Original Paper

Cyclin-Dependent Kinase Inhibitor p21^{WAF1/CIP1} Facilitates the Development of Cardiac Hypertrophy

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Key Words

Cardiac hypertrophy • Cell cycle • Cyclin-dependent kinase inhibitor 1a • p21^{WAF1/CIP1}

Abstract

Background/Aims: Adult cardiomyocytes can re-enter cell cycle as stimulated by prohypertrophic factors although they withdraw from cell cycle soon after birth. p21^{WAF1/CIP1}, a cyclin-dependent kinase inhibitor, has been implicated in cardiac hypertrophy, however, its precise contribution to this process remains largely unclear. **Methods:** The gene expression profile in left ventricle (LV) of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats was determined using quantitative PCR array and verified by real-time PCR and Western blotting. Hypertrophic response of H9c2 cells and neonatal rat ventricular myocytes (NRVM) were induced by angiotensin II (1 µmol/L). Cardiac hypertrophy of mice was elicited by isoproterenol (ISO) infusion (40 mg/kg per day for 14 days). p21-adenovirus and p21-siRNA were employed to transfect NRVM, and sterigmatocystin (STE, 3 mg/kg, ip, qd) was used to inhibit p21 activity. mRNA and protein expression levels of α- and β-myosin heavy chain (MHC), p21^{WAF1/CIP1}, calcineurin (CaN) and atrial natriuretic peptide (ANP) were assayed by real-time PCR and WB, respectively. **Results:** Sixteen genes showed two-fold or greater changes between SHR and WKY rats, in which the expression of p21^{WAF1/CIP1} was upregulated by 4.15-fold ($P=0.002$) and reversed by losartan. Surface area, protein content, mRNA and protein expressions of β-MHC, ANP and p21^{WAF1/CIP1} in H9c2 cells treated with AngII elevated significantly compared with control group. p21-Ad transfection markedly increased the surface area and β-MHC mRNA expression of normal NRVMs, and p21-siRNA transfection decreased them in AngII-treated NRVMs. STE treatment decreased HW/BW and cross-sectional area, expression levels of β-MHC, ANP and p21 significantly in ISO-treated mice. **Conclusion:** Our findings suggest that p21 facilitates the development of cardiac hypertrophy, and regulating the expression of p21 may be an approach to attenuate hypertrophic growth of cardiomyocytes.

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Introduction

Cardiac hypertrophy, a common complication and independent risk factor of a variety of cardiovascular diseases, such as hypertension, ischemic heart disease, valvular insufficiency [1, 2], is a complex and dynamic process. As is known to all, multiple neurohumoral molecules, receptors, intracellular signaling pathways and their crosstalk are involved in the regulation of cardiac hypertrophy [3-5], while detailed mechanisms remain largely unclear. The main feature of cardiac hypertrophy is an increase in cardiac myocyte volume rather than cell number. Although the differentiation of mammalian cardiomyocytes is extremely active during embryonic period, it will stop proliferation and withdraw from the cell cycle soon after birth [6, 7]. However, adult cardiac myocytes still retain the ability to respond to a variety of stimuli by hypertrophic growth [6-8]. Accordingly, adult cardiomyocytes can re-enter into cell cycle manifested as an increase in synthesis of protein and nucleic acid, activation of a program of fetal gene expression as well as cell division arrest, which in turn result in enlargement of cell size, cardiac hypertrophy and eventually heart failure. Therefore, controlling cell cycle should provide greater insight into the underlying mechanism of cardiac hypertrophy.

Cell cycle is strictly controlled by a series of regulatory molecules, including cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors [9]. CDK inhibitor 1a (CDKN1a), also known as p21, WAF1 (wild type p53-activated fragment 1), Cip1 (Cdk-interacting protein 1), or Sdi1 (senescent cell-derived inhibitor 1), is the negative regulator and halts the cell cycle progression in G₁/S and G₂/M transitions by inhibiting CDK4,6/cyclin-D and CDK2/cyclin-E, respectively [10]. For normal mammal, p21^{WAF1/CIP1} is lowly expressed in embryonic and neonatal hearts, however, highly expressed in adult heart [11]. p21^{WAF1/CIP1} has been implicated in cardiac hypertrophy, nevertheless, it is interesting to find there are distinct reports. It was showed that transverse aortic constriction (TAC) elicited a concomitant increase in cardiac expression of p21^{WAF1/CIP1} in rats and mice [12, 13]. The p21^{WAF1/CIP1} up-regulation in cardiomyocytes also was detected in mice following doxorubicin (Dox) treatment [14]. In contrast, Hauck L et al demonstrated that p21 functioned as FoxO3a downstream target to mediate a statin-derived anti-hypertrophic response [15]. Engel and coworkers stated that forced expression of p21 in isolated adult rat cardiomyocytes could prevent serum-induced increase in cell size [16]. Additionally, p21 knockout mice did not show myocardium injury and cardiac hypertrophy in response to Dox treatment [14]. These different data prompted us to extensively explore the role of p21 in cardiac hypertrophy.

In this study, we uncovered that expression of p21^{WAF1/CIP1} elevated significantly in the left ventricle of spontaneously hypertensive rats (SHR) accompanied with dramatic cardiac hypertrophy. Subsequently, various models of cardiac hypertrophy *in vitro* and *in vivo* were employed to demonstrate the upregulation of p21^{WAF1/CIP1}, which could facilitate the development of cardiac hypertrophy.

Material and Methods

Animals and treatments

Male spontaneously hypertensive rats (SHR) and age-matched normotensive Wistar-Kyoto (WKY) control rats (12-weeks old; weighing 200–250 g) were purchased from Beijing Weitong Lihua Experimental Animal Technology (Beijing, China). Neonatal Sprague-Dawley rats (1–2 days old) and adult male C57BL/6J mice weighing 18–22 g were obtained from the Experimental Animals Center of the Third Military Medical University (Chongqing, China). All the experimental procedures followed *The Guide for the Care and Use of Laboratory Animals*, Eighth Edition (National Research Council, 2011) and were approved by the Ethical Committee for Animal Experimentation of Third Military Medical University.

Twelve SHR were randomly divided into cardiac hypertrophy model and losartan-treated group (Los), and were administrated with normal saline or losartan (6 mg/kg, i.g., qd) for 8 weeks, respectively. Another six WKY rats were used as normal controls and administrated with saline. Twenty male mice were randomly

divided into four groups, i.e., control, isoproterenol (ISO) treatment, p21 inhibitor- and p21 adenovirus-treated group; and administrated with buffer (1% ascorbic acid in PBS) infusion, continuous infusion of ISO using osmotic minipump infusion (Alzet, Model 1002; Durect, Cupertino, CA) at 40 mg/kg/d for 14 days [17], ISO infusion and sterigmatocystin (STE, Sigma-Aldrich, St. Louis, MO, USA) at 3 mg/kg (ip, qd) [18, 19], 2×10^7 TU Ad-p21 adenovirus (Vector Biolabs, Philadelphia, PA) via femoral vein injection, respectively.

At the end of treatments, all animals were weighed and anaesthetized with pentobarbital sodium (50 mg/kg, ip). The animals were decapitated and the hearts were excised and weighed to calculate the ratio of heart weight to body weight (HW/BW) and the ratio of left ventricular weight to body weight (LVW/BW). The mid ventricle was fixed with a formalin neutral buffer solution and embedded in paraffin. The apex of the ventricle was stored in liquid nitrogen for future use.

Cell cultures

The hypertrophic response of H9c2 cells was induced by angiotensin II ($1 \mu\text{mol/L}$) incubating for 24h [20]. Neonatal rats' ventricular myocytes (NRVM) from 1-2 days old Sprague-Dawley rats were cultured as described previously [21, 22]. NRVMs were incubated in DMEM supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA) for 18 hours after preparation and subsequently treated with an adenoviral vector expressing p21^{WAF1/CIP1} (Ad-p21, Vector Biolabs, Philadelphia, PA) at a multiplicity of infection (MOI) of 10 for 12 hours in DMEM, and then the media were replaced with fresh DMEM without adenovirus. Simultaneously, NRVMs were transfected with rat small interfering RNAs (siRNAs) targeting p21 (Santa Cruz Biotechnology, CA) by siRNA Transfection Reagent (Santa Cruz) according to the manufacturer's protocol. Seven hours after transfection, NRVMs were cultured in serum-free DMEM for 12 h and incubated for 24 h in a non-serum medium containing $1 \mu\text{mol/L}$ AngII. Cells treated with only Ad-p21 or p21 siRNA and saline were used as controls.

Real-time PCR array

Total RNA was extracted from left ventricular tissue of SHR and WKY rats using TRIzol Reagent (Invitrogen, Carlsbad, CA) [23]. RNA was converted to cDNA using the RT² First Strand Kit (SA Biosciences, Valencia, CA). mRNA expression was determined with GPCR Signaling Pathway Finder Array (PTRN-071A, SA Biosciences, Valencia, CA, USA) [23, 24]. Data were analyzed using the comparative Ct method with normalized to the housekeeping genes Rplp1, Hprt1, Rpl13a, Ldha and β -actin. Controls including rat genomic DNA contamination, reverse transcription and positive PCR were performed simultaneously.

Cell size determination

H9c2 cells and NRVM were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature, washed with PBS, permeabilized with 0.1% Triton-X 100 (Shanghai Bioengineering, China) for 45 min. To visualize the cell morphology, cells were incubated with rhodamine-labeled phalloidin (Sigma-Aldrich, St. Louis, MO, USA) in 1:100 dilution for 1 h and nuclei were stained with DAPI for 10 min. The microscopic images of cell morphology were quantitatively analyzed using ImageJ software [21].

Left ventricle tissue was fixed in 10% formalin for 48 hours and was embedded in paraffin. Sections ($5 \mu\text{m}$) were stained with fluorescein isothiocyanate (FITC)-labeled lectin wheat germ agglutinin (Sigma, MO, USA) [25]. Cross-sectional areas (CSA) were measured using the Image Pro Plus 5.1 image analysis program (Media Cybernetics, Silver Spring, MD) [21].

Immunofluorescence Staining

Double-immunofluorescence staining in NRVMs was performed as previously reported [21]. Primary α -actinin polyclonal rabbit antibody and p21 mouse monoclonal antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), and secondary anti-rabbit antibody conjugated to Alexa Fluor 555 and anti-mouse antibody conjugated to fluor 488 was used, respectively. A negative control was carried out by replacing the primary antibody with isotype IgG. The expression of p21 was observed by laser scanning confocal microscopy and analyzed using ZEN imaging software (2012, blue edition, Carl Zeiss).

Real-time reverse transcription PCR

Total RNA was extracted from cells and left ventricular tissue and reversely transcribed with the Prime Script RT reagent kit (TaKaRa, Japan). The primers were designed by Premier 5.0 (Premier Biosoft

Table 1. Primers used for Real-time RT-PCR

Sample from	Gene	Forward (5'→3')	Reverse (5'→3')
Rats	α-MHC	TATGCTGGCACCCTGGACTA	GAGTTTGAGGGAGGACTTCTGG
	β-MHC	GAGTGGACGTTTATTGACTTCGG	GGCTTCTGCTTTCCCTTGA
	CaN	CCCTAAGAAGAGGTAGCGAGTG	GTGCTTCGATTCTCCGACAG
	Cdkn1a	TGTGCGCTGTCTTGA CTCT	GGCACTTCAGGGCTTTCTC
	ANP	GAGGAGAAGATGCCGGTAG	CTAGAGAGGGAGCTAAGTG
Mice	β-actin	CGTAAAGACCTCTATGCCAACA	TAGGAGCCAGGGCAGTAATC
	α-MHC	AACAACCCATACGACTACGCC	CAGCATCTTCTGTGCCATCA
	β-MHC	TGCCAATGACGACCTGAAAGA	CGCTCGCTGGTCTCAATCAG
	Cdkn1a	TACTTCCTCTGCCCTGCTGC	TGGTCTGCCTCCGTTTTTCG
	ANP	CTGGGCTTCTTCTCTCGTCTT	TCCAGGTGGTCTAGCAGGTTTC
	β-actin	CCGTAAAGACCTCTATGCCAACA	CTGCTGGAAGGTGGACAGTGAG

International, Palo Alto, CA, USA) and synthesized by Sangon Biotech (Shanghai, China) (Table 1). qPCR was carried out on the CFX Connect Real-time PCR instrument (Bio-Rad, Hercules, CA). Gene expression was normalized to β-actin and presented using a modification of the $2^{-\Delta\Delta Ct}$ method [26].

Western blotting assay

The protein expression levels of α-, β-myosin heavy chain (MHC), Cdkn1a, calcineurin (CaN) and atrial natriuretic peptide (ANP) were assayed by Western blot. Total homogenates from cells and left ventricular tissue were prepared, and equal amounts (30 μg) of the denatured proteins were loaded and separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were transferred onto a PVDF membrane (Millipore, Bedford, MA) and incubated with primary antibody (α-MHC mAb, 1:600, Santa Cruz Biotechnology, CA, USA; β-MHC mAb, 1:600, Santa Cruz; CaN mAb, 1:1000, Millipore, Bedford, MA; Cdkn1a mAb, 1:1000, Millipore; ANP pAb, 1:600, Santa Cruz; β-actin mAb, 1:10000, Sigma, St. Louis, MO) at 4°C overnight, followed by horseradish peroxidase (HRP)-conjugated anti-goat IgG or HRP conjugated anti-mouse IgG. Chemiluminescence was detected with an ECL western blot detection kit (Millipore, Bedford, MA).

Statistical analysis

The data are expressed as the mean ± SEM. The statistical significance of differences between the group means was determined by a one-way ANOVA (SPSS 13.0). $P < 0.05$ was considered significant.

Results

Gene expression profile in SHR left ventricular myocardium

After one-week adaptation and 8-week treatment, the ratio of HW/BW and LVW/BW significantly increased in SHR by comparison of those in WKY rats, while losartan treatment obviously decreased these indexes compared with SHR (Fig. 1B and C). Simultaneously, no significant difference of body weight was observed among these groups (Fig. 1A). In addition, mRNA and protein expression of α-MHC both decreased, while mRNA and protein expression levels of β-MHC and CaN increased significantly in SHR by comparison of those in WKY rats ($P < 0.01$). In contrast, losartan treatment elevated expression of α-MHC and lowered that of β-MHC and CaN markedly ($P < 0.01$) (Fig. 1D-F).

To investigate the potential involvement of genes in cardiac hypertrophy, we performed microarray analysis in SHR and WKY rats. There were 16 genes showed two-fold or greater changes between SHR and control WKY rats (Fig. 2A, Table 2), in which the expression changes of Cdkn1a (p21), Ctgf (connective tissue growth factor), Fgf2 (fibroblast growth factor 2), and Serpine1 (serine or cysteine peptidase inhibitor, member 1) were more noticeable, showing upregulation by 4.15-fold ($P = 0.002$), 4.71-fold ($P = 0.005$), 11.33-fold ($P = 0.015$) and 4.82-fold ($P = 0.003$), respectively. However, as the cardiac hypertrophy reversed by losartan, only two genes were downexpressed significantly, namely Cdkn1a (-2.00-fold, $P = 0.02$) and Ctgf (-3.16-fold, $P = 0.008$) (Fig. 2B). Given it has been well known about the importance role of growth factors during cardiac hypertrophy, we concerned p21 for the next experiments. We verified the result using quantitative RT-PCR and Western blotting, respectively. Compared

Fig. 1. Cardiac hypertrophy was developed in spontaneously hypertensive rats (SHR) and reversed by losartan treatment. Rats were administrated intragastrically with normal saline (for WKY and SHR group) or losartan (6 mg/kg, i.g., for Losartan group) once daily for 8 weeks. (A) body weight (BW); (B) heart index, value was determined as the ratio of heart weight (HW) to BW; (C) left ventricular index, value was determined as the ratio of left ventricular weight (LVW) to BW. D, mRNA expression levels, determined with real-time PCR. (E) Western blots, (F) Histograms representing protein expression levels. The data are presented as mean $\pm$ SEM ($n=6$ in each group) and analyzed with one-way ANOVA. WKY, Wistar-Kyoto rats; MHC, myosin heavy chain; CaN, calcineurin.

Fig. 2. Differentially expressed genes in left ventricle of rats. (A) differentially expressed genes between SHR and WKY rats; (B) differentially expressed genes between losartan- and saline-treated SHR. Differentially expressed genes detected by RT-PCR array. 2-fold difference and p value = 0.05 were marked as vertical and horizontal lines, respectively. $n=4$ in each group. (C) and (D), Verification of the expressions of Cdkn1a in array. mRNA and protein expressions of Cdkn1a was measured using real-time PCR (C) and Western blotting (D). The data are presented as mean $\pm$ SEM ($n=4$ in each group) and analyzed with one-way ANOVA.

with WKY rats, mRNA and protein expression level of p21 increased significantly in SHR ($P < 0.01$), while losartan treatment markedly lowered mRNA and protein expression level of p21 ($P < 0.01$) (Fig. 2C and D). Hence, p21^{WAF1/CIP1} may be related to cardiac hypertrophy in SHR model.

Table 2. Gene expression changed significantly by at least 2-fold between SHR and WKY rats

Gene	Fold changed	p value
Adcy5 (adenylate cyclase 5)	2.33	0.0040
Bcl2l1 (Bcl2-like protein 1)	2.99	0.0015
Cdkn1a (cyclin-dependent kinase inhibitor 1A, p21, Cip1)	4.15	0.0020
Chhr2 (corticotropin releasing hormone receptor 2)	2.11	0.0212
Ctgf (connective tissue growth factor)	4.71	0.0052
S1pr1 (sphingosine-1-phosphate receptor 1)	2.91	0.0002
Edn1 (endothelin 1)	2.67	0.0063
Fgf2 (fibroblast growth factor 2)	11.33	0.0152
Gcgr (glucagon receptor)	-2.67	0.0107
Grm1 (glutamate receptor, metabotropic 1)	2.31	0.0364
Jun (Jun oncogene)	2.03	0.0197
S1pr3 (sphingosine-1-phosphate receptor 3)	2.40	0.0201
Nos2 (nitric oxide synthase 2, inducible)	-2.00	0.0020
Ptgs2 (prostaglandin-endoperoxide synthase 2, COX-2)	2.50	0.0184
Serpine1 (serine or cysteine peptidase inhibitor, clade E, member 1)	4.82	0.0026
Tnf (tumor necrosis factor; TNF superfamily, member 2)	-2.00	0.0234

Fig. 3. Expression of p21^{WAF1/CIP1} (Cdkn1a) in hypertrophic H9c2 cardiomyocytes. Hypertrophic response of H9c2 cells was induced by treatment of 1 μ mol/L angiotensin (Ang) II for 24 hours. (A) H9c2 cells stained with rhodamine-labeled phalloidin and DAPI (bar = 50 μ m); (B) Cell size of H9c2 cells (n = 20 cells in each group); (C) Protein content (n = 5); (D) mRNA expression of p21, α -MHC, β -MHC and atrial natriuretic peptide (ANP), determined using real-time PCR (n = 5); (E) Western blots; (F) Histograms representing protein expression level (n = 4). The data are presented as mean $\pm$ SEM and analyzed with student's T test.

p21 expression in hypertrophic H9c2 cells

We then examined the expression of p21 in hypertrophy cardiomyocytes. As shown in Fig. 3A-C, both surface area and protein content in AngII-treated group elevated significantly compared with control group. Moreover, the mRNA and protein expression levels of β -MHC and ANP increased, whereas α -MHC decreased obviously in AngII-treated group ($P < 0.01$). Noticeably, the mRNA and protein expression levels of p21 also obviously increased in hypertrophic H9c2 cells ($P < 0.01$) (Fig. 3D-F).

p21 expression in left ventricular myocardium of mice

ISO infusion increased the ratio of HW/BW and CSA significantly compared with control group, while STE, a p21 inhibitor, decreased them obviously by comparison of those in ISO group, respectively (Fig. 4A-C). Meanwhile, compared with controls, the mRNA and protein expression levels of myocardial α -MHC decreased and those of β -MHC, ANP, p21 increased significantly in ISO group ($P < 0.01$). After treatment with STE, expression of α -MHC increased

Fig. 4. Expression of p21^{WAF1/CIP1} in hypertrophic myocardium in mice. Cardiac hypertrophy was induced by isoproterenol (ISO) osmotic minipump infusion (40 mg/kg per day) subcutaneously for 14 days. Sterigmatocystin (STE, 3 mg/kg) was administrated intraperitoneally once daily for 14 days to inhibit p21^{WAF1/CIP1}. Ad-p21 adenovirus was administrated intravenously to normal mice. (A) heart index, value was determined as the ratio of heart weight (HW) to body weight (BW); (B) Fluorescein isothiocyanate (FITC) conjugated lectin staining of myocardium. (C) Cell area; (D)-(G) mRNA expression levels of α-MHC, β-MHC, ANP and p21^{WAF1/CIP1}, determined by real-time PCR. (H) Western blots; (I)-(L) Protein expression levels of α-MHC, β-MHC, ANP and p21. The data are presented as mean ± SEM (n=4-5 in each group) and analyzed with one-way ANOVA.

and that of β-MHC, ANP, p21 decreased markedly (Fig. 4D-L). Although Ad-p21 modified the mRNA expression of α-MHC, β-MHC, ANP and p21 as ISO treatment, however, it had no significant effects on the protein expression of α-MHC and ANP.

Expression of p21 in NRVM

Immunofluorescence staining was used to co-localize p21 and cardiomyocytes (anti-α-actinin). p21 was expressed in both the cytoplasm and nuclei (Fig. 5A). Compared with the control group, the expression level of p21 in myocytes treated with AngII elevated markedly (Fig. 5B). And the ratio of nuclear to cytoplasmic expression of p21 was higher after AngII treatment ($P < 0.01$) (Fig. 5C), suggesting an increase of p21 translocation from cytoplasm to nuclei.

p21 promote hypertrophy of NRVM

Next, we tested the influence of p21 on the hypertrophic response in NRVM, and it was found that Ad-p21 transfection increased p21 expression while p21 siRNA decreased it significantly (Fig 6A and B). In addition, Ad-p21 transfection markedly elevated the surface

Fig. 5. Expression of p21^{WAF1/CIP1} in neonatal rat ventricular myocytes (NRVMs). Hypertrophic response of NRVM was induced by treatment of 1 μ mol/L angiotensin (Ang) II for 24 hours. (A) Expression of p21^{WAF1/CIP1} was detected using double-label immunofluorescence staining with α -actinin. Cells were co-stained with DAPI to visualize their nuclei. Images from the same field were merged. (bar=20 μ m); (B) Expression intensity level of p21^{WAF1/CIP1}; C, Ratio of p21^{WAF1/CIP1} expression in nucleus to that in cytoplasm. The data are presented as mean $\pm$ SEM (n=20 in each group) and analyzed with one-way ANOVA.

Fig. 6. Effects of p21^{WAF1/CIP1} expression level on hypertrophic response of neonatal rat ventricular myocytes (NRVM). (A) mRNA expression level of p21^{WAF1/CIP1} in NRVM transfected with Ad-p21 adenovirus or p21-siRNA, determined by real-time PCR (n = 4); (B) Western blots and expression level of p21^{WAF1/CIP1} in NRVMs; (C) NRVMs stained with rhodamine-labeled phalloidin and DAPI (bar = 50 μ m); (D) Cell size of NRVMs (n = 50 cells in each group); (E) mRNA expression of β -MHC in NRVM, determined using real-time PCR (n = 5). The data are presented as mean $\pm$ SEM and analyzed with one-way ANOVA.

area and the β -MHC mRNA expression in normal NRVMs, in contrast, p21 siRNA did not produce obvious effects on them. Accompanying with the stimulation of AngII, cell size of NRVMs was increased by 2.68-fold compared with control, and deranged actin microfilaments could be seen. Although there was no significant difference in cell size and the expression of β -MHC mRNA between AngII-treated and Ad-p21 transfected cells, Ad-p21 transfection caused the microfilaments disorganized obviously. However, p21 siRNA transfection in AngII treated-NRVMs decreased cell size ($P<0.01$) and β -MHC mRNA expression ($P<0.05$) remarkably (Fig. 6C-E). Thus, these data supported a critical role for p21 in the regulation of cardiac hypertrophy.

Discussion

p21 protein, which binds to and inhibits the activity of various cyclin-CDK complexes [27, 28], subsequently inhibits the phosphorylation of retinoblastoma (Rb) protein and release of early gene 2 transcription factor 1 (E2F1). Accordingly, p21 is defined as a regulator of cell cycle arrest [11, 27, 29, 30]. Although the role of p21^{WAF1/CIP1} in tumor cells has been well documented, the underlying function and mechanism of p21 in the developing of cardiac hypertrophy remains an enigma. In the screening for target genes related to cardiac hypertrophy, it is interesting to note the dramatically overexpressed p21 in hypertrophied left ventricle of SHR. To address this issue, H9c2 cells, NRVM, and mice was induced by prohypertrophic agonist, respectively, to further identify the high expression of p21 during cardiac hypertrophy.

Myocytes of mammalian heart display three forms from fetal to adult stage, namely, proliferation, binucleation, and hypertrophy [31]. Cardiac myocytes proliferate rapidly during embryo stage, whereas withdraw from cell cycle in the perinatal period even though some myocytes undergo an additional round of DNA synthesis and nuclear mitosis without cytokinesis [31, 32]. Generally, increase in cardiac mass is achieved through enlarged cell size or hypertrophy rather than myocytes proliferation during adult life. However, accumulated studies have elucidated adult cardiomyocytes should reenter into cell cycle and transit from G₁ to S phase during the process of cardiac hypertrophy.

Cell-cycle regulation is effected by the ordered activation of a group of cyclins and related enzymes known as the cyclin-dependent kinases (CDK) [27]. Cyclin-CDK complexes are precisely and strictly regulated to prevent abnormal proliferation. There are two families of inhibitors that block the catalytic activity of cyclin-CDK complexes, namely inhibitor of CDK4 (INK4) family which only bind to CDK4/6, and KIP/CIP family including p21^{WAF1/CIP1} and p27^{KIP1}, which can inhibit all cyclin-CDK complexes [11, 27, 33, 34]. A gene knockout study in mice confirmed that p21 and p27 are important partners in mammalian cardiomyocyte [35]. p21 and p27 coordinately force the cell cycle exit by blocking all the phases of the cell cycle. Both p21 and p27 inhibit G₁ phase entry, while p21 also plays a crucial role in the inhibition of M-phase entry [35-37]. In hypertrophied cardiomyocytes, increased expression of p21 can inhibit the activity of cyclin A-CDK1 complex in G₂ phase and cyclin B-CDK1 complex following cells arrest in G₂/M phase [38, 39]. Our current study showed that p21 mRNA expression increased 4.15 folds in SHR by comparison with WKY rats, while p27 gene expression had no significant change (1.56-fold, p>0.05), suggesting that p21 may be the key regulator during the development of cardiac hypertrophy in SHR.

H9c2 cell line, which has been used widely in the research of cardiac hypertrophy, is originally derived from embryonic rat ventricular tissue and has the advantage of being an animal-free alternative. Watkins et al reported that H9c2 cells showed almost identical hypertrophic responses to those observed in primary cardiomyocytes [40]. In this study, surface area of H9c2 cells and NRVMs increased 2.51- and 3.67-fold, respectively, following stimulation with AngII, which was accompanied by the increase in mRNA and protein expression of p21. Moreover, overexpression of p21 through transfection of p21 adenovirus elevated the cell size, while downregulation of p21 had no significant influence on normal NRVMs. In AngII-treated cells, however, downexpression of p21 using siRNA transfection decreased cell size and β -MHC mRNA expression significantly, while overexpression of p21 had no significant influence. These results implied that in normal myocytes, elevated expression of p21 should induce effectively hypertrophic response whereas have no synergistic effects with AngII stimulation. On the other hand, inhibition of p21 expression can play roles in hypertrophic cardiomyocytes rather than the normal cardiomyocytes. Similar results were also detected in mice treated with isoproterenol infusion, supporting the idea that inhibition of p21 should decrease CSA, expression of ANP and β -MHC notably and then ameliorate the development of cardiac hypertrophy.

Considering the distinct reports about p21 expression during cardiac hypertrophy, we can imagine the possible explanations. Brooks et al. [11, 41] found that there was a transient

down-regulation of p21 expression in left ventricle tissue of rats during the 3-14 day post-operative period undergone aortic constriction compared with sham-operated animals. Downexpression of p21 in G₁/S phase induce the increase of cyclin-CDK complexes and promote the synthesis of DNA and protein, and upexpression of p21 in G₂/M phase induce cell cycle arrest and hypertrophy. Furthermore, recent studies stated p21 can exert dual role depending on subcellular location. For example, p21 inhibits proliferation by blocking the cyclin dependent kinases in the nucleus while it acts inhibiting pro-apoptotic protein determining cell death inhibition in the cytoplasm [39]. Therefore, different pathological stage of cardiac hypertrophy and subcellular location of p21, which should be responsible for distinct role of p21 in cardiac hypertrophy.

The present study still has certain limitations. Sterigmatocystin was used as an inhibitor of p21 activity in this study. However, it is also a known inhibitor of G₁ Phase and DNA synthesis. In this case, the present results do not allow us to distinguish whether the inhibition of hypertrophy is p21 dependent or independent manner. As known that p21 expression pattern is correlated to hypertrophic stage and subcellular location, it would be necessary for us to excavate more detailed temporal and spatial expression pattern of p21. In addition, p21KO mice should be helpful to understand thoroughly the role and mechanisms of p21 in the development of cardiac hypertrophy.

Collectively, our findings supported that p21 should facilitate the development of cardiac hypertrophy, and regulating the expression of p21 may be an approach to attenuate hypertrophic growth of cardiomyocytes.

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Disclosure Statement

None.

Reference

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