG G-3',  $\alpha$ -SMA ( $\alpha$ -SMA), forward 5'-ACT GGG ACG ACA TGG AAA AG-3' and reverse 5'-CAT CTC CAG AGT CCA GCA CA-3', and GAPDH, forward 5'-CAA GAT CAT TGC TCC TCC TG-3' and reverse 5'-TCA TCG TAC TCC TGC TTG CT-3'.

## Statistical analysis

All data are presented as the mean  $\pm$  SEM of at least three independent experiments. Differences between groups were analyzed with *t*-tests or ANOVAs using SPSS software version 18.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism (version 5.00 for Windows, GraphPad Software, Inc., La Jolla, CA, USA). *p* < 0.05 was considered statistically significant.

## Results

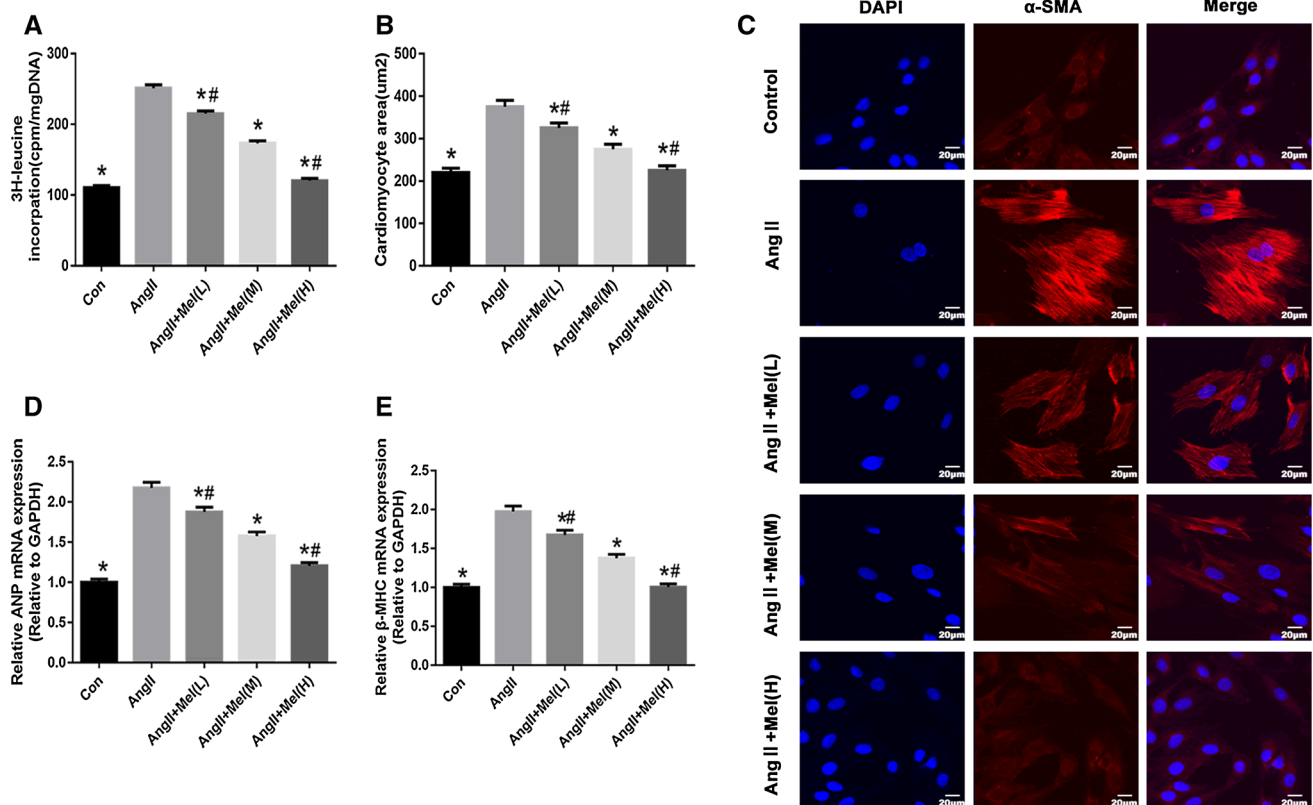
### Melatonin inhibits Ang II-induced H9C2 cell hypertrophy

To determine whether melatonin has an effect on Ang II-induced hypertrophy in H9C2 cells, we pretreated H9C2 cells with different concentrations of melatonin (125, 250, or 500  $\mu$ mol/L) for 60 min and then exposed them to 100 nmol/L of Ang II for 24 h. We found that pretreatment with melatonin attenuated Ang II-induced increases in [<sup>3</sup>H] leucine incorporation in a concentration-dependent manner, with the greatest effect at 500  $\mu$ mol/L (Fig. 1a). Moreover, melatonin significantly reduced the surface area of these cells in the presence of Ang II in a concentration-dependent manner (Fig. 1b, c) and downregulated the mRNA expression levels of ANP and  $\beta$ -MHC, known as fetal genes expressed in hypertrophic cardiac tissues (Fig. 1d, e).

Immunofluorescence analyses revealed that  $\alpha$ -SMA was strongly expressed in H9C2 cells under Ang II stimulation. However, melatonin pretreatment reduced  $\alpha$ -SMA protein and mRNA expression in a concentration-dependent manner (Fig. 1c), as demonstrated by western blot and PCR analyses (Fig. 2a, b, d).

### Melatonin decreases Ang II-induced ROS generation in H9C2 cells

To determine whether melatonin pretreatment inhibits ROS generation in H9C2 cells, we observed DCFH fluorescence in these cells in response to different treatments, as described above, and noted that melatonin blocked ROS generation in a



**Fig. 1** Melatonin inhibits Ang II-induced H9C2 cell hypertrophy. **a** Effects of melatonin on Ang II-induced [<sup>3</sup>H] leucine incorporation. **b** Quantification of cell surface area. **c** Immunofluorescence analysis of  $\alpha$ -SMA, red (magnification  $\times 400$ ). Scale bar 20  $\mu$ m. **d** and **e** ANP and  $\beta$ -MHC mRNA expression levels. Data are expressed as the

concentration-dependent manner (Fig. 2e). We also explored the effects of melatonin on the protein level of NOx2, which is a major marker of oxidative stress injury. As shown in Fig. 2a, c, pretreatment with melatonin significantly and concentration-dependently decreased the level of NOx2.

### Ang II-induced H9C2 cell hypertrophy through CyPA/CD147 signaling pathway

Previous studies have demonstrated that Ang II induces cardiac hypertrophy and simultaneously increases CyPA generation. To investigate the role of the CyPA/CD147 signaling pathway in Ang II-induced H9C2 cell hypertrophy, we subjected H9C2 cells to 24 h of Ang II treatment in either the absence or presence of 60 min of CsA pretreatment (100 nmol/L). Subsequent western blot analyses revealed that Ang II treatment significantly increased the levels of  $\alpha$ -SMA and CyPA, as well as that of the CyPA receptor, CD147, as shown in Fig. 3a–d. Immunofluorescence analyses revealed that  $\alpha$ -SMA was strongly expressed in H9C2 cells during Ang II stimulation (Fig. 3e). However, these increases were markedly suppressed by CsA pretreatment (Fig. 3).

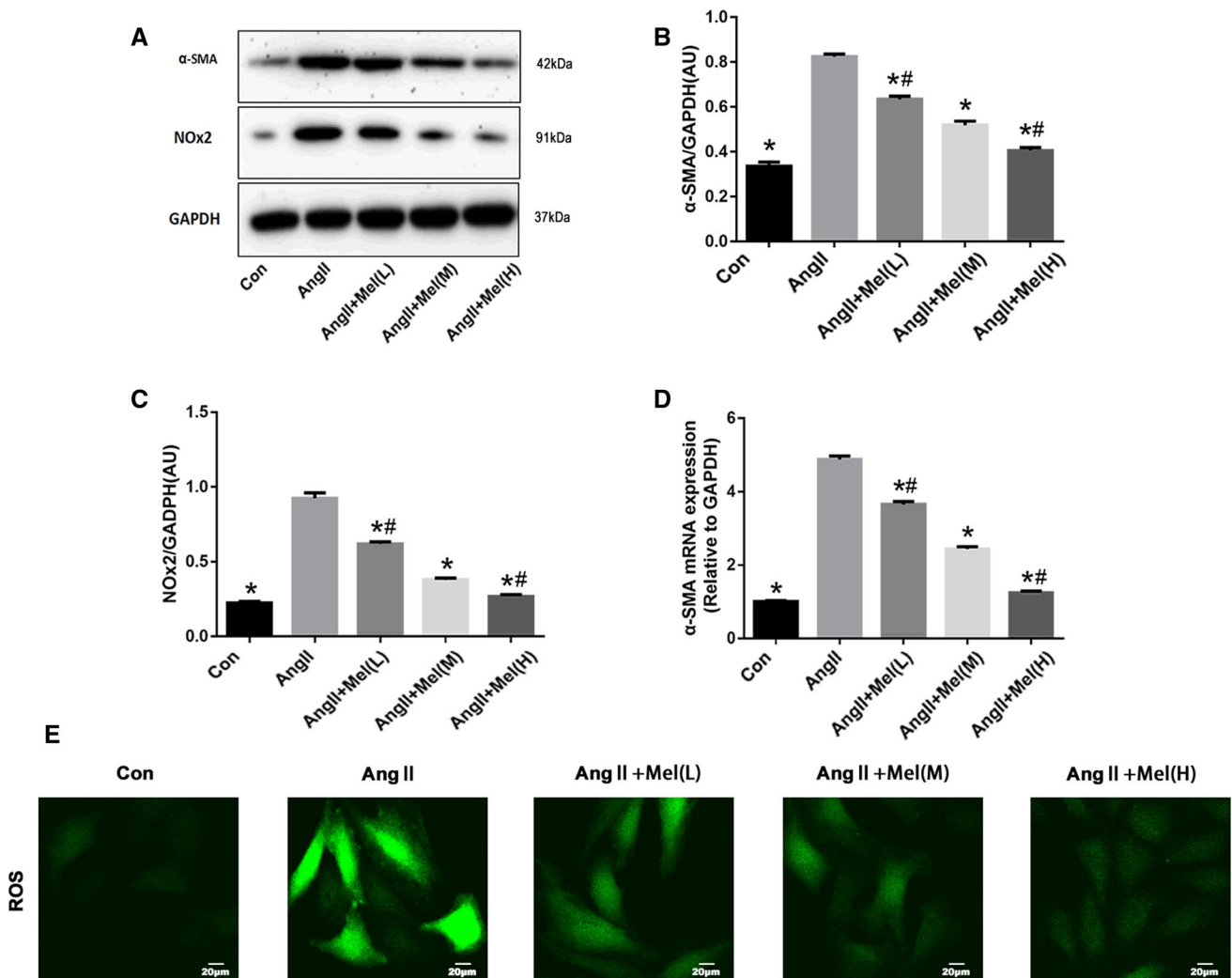
mean  $\pm$  SEM ( $n = 3$ ). \* $p < 0.05$  versus Ang II, # $p < 0.05$  versus Ang II + Mel(M). Con 0.01 % DMSO, Ang II 100 nmol/L angiotensin II, Mel(L) 125  $\mu$ mol/L melatonin, Mel(M) 250  $\mu$ mol/L melatonin, Mel(H) 500  $\mu$ mol/L melatonin. (Color figure online)

### Melatonin blocks CyPA/CD147 signaling pathway-related Ang II-induced H9C2 cell hypertrophy

To investigate the role of the CyPA/CD147 signaling pathway in the antioxidative effects of melatonin, we detected the expression levels of CyPA and its receptor, CD147, in response to Ang II stimulation via immunofluorescence and western blot analyses, which demonstrated that Ang II treatment significantly increased CyPA and CD147 levels, which were markedly and concentration-dependently suppressed by melatonin pretreatment (Fig. 4). Together with the results presented in Figs. 1 and 2, these findings indicate that melatonin protects against Ang II-induced cardiomyocyte hypertrophy, at least in part, via CyPA/CD147 signaling pathway inhibition.

### Melatonin inhibits CyPA-induced cardiac hypertrophy

We tested whether CyPA stimulation alone induces H9C2 cell hypertrophy and investigated the effects of melatonin pretreatment on CyPA-induced cardiac hypertrophy. Our results show that CyPA alone induced H9C2 cell



**Fig. 2** Melatonin decreases Ang II-induced  $\alpha$ -SMA expression and ROS generation in H9C2 cells. **a** Western blot analysis of  $\alpha$ -SMA and NOx2 expression. **b, c** Quantitative analysis of the results in **a**. **d**  $\alpha$ -SMA mRNA expression levels. **e** Representative pictures of DCFH-DA fluorescent staining in H9C2 cells (magnification  $\times 400$ ). Scale

bar 20  $\mu$ m. Data are expressed as the mean  $\pm$  SEM ( $n = 3$ ). \* $p < 0.05$  versus Ang II, \*\* $p < 0.05$  versus Ang II + Mel(M). Con 0.01 % DMSO, Ang II 100 nmol/L angiotensin II, Mel(L) 125  $\mu$ mol/L melatonin, Mel(M) 250  $\mu$ mol/L melatonin, Mel(H) 500  $\mu$ mol/L melatonin

hypertrophy and that pretreatment with melatonin was able to inhibit CyPA-induced cardiac hypertrophy, as revealed by the downregulation of mRNA expression of ANP and  $\beta$ -MHC (Figs. 5, 6a, b, d).

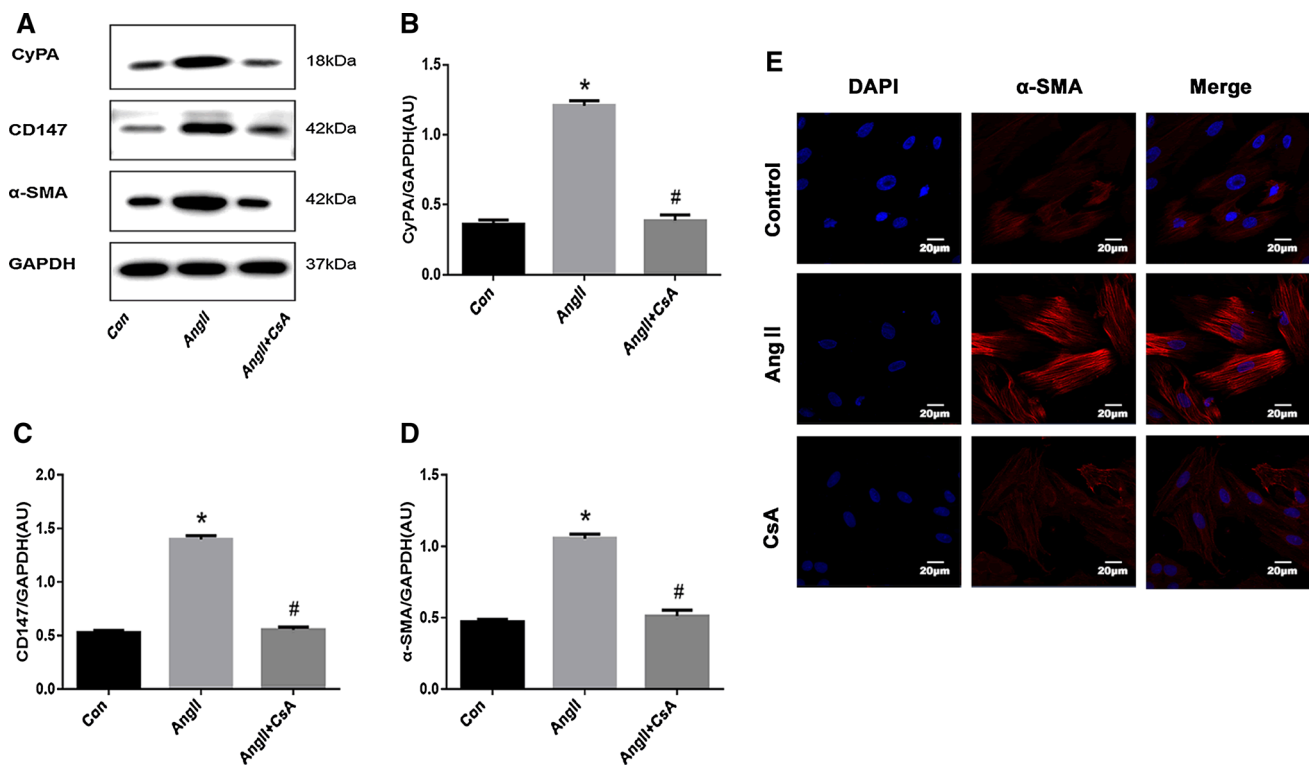
### Melatonin decreases ROS generation in CyPA-induced H9C2 cell hypertrophy

It has been reported that CyPA directly augments Ang II-induced ROS production in cardiac tissue. We further investigated whether CyPA enhances ROS generation in H9C2 cells and whether melatonin could decrease ROS production. The results indicate that CyPA stimulation alone increases ROS generation in H9C2 cells and that melatonin blocks ROS generation in a concentration-

dependent manner in CyPA-induced cardiac hypertrophy (Fig. 6e). We simultaneously observed the effects of melatonin on NOx2 expression in CyPA-induced cardiac hypertrophy. As shown in Fig. 6a, c, western blot results suggest that melatonin significantly decreased the levels of NOx2 in a concentration-dependent manner.

### Discussion

The present study was designed to investigate the role and mechanism of action of melatonin in Ang II-induced cardiac hypertrophy. We demonstrated that CyPA contributes to the development of cardiac hypertrophy in H9C2 cells. CyPA and CD147 were both upregulated in response to



**Fig. 3** Ang II induces H9C2 cell hypertrophy through the CyPA/CD147 signaling pathway. **a** Western blot analysis of CyPA, CD147, and  $\alpha$ -SMA expression. **b–d** Quantitative analysis of the results in **a**. **e** Immunofluorescence analysis of  $\alpha$ -SMA (magnification  $\times 400$ ).

Ang II, and this response was attenuated by melatonin. Our experiments show that the potential effect of melatonin against oxidative stress is partially mediated by the CyPA/CD147 signaling pathway in Ang II-induced hypertrophy.

### Melatonin protects against Ang II-induced cardiac hypertrophy in H9C2 cells

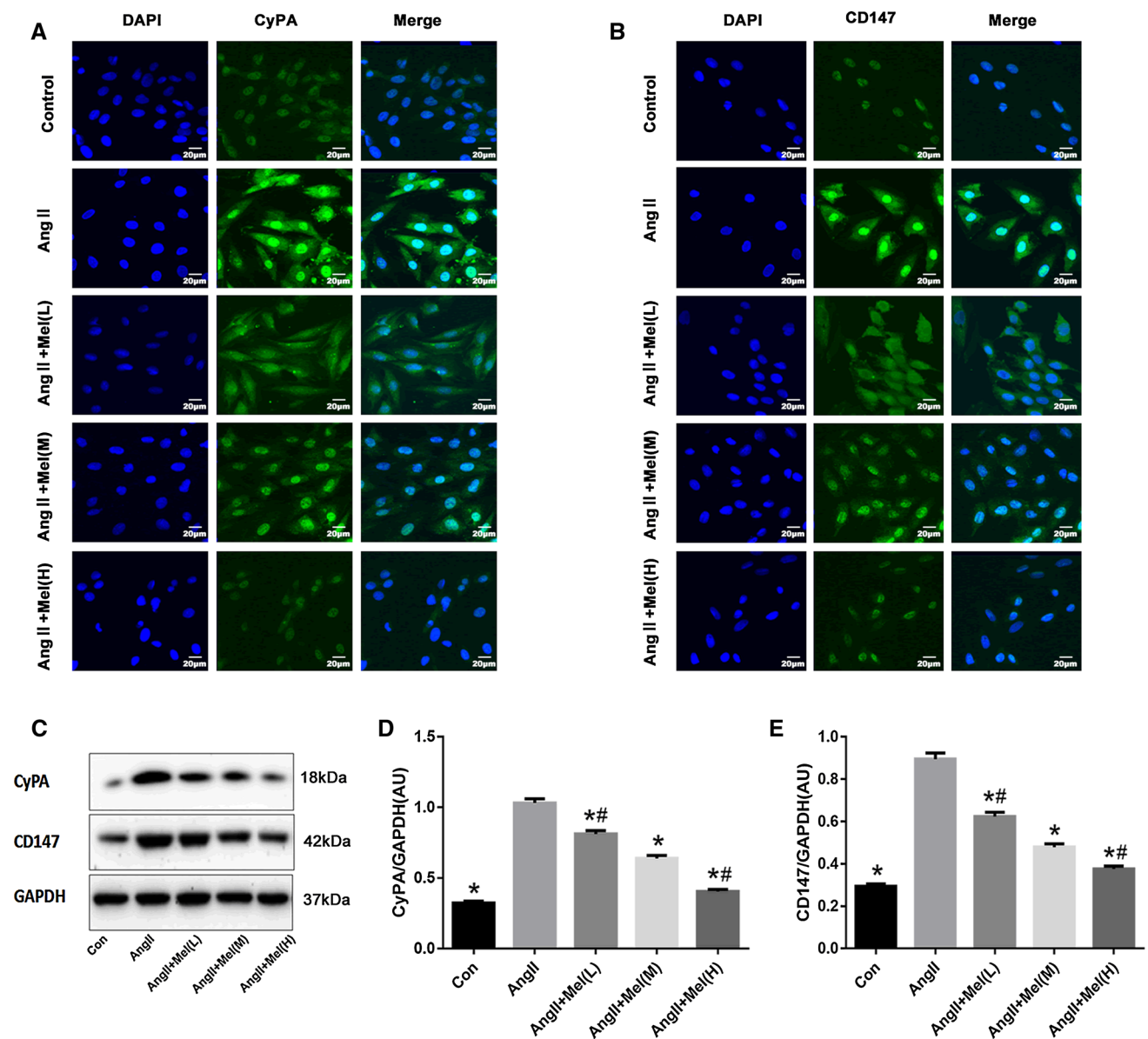
In recent years, a growing body of evidence has demonstrated that Ang II plays an important role in the pathological process of cardiac hypertrophy [39]. Ang II is a key determinant of ROS generation and calcium regulation [40], and can directly increase cardiomyocyte size and initiate a hypertrophic gene program. Accumulating evidence suggests that oxidative stress, which is characterized by an imbalance in the cellular capacity to regulate ROS levels, is responsible for the development of hypertension and several cardiovascular diseases [41]. Numerous studies have supported the notion that the overproduction of ROS is one of the major mechanisms involved in the pathophysiology of Ang II-induced hypertrophy [5, 6], and several recent studies have demonstrated that melatonin is beneficial to the treatment of several cardiovascular diseases, including hypertension, ischemic heart disease, cardiac remodeling, and heart failure [28, 42, 43]. Melatonin has potent antioxidant

Scale bar 20  $\mu$ m. Data are expressed as the mean  $\pm$  SEM ( $n = 3$ ). \* $p < 0.05$  versus Con, # $p < 0.05$  versus Ang II. Con 0.01 % DMSO, Ang II 100 nmol/L angiotensin II, Csa 100 nmol/L cyclosporine A

properties in a variety of pharmacological and physiological concentrations [44, 45]. Therefore, we investigated the cardioprotective role of melatonin against Ang II-induced cardiac hypertrophy. Our results confirm that pretreatment with melatonin remarkably inhibits Ang II-induced cardiac hypertrophy in a cellular model, as melatonin directly decreased cardiomyocyte surface area and the expression of cardiac hypertrophy markers in the presence of Ang II in a concentration-dependent manner and also inhibited Ang II-induced ROS production.

### Ang II-induced H9C2 cell hypertrophy through the CyPA/CD147 signaling pathway

Ang II plays a key role in the physiological and pathological processes of many cardiovascular diseases, including cardiac hypertrophy [39]. Therefore, understanding the molecular mechanisms underlying Ang II-induced cardiac hypertrophy is critical to the development of new cardiac dysfunction therapies [46]. Satoh et al. [47] provided strong mechanistic evidence indicating that CyPA and Ang II synergistically increase ROS generation, thereby stimulating myocardial hypertrophy, matrix remodeling, and cellular dysfunction. As outlined above, Csa, a CyPA inhibitor and the most well studied and



**Fig. 4** Melatonin blocks the CyPA/CD147 signaling pathway in Ang II-induced H9C2 cell hypertrophy. **a, b** Immunofluorescence staining for CyPA and CD147. Scale bar 20  $\mu\text{m}$ . **c** Western blot analysis of CyPA and CD147 expression. **d** and **e** Quantitative analysis of the results in **c**. Data are expressed as the mean  $\pm$  SEM ( $n = 3$ ).

\* $p < 0.05$  versus Ang II, # $p < 0.05$  versus Ang II + Mel(M). Con 0.01 % DMSO, Ang II 100 nmol/L angiotensin II, Mel(L) 125  $\mu\text{mol/L}$  melatonin, Mel(M) 250  $\mu\text{mol/L}$  melatonin, Mel(H) 500  $\mu\text{mol/L}$  melatonin

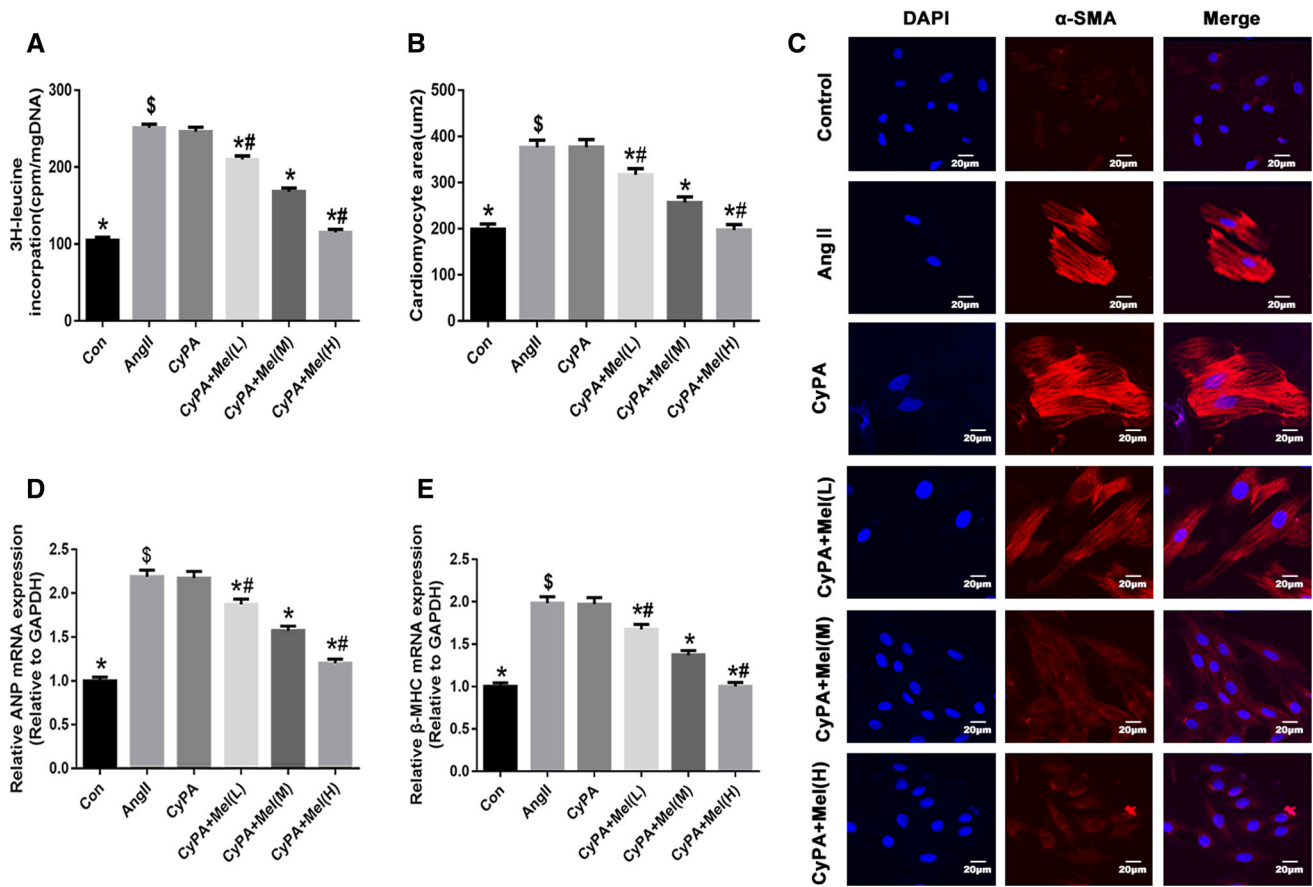
tightest binding CyPA ligand identified to date [48], inhibits the PPIase activity of both extracellular and intracellular CyPA. Yurchenko et al. [49] reported that CD147 also interacts with extracellular CyPA in a CsA-dependent manner. Thus, we studied the inhibitory role of CsA and the role of the CyPA/CD147 signaling pathway in Ang II-induced cardiac hypertrophy.

We observed that Ang II treatment significantly increased  $\alpha$ -SMA, CyPA, and CD147 levels compared with the control group in this study. However, these increases were markedly suppressed by CsA pretreatment in a

cellular model, confirming the pivotal role played by CsA in the inhibitory effects of CyPA and indicating that Ang II induced H9C2 cell hypertrophy through the CyPA/CD147 signaling pathway.

#### Melatonin blocks Ang II-induced cardiac hypertrophy by inhibiting the CyPA/CD147 signaling pathway

Satoh et al. [15] showed that ApoE<sup>-/-</sup> mice developed hypertrophic cardiomyopathy after Ang II treatment and



**Fig. 5** Melatonin inhibits CyPA-induced cardiac hypertrophy. **a** Effects of melatonin on CyPA-induced [<sup>3</sup>H] leucine incorporation. **b** Quantification of cell surface area. **c** Immunofluorescence analysis of  $\alpha$ -SMA (magnification  $\times 400$ ). Scale bar 20  $\mu$ m. **d** and **e** ANP and  $\beta$ -MHC mRNA expression levels. Data are expressed as the

mean  $\pm$  SEM ( $n = 3$ ).  $*p < 0.05$  versus CyPA,  $^{\#}p < 0.05$  versus CyPA + Mel(M),  $^{\$}p > 0.05$  versus CyPA. Con 0.01 % DMSO, Ang II 100 nmol/L angiotensin II, CyPA 100 nmol/L cyclophilin A, Mel(L) 125  $\mu$ mol/L melatonin, Mel(M) 250  $\mu$ mol/L melatonin, Mel(H) 500  $\mu$ mol/L melatonin

that cardiac hypertrophy was significantly reduced in ApoE<sup>-/-</sup> Ppia<sup>-/-</sup> mice compared with ApoE<sup>-/-</sup> Ppia<sup>+/+</sup> mice. Cardiac fibroblasts lacking CyPA exhibited reductions in ROS production, proliferation, and migration upon Ang II stimulation [14]. We demonstrated Ang II-induced H9C2 cell hypertrophy through the CyPA/CD147 signaling pathway in a cellular model (Fig. 3). Taken together, these data illustrate that CyPA/CD147 plays an important role in Ang II-induced cardiac hypertrophy.

The authors of previous studies found that archetypal CyPA exists primarily in tissue cell cytoplasm and seldom localizes in the nucleus or endoplasmic reticulum; more recent studies have demonstrated that CyPA is widely expressed in almost all tissues [50] and can be released into the extracellular space in response to ROS. CD147 is a highly glycosylated transmembrane protein consisting of two immunoglobulin extracellular domains, a transmembrane and a short cytoplasmic domain [51]. Notably, we observed that CyPA and CD147 were localized not only in the cytoplasm but also in the nucleus, especially in Ang II-

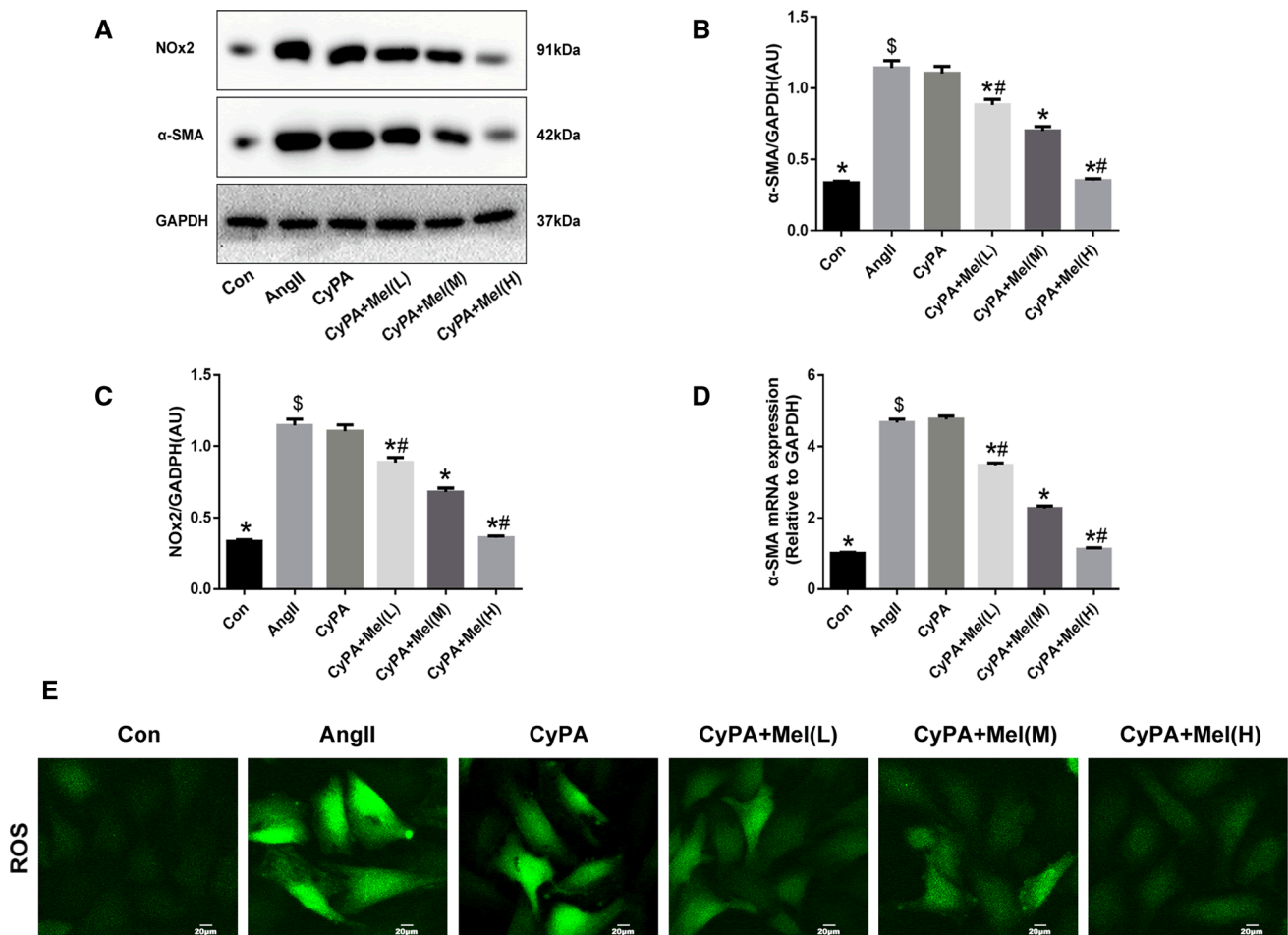
treated cells (Fig. 4a, b). We therefore inferred that Ang II induces CyPA and CD147 expression in H9C2 cells. We also observed that CyPA was transported to the nucleus and that CD147 was transported to the nucleus and cytoplasm, and thus elected to compare each group with the whole cell staining of CyPA and CD147.

We observed that melatonin reduced CyPA and CD147 expression (Fig. 4) and attenuated Ang II-induced cardiac hypertrophy in H9C2 cells in a concentration-dependent manner, suggesting that melatonin has potent, concentration-dependent antioxidant effects that may at least partially prevent CyPA/CD147 signaling pathway-mediated Ang II-induced cardiac hypertrophy.

### Melatonin attenuates CyPA-induced cardiac hypertrophy in H9C2 cells

It is well known that CyPA, a ubiquitous intracellular form of cyclophilin in humans that functions as a chaperone protein, contributes to cellular responses to various





**Fig. 6** Melatonin decreased  $\alpha$ -SMA expression and ROS generation in CyPA-induced H9C2 cells hypertrophy. **a** Western blot analysis of  $\alpha$ -SMA and NOx2 expression. **b, c** Quantitative analysis the results in **a**. **d**  $\alpha$ -SMA mRNA expression levels. **e** Representative pictures of DCFH-DA fluorescent staining in H9C2 cells (magnification  $\times 400$ ). Data are expressed as the mean  $\pm$  SEM ( $n = 3$ ). Scale bar 20  $\mu$ m.

\* $p < 0.05$  versus CyPA, # $p < 0.05$  versus CyPA + Mel(M), \$ $p > 0.05$  versus CyPA. Con 0.01 % DMSO, Ang II 100 nmol/L angiotensin II, CyPA 100 nmol/L cyclophilin A, Mel(L) 125  $\mu$ mol/L melatonin, Mel(M) 250  $\mu$ mol/L melatonin, Mel(H) 500  $\mu$ mol/L melatonin

pathological conditions, including inflammatory diseases, oxidative stress, and cardiovascular diseases [10, 11]. A previous study conducted by Satoh et al. demonstrated that CyPA plays a crucial role in cardiac hypertrophy pathophysiology and that it is a key determinant of ROS production in cardiomyocytes [14]. However, it has not previously been determined whether melatonin can protect against CyPA-induced cardiac hypertrophy.

Our results show that melatonin pretreatment clearly attenuated CyPA-induced cardiac hypertrophy in a concentration-dependent manner by reducing cell surface area, downregulating hypertrophic marker expression, and inhibiting CyPA-induced ROS production, supporting the hypothesis that melatonin plays an important role in protecting cardiac tissue via its antioxidative properties.

Previous studies found that melatonin-induced cardioprotection was associated with activation of protein kinase

B, Notch1/Mfn2 pathway, extracellular signal-regulated kinase (the reperfusion injury salvage kinase pathway), and signal activator and transducer 3 (the survivor activating factor enhancement pathway) during ischemia/reperfusion and inhibition of the mitochondrial permeability transition pore [29]. Lu et al. [52] reported that melatonin had the potential to protect against myocardial hypertrophy induced by LPS in vitro through downregulation of the TNF- $\alpha$  expression and retaining the intracellular  $Ca^{2+}$  homeostasis. However, the role and the underlying mechanism of melatonin in Ang II-induced cardiac hypertrophy were unknown.

Our study suggests that the CyPA/CD147 signaling pathway is activated in Ang II-induced cardiac hypertrophy and that melatonin attenuates Ang II-induced cardiac hypertrophy via its potential antioxidant properties. We also show that the inhibition of the CyPA/CD147 signaling

pathway is at least partially involved in this process; meanwhile some other signal pathway may be also involved in it. Therefore, we propose that the CyPA/CD147 pathway may represent a novel therapeutic target for treating cardiac hypertrophy and that the potential cardio-protective effects of melatonin might provide a new approach for cardiac hypertrophy therapies. Further studies are needed to elucidate the downstream signaling targets of the CyPA/CD147 pathway during the development of cardiac hypertrophy as well as the influence of melatonin on these targets.

**Acknowledgments** The author(s) disclose receipt of the following financial support for this research and the authorship and/or publication of this article: funding support was provided by the National Natural Science Foundation of China (Nos. 81070076, 81170135), the National 973 Basic Research Program of China (No. 2012CB722406), and the Science Foundation of Qilu Hospital of Shandong (No. 2016QLQN17).

**Author Contributions** This Project was conceived by Hongyan Su. The experiments were performed by Hongyan Su, Jingyuan Li, Tongshuai Chen, and Na Li. The results were interpreted by Hongyan Su, Jingyuan Li, Tongshuai Chen, Na Li, and Peili Bu. The manuscript was written and approved by all authors.

#### Compliance with ethical standards

**Conflict of Interest** The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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