FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 49, No. 3, 2011
pp. 445–451

ORIGINAL STUDY

VIA MEDICA

p21^{WAF1} and hypoxia/reoxygenation-induced premature senescence of H9c2 cardiomyocytes

Dan Wang, Yu-Zhen Zhang, Bing Yang, Feng-Xiang Zhang, Ming-Yong Cao, Cheng Wang, Ming-Long Chen

Department of Cardiology, First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China

Abstract: We have previously reported on hypoxia/reoxygenation-induced premature senescence in neonatal rat cardiomyocytes. In this research, we investigated the effects of p21^{WAF1} (p21) in hypoxia/reoxygenation-induced senescence, using H9c2 cells. A plasmid overexpressing wild type p21^{WAF1} and a plasmid expressing small hairpin RNA (shRNA) targeting p21^{WAF1} were constructed, and transfected into H9c2 cells to control the p21 expression. Hypoxia/reoxygenation conditions were 1% O_2 and 5% CO_2, balancing the incubator chamber with N_2 for 6 h (hypoxia 6 h), then 21% oxygen for 8 h (reoxygenation 8 h). Cell cycle was examined using flow cytometry. Senescence was assessed using β-galactosidase staining. The expression of p53, p21, p16^{INK4a}, and cyclin D1 was assayed using Western blotting. At hypoxia 6 h, cells overexpressing p21 had a larger G1 distribution, stronger β-galactosidase activity, and lower cyclin D1 expression compared to control cells, while the opposite results and higher p53 expression were obtained in p21-knockdown cells. At reoxygenation 8 h, p21-silenced cells had a smaller percentage of G1 cells, weaker β-galactosidase activity and lower 16^{INK4a} expression, and higher cyclin D1 expression, but the overexpression group showed no difference. Taken together, this data implies that p21^{WAF1} is important for the hypoxia phase, but not the reoxygenation phase, in the H9c2 senescence process. *(Folia Histochemica et Cytobiologica 2011, Vol. 49, No. 3, 445–451)*

Key words: H9c2, hypoxia reoxygenation, p21^{WAF1}, β-galactosidase, senescence

Introduction

Cellular senescence is defined as cell cycle arrest caused by DNA damage or other stress [1]. Various types of stimuli such as telomere shortening, oncogenic or tumor suppressor signals, and DNA damage can trigger cellular senescence [2]. Cellular senescence is characterized by an increase in senescence-associated β-galactosidase activity, cell cycle arrest and expression of gene-associated senescence [2]. Cellular senescence has been observed in many cell types [3], including cardiomyocytes [4]. Cardiomyocytes were thought to be a terminally differentiated cell type unable to undergo mitosis; however, recent evidence of cardiac regeneration has been reported. Anversa et al. [5] demonstrated cardiomyocyte cell division in adult human hearts, and proliferation and cell division in a fraction of cardiomyocytes were discovered in young, as well as in adult and elderly, hearts. Cardiac stem cell senescence has also been reported [6].

Oxidative stress is the most important factor in the aging process of cardiomyocytes [7].

Zhang et al. [8] reported hypoxia-reoxygenation induced premature senescence in Fanconi anemia bone marrow hematopoietic cells. Our previous study [9] also found that exposure to hypoxia/reoxygenation induced premature senescence in neonatal Sprague-Dawley rat cardiomyocytes. Currently, aging *per se* is considered a risk factor for reduced cardiac function and heart diseases [7], and senescent cardiomyocytes have been found in patients with heart failure, suggesting that cellular senescence plays a role in the

Correspondence address: M.-L. Chen, Department of Cardiology, First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China;
e-mail: chenminglong2001@163.com

course of heart failure [4]. Jugdutt et al. reported [10] that aging is related to reperfusion injury and left ventricular remodeling after reperfused ST-segment-elevation myocardial infarction. Therefore, investigating the molecular mechanism of cardiomyocyte aging is important for both clinical and basic cardiology research.

A universal inhibitor of cyclin-dependent kinases (CDK), $p21^{WAF1}$ also negatively regulates the cell cycle by binding and inactivating most cyclin-CDK complexes. Being closely related to $p16^{INK4a}$ (p16) and p53, $p21^{WAF1}$ plays an essential role for cells, not only with regards to physiological conditions, but also in response to DNA damage by regulating cell cycle progression, apoptosis, and transcription [11]. Senescent cardiomyocytes enter a stage characterized by irreversible cell-cycle arrest, upregulation of $p16^{INK4a}$ and p53, and reduction in telomere length after exposure to stress [7]. However, the function of $p21^{WAF1}$ in cardiomyocyte aging induced by hypoxia/reoxygenation is not clear. Therefore, in the current study, we investigated the role of $p21^{WAF1}$ in hypoxia/reoxygenation-induced aging of cardiomyocytes.

Material and methods

Experimental animals and cell culture. All animal experiments were performed using protocols approved by our Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals of Nanjing Medical University, China. Rat cardiomyocyte H9c2 cells were from American Type Culture Collection, and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml).

Preparation of RNA and RT-PCR. Total RNA was isolated from cultured cells with TRIZOL (Invitrogen, USA) according to the manufacturer's protocol. RNA was concentrated by Biophotometer (Eppendorf, Germany), and cDNA synthesized using M-MuLV reverse transcriptase (RT) and random primers according to the manufacturer's recommendations (Fermentas, USA). Semiquantitative PCR analysis used PCR Master Mix (Fermentas, USA).

Western blotting. Cultured cells in three groups were washed with iced PBS and lysed with 2 × SDS lysis buffer. Protein concentration was determined by the Lowry method and 50 μg of whole cell lysate was loaded and electrophoresed on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes (Bio-Rad, USA). Membranes were blocked with 5% skim milk in TBST and incubated with anti-$p16^{INK4a}$ (1:1,000, Abcam, UK), or anti-$p21^{WAF1}$ monoclonal antibodies (1:300 Lifespan Biosciences, USA), or anti-p53 (1:800, Abcam, UK), or anti-cyclin D1 (1:1,000, GenWay Biotech, USA) polyclonal antibodies for 16 h at 4°C. A GAPDH antibody (Sigma, USA) was used as a protein loading control. After 1-h incubation at room temperature with secondary antibody linked to horseradish peroxidase, the membranes were washed and the membrane blots were developed with EZ/ECL reagents (Pierce). The quantity of protein was determined using 'Quantity One' (Bio-Rad, USA).

Construction of plasmids PRNAT-$p21^{WAF1}$-cGFP and PRNAT-silencer. ICR mice (4–6 weeks old, 23.5 ± 2.4 g) were given 50% alcohol at 12 ml/kg body weight, and sacrificed 12 h later without feeding. Total RNA was extracted from liver tissues, and cDNAs encoding full-length mouse $p21^{WAF1}$ obtained by RT-PCR using primers: F: 5'-GG GGATTC ATGTCCAATCCTGGTGATGTCCGAC -3'
R: 5'-GG GGTACC TCAGGGTTTTCTCTTGCAGAAGACC -3'

After sequencing, full-length $p21^{WAF1}$ cDNA was subcloned into plasmid PRNAT-U6.1/neo-cGFP (GenScript Corporation, USA) to generate PRNAT- $p21^{WAF1}$-cGFP. Small hairpin RNA (shRNA) against $p21^{WAF1}$ was designed and synthesized by GenScript Corporation (USA) with the sequence: 5'-CGTGTCGCTGTCTTGCACTCTGGTTCAAGAGACCAGAGTGCAAGACAGCGACATTTTTTCCAA -3'. Cloning into plasmid PRNAT-U6.1/neo-cGFP, generated plasmid PRNAT-silencer. Plasmid PRNAT-U6.1/Neo/CTL was used as a control and contained the noncoding sequence: 5'-CGTCGCTTACCGATTCAGAATGGTTGATATCCGCCATTCTGAATCGGTAAGCGATTTTTTCCAACG-3'.

H9c2 transfection and verification. The three plasmids (PRNAT-$p21^{WAF1}$-cGFP, PRNAT-silencer, and PRNAT--U6.1/Neo/CTL) were transfected into H9c2 cells with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions, to generate p21-overexpressing, p21-silenced, and control group cells. Cells were cultured with G418 (400 μg/ml) for two weeks after transfection, and positive clones were picked. $p21^{WAF1}$ expression was observed by RT-PCR and Western blotting methods as described above.

Hypoxia/reoxygenation. As illustrated in our previous study [9], suspended cells were diluted to 1.0×10^5 cells/ml, and 10 ml seeded into 75 cm^2 plastic culture flasks and cultured for 48 h. The treatment conditions for the three group cells were as follows: normoxia, cells were continuously incubated in 5% CO_2 and 95% air at 37°C; hypoxia/reoxygenation, cells were cultured with D-Hanks solution, and placed in a modular incubator chamber (BioSpherix, USA) with 1% O_2, 5% CO_2, balanced with N_2 for 6 h (hypoxia 6 h), then exposed to 21% oxygen for 8 h (reoxygenation 8 h).

Cells cycle analysis. Cells were harvested, fixed in 70% ethanol, and stored at –20°C before washing twice with ice-cold phosphate-buffered saline (PBS) and incubating with RNase and propidium iodide, a DNA-intercalating dye. Cell cycle phase analysis was performed using a Becton Dickinson Facstar flow cytometer equipped with Modfit 3.0 software.

***β*-galactosidase staining.** A *β*-galactosidase colorimetric assay kit (Cell Signaling Technology, USA) was used according to the manufacturer's protocol. The percentage of senescence-associated-*β*-galactosidase positive cells was determined by counting blue cells under bright-field illumination, using the total number of control cells in the same phase as a standard. The percentage of positive cells represented the intensity of the *β*-galactosidase activity.

Statistical analysis. All experiments were performed in triplicate and results analyzed by SPSS 13.0 software (SPSS Inc, USA). Data was expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used and an least significant difference (LSD) test performed for group comparisons. A p value of less than 0.05 was considered significant.

Results

Expression of $p21^{WAF1}$ increased at the mRNA and protein levels in cells in the p21-overexpressing group, and decreased in the p21-silenced group (Figure 1). At hypoxia 6 h and reoxygenation 8 h, $p21^{WAF1}$ expression showed a lower level in the p21-silenced group and a higher level in the p21-overexpressing group (Figures 2A, 2B). Both plasmids affected $p21^{WAF1}$ expression in H9c2 cells.

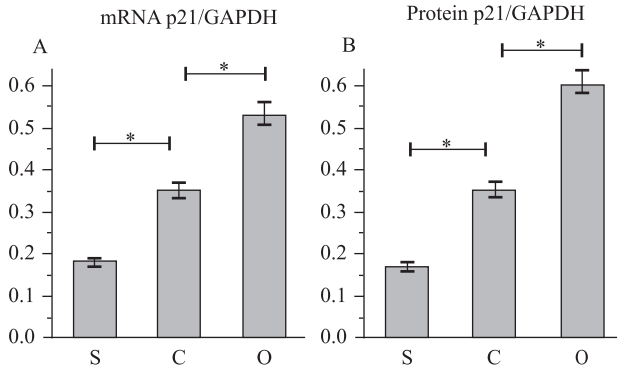

Figure 1. The $p21^{WAF1}$ expression of H9c2 cells at the mRNA level (**A**) and the protein level (**B**). Total mRNA and proteins of the p21-silenced group (S), control group (C) and p21-overexpressing (O) group were extracted and tested by RT-PCR (**A**) and Western blotting methods (**B**); *means significantly different at $p < 0.05$

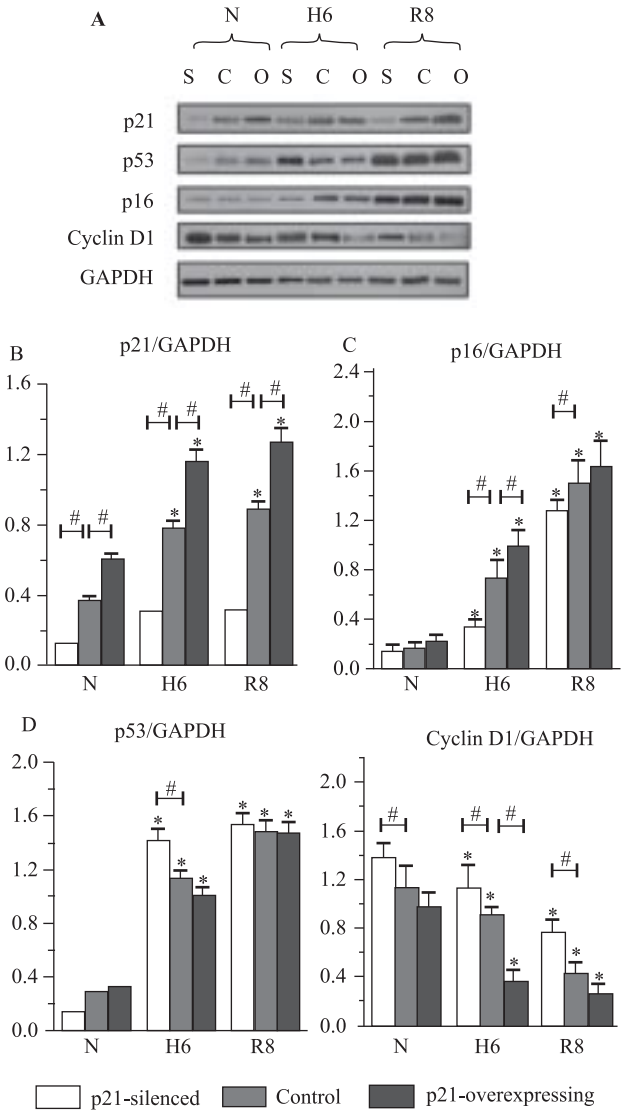

Figure 2. The p21-silenced group (S), control group (C), and p21-overexpressing group (O) were treated with hypoxia/reoxygenation. Total proteins were extracted and assayed by Western blotting (**A**) method at normoxia (N), hypoxia 6 h (H6), and reoxygenation 8 h (R8) as described in 'Material and methods'. The expressions of protein $p21^{WAF1}$(**B**), $p16^{INK4a}$ (**C**), p53 (**D**), and cyclin D1 (**E**) were analyzed respectively, and relative value was determined by normalization with GAPDH; * means significantly different at $p < 0.05$ compared to the same group itself in normoxia condition; # means significantly different at $p < 0.05$ compared to the control group in the same treatment condition

Compared to cells in normoxia condition, senescence markers changed substantially in cells exposed to hypoxia/reoxygenation. At both hypoxia and reoxygenation phases, all three groups of cells had larger distribution of G1 cells (Figure 3), stronger *β*-galactosidase activity (Figure 4), higher $p16^{INK4a}$ and p53

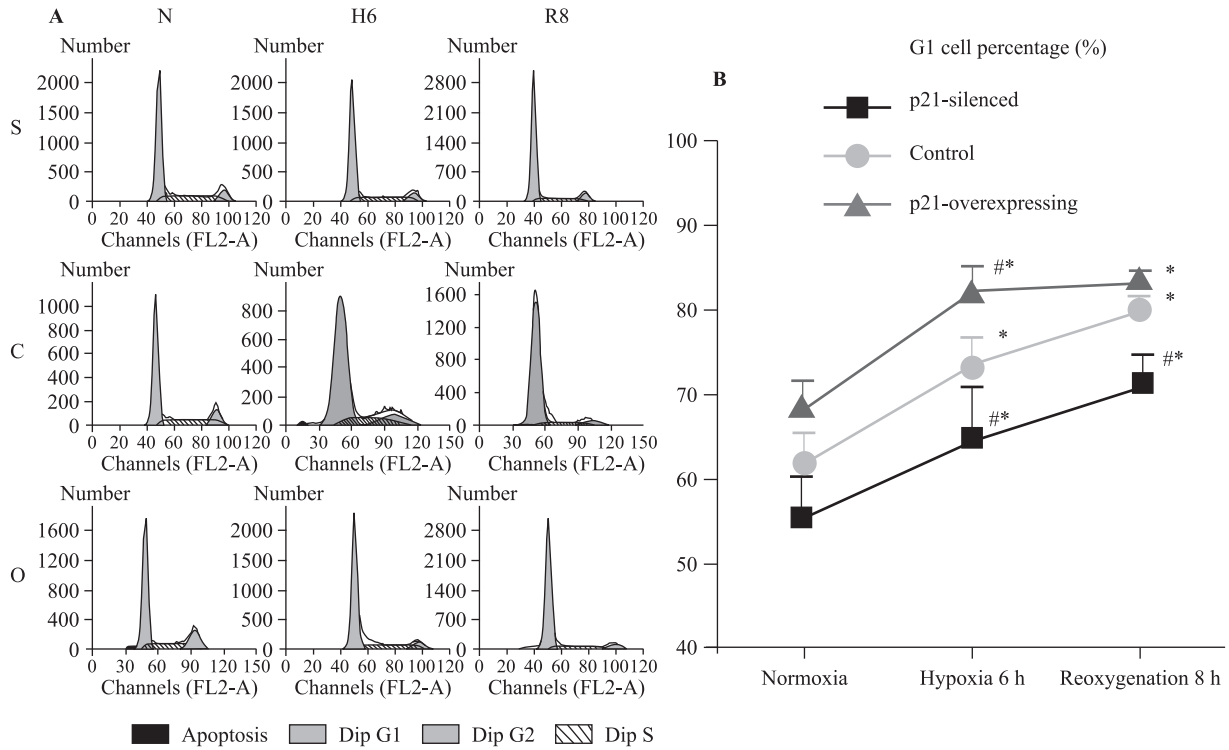

Figure 3. After being treated with hypoxia/reoxygenation, cells of the p21-silenced group (S), control group (C) and p21-overexpressing group (O) were collected, and at least 1×10^4 cells were tested by flow cytometry (**A**). The G1 phase percentage of the three groups at normoxia (N), hypoxia 6 h (H6) and reoxygenation 8 h (R8) were described (**B**); *means significantly different at $p < 0.05$ compared to the same group itself in normoxia condition; # means significantly different at $p < 0.05$ compared to the control group in the same treatment condition

expression, and lower cyclinD1 (Figures 2A, 2C–E) expression compared to those in normal conditions. The results show that senescence was induced in all groups by hypoxia/reoxygenation.

At hypoxia 6 h, senescence markers in the p21--overexpressing group, G1 cell percentage, and percentage of β-galactosidase staining and $p16^{INK4a}$ expression were much higher than in the control group (Figures 2C, 3, 4), whereas cyclin D1 expression was much lower (Figure 2E). The opposite results were observed in the p21-silenced group (Figures 2C, 2E, 3, 4). This data shows that $p21^{WAF1}$ expression was involved in the hypoxia phase of the hypoxia/reoxygenation-induced senescence of H9c2 cells.

At reoxygenation 8 h, although $p21^{WAF1}$ expression was depressed in the p21-silenced group, the senescence was still induced, and senescence markers were still higher than in the normoxia condition, and still lower than in both the control group and the p21--overexpressing group (Figures 3, 4). However, when $p21^{WAF1}$ was overexpressed, no significant differences were seen in G1 cell percentage, β-galactosidase staining, and $p16^{INK4a}$, cyclinD1, or p53 expression between the p21-overexpressing group and the control group (Figures 2C–E, 3, 4), indicating that the effect of $p21^{WAF1}$ was attenuated in the reoxygenation phase. Thus, $p21^{WAF1}$ affected the senescence process mainly in the hypoxia phase and not in the reoxygenation phase.

Discussion

Expression of $p21^{WAF1}$ is elevated in many aging cells [12–13], and this protein protects adult stem cells from acute genotoxic stress, and impairs stem cell function in stem cell aging [14]. Accumulation of $p21^{WAF1}$ leads to growth arrest, and its suppression causes a delay in senescence [15]. The plasmids PRNAT- $p21^{WAF1}$-cGFP and PRNAT-silencer were constructed to control $p21^{WAF1}$ expression. Using those plasmids, and via hypoxia/reoxygenation treatment of H9c2 cells, $p21^{WAF1}$ expression was found to be increased in all three group cells after hypoxia/reoxygenation, indicating that $p21^{WAF1}$ was induced for its DNA repair function when the damage had occurred because of the oxidative stress. However, because of the plasmids PRNAT--$p21^{WAF1}$-cGFP and PRNAT-silencer, $p21^{WAF1}$ was effectively controlled in p21-overexpressing group and p21-silenced group at different treatment conditions.

Activity of β-galactosidase, as well as expression of $p16^{INK4a}$ and p53, increased with hypoxia/reoxygenation, and was accompanied by cell cycle arrest, suggesting that cellular senescence occurred in all three cell groups.

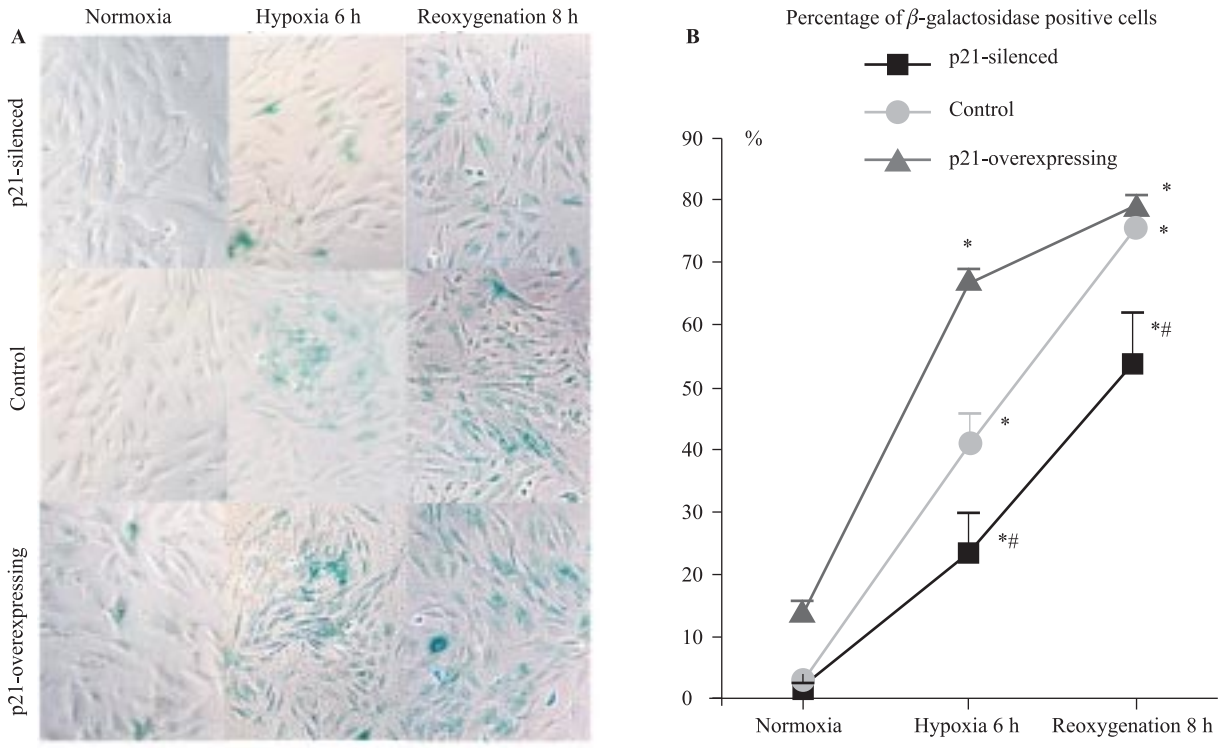

Figure 4. (**A**) β-galactosidase staining in the three groups and positive cells (senescent cells) showed blue in the pictures ($\times$ 200). The percentage of positive cells represented the β-galactosidase activity of the three groups, and was analyzed (**B**); * means significantly different at $p < 0.05$ compared to the same group itself in normoxia condition; # means significantly different at $p < 0.05$ compared to the control group in the same treatment condition

Studies have shown that β-galactosidase activity is a marker of premature senescence in many types of cells [2], and adult mouse cardiomyocytes [16]. The activity of β-galactosidase increases with expression of the senescence-associated protein p21 [9]. The findings of the present study conform to these previously reported results. At the hypoxia phase, the number of β-galactosidase-positive cells differed according to p21 expression, indicating that p21 affected senescence in the hypoxia phase of hypoxia/reoxygenation-treated cardiomyocytes; however, the effect was weakened at reoxygenation phase.

Cell cycle arrest is an important characteristic of cellular senescence. First, $p21^{WAF1}$ induces G1 arrest by directly inhibiting cyclin D1 [17]. Yoshiba et al. [18] reported that hypoxia induces G1 phase cell cycle arrest in oral cancer cells. In addition, p21 mediates cell cycle arrest in response to DNA damage, not only by inactivating G1-phase cyclins/CDKs complexes, but also through direct interaction with PCNA to inhibit DNA replication [11]. Consistent with these results, the findings of the current study suggest that p21 expression might control the cell cycle by inhibiting cyclin D1. Similar results were obtained by Waldman et al. [19], who also found that $p21^{WAF1}$ is necessary for p53-mediated G1 arrest in human cancer cells. However, the results of the current study show that, at reoxygenation phase, p21's effect on the cell cycle was attenuated, and other factors had minor or partial effects.

When cellular senescence occurred, the expression of senescence-related proteins such as p53, p21, and $p16^{INK4a}$ changed. As described in a previous report [20], during chronic hypoxia, cells would lower their normoxic set-point such that the return to normoxic pO_2 after chronic hypoxia results in perceived hyperoxia. DNA was damaged by the oxygen toxicity after hypoxia/reoxygenation and induction of p53, which is necessary for the signaling to p53-dependent senescence [2]. The p21 protein, a target of p53, is closely involved in cellular senescence, and can be activated by p53-dependent [21] and p53-independent [22] pathways. As can be seen in the current study, p21 expression was not completely parallel with p53. That meant that p21 was induced both by p53 indirectly and by DNA damage directly. Cells reportedly enter into senescence, in which repair to phase damages take place; $p21^{WAF1}$ may be directly involved in DNA repair [11]. At the hypoxia phase, DNA repair could be partly delayed in the p21 silenced cell group, and as a reaction to feedback, p53 expression was higher than the other two groups accordingly.

The different senescent extent of the three groups suggested another factor could contribute to the senescence process both independently and by co-operation with $p21^{WAF1}$.

We think p16^{INK4a} could be the one. Close interaction between p21^{WAF1} and p16^{INK4a} has been suggested in articles. Huang et al. [23] reported down-regulation of p21^{WAF1} that caused cells to express less p16^{INK4a} mRNA and p16^{INK4a}, possibly programming the cell senescence process by triggering senescence when p16^{INK4a} protein accumulates. Recently, an intrinsic cooperation between p16^{INK4a} and p21^{WAF1} in the onset of cellular senescence was established [24, 25]. In the present study, p16^{INK4a} increased in all three groups, and the results imply that p16^{INK4a} was induced in H9c2 cells after hypoxia/reoxygenation, and was further affected by p21^{WAF1} levels. At the reoxygenation phase, senescence markers still increased, such as G1 phase percentage and β-galactosidase activity, which meant that senescence might still proceed when p16^{INK4a} was expressed, despite p21^{WAF1} repression. Some reports indicated that [26] senescence delayed when p16^{INK4a} was repressed, and p16^{INK4a} could initiate the cellular senescence [27] and also induced the G1 phase arrest accompanied by increment of β-galactosidase activity [28].

Hence, the results suggest that the effects of p21^{WAF1} in this process were attenuated for the increase of p16^{INK4a} induced by both oxidative stress and p21^{WAF1} at reoxygenation. But when p21was knocked down, p16^{INK4a} expression was lower, and the senescent markers were also weaker. That meant that p21 played a role, partly at least, in the reoxygenation phase. Otherwise, some reports suggested p21^{WAF1} mainly adjusted the early stage of cellular senescence [29], and p16^{INK4a} mainly adjusted the late stage of cellular senescence [30]; and another [31] suggested that at late-passage of senescence, cells consistently exhibited increased expression of p16^{INK4a} seen at both the mRNA and protein level, but the expression of p21^{WAF1} mRNA did not differ significantly between early- and late-passage.

In short, p21^{WAF1} is important for the hypoxia phase of senescence; however, during reoxygenation, the increase in p16^{INK4a} induced by cellular damage controls the cellular senescence processes associated with p21^{WAF1}.

Acknowledgement

This work was supported by a grant from the National Natural Science Foundation of China (No 30671073).

References

1. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res.* 1961;25:585–621.
2. Saab R. Cellular senescence: many roads, one final destination. *Sci World J.* 2010;10:727–741.
3. Chen Q, Ames BN. Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells. *Proc Natl Acad Sci USA.* 1994;91:4130–4134.
4. Chimenti C, Kajstura J, Torella D et al. Senescence and death of primitive cells and myocytes leads to premature cardiac aging and heart failure. *Circ Res.* 2003;93:604–613.
5. Anversa P, Leri A, Kajstura J. Cardiac regeneration. *J Am Coll Cardiol.* 2006;47:1769–1776.
6. Torella D, Rota M, Nurzynska D et al. Cardiac stem cell and myocyte aging, heart failure, and insulin-like growth factor-1 overexpression. *Circ Res.* 2004;94:514–524.
7. Bernhard D, Laufer G. The aging cardiomyocyte: a mini--review. *Gerontology.* 2008;54:24–31.
8. Zhang X, Li J, Sejas DP et al. Hypoxia-reoxygenation induces premature senescence in FA bone marrow hematopoietic cells. *Blood.* 2005;106:75–85.
9. Zhang FX, Chen ML, Shan QJ et al. Hypoxia reoxygenation induces premature senescence in neonatal SD rat cardiomyocytes. *Acta Pharmacol Sin.* 2007;28:44–51.
10. Jugdutt BI, Jelani A, Palaniyappan A et al. Aging-related early changes in markers of ventricular and matrix remodeling after reperfused ST-segment elevation myocardial infarction in the canine model. Effect of early therapy with an Angiotensin II Type 1 receptor blocker. *Circulation.* 2010.
11. Cazzalini O, Scovassi AI, Savio M et al. Multiple roles of the cell cycle inhibitor p21(CDKN1A) in the DNA damage response. *Mutat Res.* 2010;704:12–20.
12. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* 1999; 13:1501–1512.
13. Besson A, Dowdy SF, Roberts JM. CDK inhibitors: cell cycle regulators and beyond. *Dev Cell.* 2008;14:159–169.
14. Ju Z, Choudhury AR, Rudolph KL. A dual role of p21 in stem cell aging. *Ann NY Acad Sci.* 2007;1100:333–344.
15. Dyson N. The regulation of E2F by pRB-family proteins. *Genes Dev.* 1998;12:2245–62.
16. Oku T, Ikeda S, Sasaki H et al. Functional sites of human PCNA which interact with p21 (Cip1/Waf1), DNA polymerase delta and replication factor C. *Genes Cells.* 1998; 3:357–369.
17. Gartel AL, Radhakrishnan SK. Lost in transcription: p21 repression, mechanisms, and consequences. *Cancer Res.* 2005; 65:3980–3985.
18. Yoshiba S, Ito D, Nagumo T et al. Hypoxia induces resistance to 5-fluorouracil in oral cancer cells via G(1) phase cell cycle arrest. *Oral Oncol.* 2009;45:109–115.
19. Waldman T, Kinzler KW, Vogelstein B. p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res.* 1995;55:5187–5190.
20. Min LJ, Mogi M, Iwanami J et al. Angiotensin II type 2 receptor deletion enhances vascular senescence by methyl methanesulfonate sensitive 2 inhibition. *Hypertension.* 2008;51: 1339–1344.
21. Antropova YG, Bryantsev AL, Kalinina NI et al. Proliferative activity and expression of cyclin-dependent kinase inhibitor p21WAF1 and p53 protein in endothelial cells of human aorta during replicative aging in vitro. *Bull Exp Biol Med.* 2002;134:81–83.
22. Aliouat-Denis CM, Dendouga N, Van den Wyngaert I et al. p53-independent regulation of p21Waf1/Cip1 expression and senescence by Chk2. *Mol Cancer Res.* 2005;3:627–634.
23. Huang Y, Corbley MJ, Tang Z et al. Down-regulation of p21WAF1 promotes apoptosis in senescent human fibroblasts: involvement of retinoblastoma protein phosphorylation and delay of cellular aging. *J Cell Physiol.* 2004;201:483–491.

24. Duan J, Zhang Z, Tong T. Senescence delay of human diploid fibroblast induced by anti-sense p16INK4a expression. *J Biol Chem*. 2001;276:48325–48331.
25. Han XL, Wu FG, Zhang ZY et al. Posttranscriptional induction of p21Waf1 mediated by ectopic p16INK4 in human diploid fibroblast. *Chin Med J (Engl)*. 2007;120: 405–409.
26. Zhou R, Han L, Li G et al. Senescence delay and repression of p16INK4a by Lsh via recruitment of histone deacetylases in human diploid fibroblasts. *Nucleic Acids Res*. 2009; 37:5183–5196.
27. Dai CY, Enders GH. p16 INK4a can initiate an autonomous senescence program. *Oncogene*. 2000;19:1613–1622.
28. Ksiazek K, Mikula-Pietrasik J, Olijslagers S et al. Vulnerability to oxidative stress and different patterns of senescence in human peritoneal mesothelial cell strains. *Am J Physiol Regul Integr Comp Physiol*. 2009;296:R374–382.
29. Weinberg RA. The retinoblastoma protein and cell cycle control. *Cell*. 1995;81:323–330.
30. Hara E, Smith R, Parry D et al. Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence. *Mol Cell Biol*. 1996;16:859–867.
31. Ksiazek K, Piwocka K, Brzezinska A et al. Early loss of proliferative potential of human peritoneal mesothelial cells in culture: the role of p16INK4a-mediated premature senescence. *J Appl Physiol*. 2006;100:988–995.

Submitted: 31 October, 2010
Accepted after reviews: 1 May, 2011