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Testosterone protects cardiomyocytes against hydrogen peroxide-induced aging by upregulating IGF1 and SIRT1 pathways

LI YAN[1†], XITING NONG[2†], JIZHAO DENG[1] and GUANG YANG[1*]

[1] Department of Cardiology, Shaanxi Provincial People Hospital, Xian, China

[2] Department of Endocrinology, Xi'an Central Hospital, Xian, China

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ABSTRACT

Objective: To investigate the role of IGF1 and SIRT1 pathways in protection of hydrogen peroxide (H_2O_2)-induced aging in H9c2 rat cardiomyocyte cells by testosterone. *Methods:* The cells were treated with testosterone or up- or down-regulated for the IGF1 and SIRT1 genes and assessed for apoptosis, aging and expression of relevant genes. *Results:* Aging was induced and the expression of SIRT1 and IGF1 was down-regulated after H_2O_2 treatment in H9c2 cells. The aging was attenuated in a dose-dependent manner after the cells were exposed to testosterone. Down-regulation of SIRT1 and IGF1expression was offset in the H_2O_2-treated cells co-treated with testosterone. Up- or down-regulation of IGF1 significantly reduced or increased senescence-associated beta-galactosidase (SA-β-gal) cells and the ROS level, respectively. In addition, SIRT1 expression was regulated by IGF1 expression. Down- or up-regulation of SIRT1 significantly decreased or increased the IGF1 levels, respectively. Furthermore, after IGF1 and SIRT1 knockdown, testosterone did not protect the cells from senescence. Testosterone, and overexpression of IGF1 and SIRT1 also up-regulated the expression of the fetal genes SERCA2 and MYH6 and down-regulated the expression of the ACTA1 and MYH7 genes. *Conclusions:* Our data indicate that testosterone can attenuate cardiomyocyte aging induced by H_2O_2 and up-regulate SIRT1 and IGF1. The IGF1and SIRT1 pathway may be new targets to treat heart aging and heart failure.

KEYWORDS

insulin-like growth factor-1, sirtestosteronein-1, cardiomyocyte senescence, testosterone, H_2O_2

* Corresponding author. Department of Cardiology, Shaanxi Provincial People Hospital, 256 Youyi West Road, Xian 710068, China. Tel./fax: +8618691885667. E-mail: ar6883g@163.com

† These authors contributed equally to the work.

INTRODUCTION

Chronic heart failure (CHF) is one of the major health issues throughout the world. The global prevalence of CHF is about 26 million, which is the main cause of population morbidity and mortality [1, 2]. Despite tremendous medical advances, the cost of care for CHF remains high and hospitalization accounts for 65% of the cost. For instance, in the United States, $980 million are estimated to be spent annually on the treatment and management of the CHF population, and the World Bank estimates that the annual global economic cost to manage CHF patients is $10.8 billion [1, 3]. The prevalence of heart failure is related to age. A survey conducted by the American College of Cardiology shows that the prevalence of heart failure in elderly people over 65 years is 10% [4, 5]. As an important organ, the heart undergoes a series of changes such as decrease in the number of myocytes and increase in accumulation of lipid and areas of fibrosis with age, called heart aging [6]. Apoptosis of cardiomyocytes is one of the characteristics of heart aging [7]. Reducing cardiomyocyte aging is of great significance for delaying heart aging and preventing the occurrence of heart failure [8].

Another significant metabolic change during the aging process is the change in the balance of sex hormones. Testosterone is the most important androgen in males. It has been found that the levels of total testosterone and bioactive testosterone in males gradually decrease after the age of 40 years [9]. Plasma testosterone level is inversely related to the incidence of multiple age-related diseases in older men [10]. A number of testosterone replacement therapy trials have found that testosterone supplementation can improve cognitive ability, increase muscle strength, enhance exercise capacity, reduce insulin resistance, and improve sexual function [11, 12]. Testosterone can also improve the heart function of the elderly and delay the occurrence of senile heart failure [13, 14]. In addition, other cellular signaling pathways such as IGF1 and SIRT1 are also involved in cardiac aging [15–17].

As a peptide hormone, IGF1 is synthesized mainly in the liver and it functions as a growth and differentiation factor in tissues such as heart muscle [18, 19]. IGF1 can activate RNA polymerase, regulate the proliferation and differentiation of different types of cells and reduce oxidative stress in heart via IGF/JNK1/SIRT1 signaling [18, 19]. IGF1 reduces cardiomyocyte apoptosis via upregulating the expression of adiponectin, uncoupling protein 1 (UCP1), and metallothionein 2 (MT-2) and down-regulation of apoptosis-related proteins such as p-ASK, p-JNK, Bax and caspase-3 [19, 20]. IGF1 protects the heart from oxidative stress via SIRT1, a member of the sirtuin family of nicotinamide adenine dinucleotide NAD-dependent deacetylases [21]. Once upregulated, it can reduce apoptosis, increase viability and stress resistance and reduce paraquat-induced oxidative stress [15].

Thus, we hypothesized that testosterone might delay cardiomyocyte aging via the IGF1 and SIRT1 pathway. To prove this, H_2O_2-treated cardiomyocyte cells were exposed to testosterone to examine the expression of IGF1 and SIRT1 and cellular aging and changes in senescent parameters after up- and down-regulating the two genes. We demonstrated that testosterone activates the IGF1 and SIRT1 pathways and alters the expression of the systolic/diastolic-associated enzymes and proteins MYH and SERCA-2 in aging cells. These findings provide new insights into the molecular mechanism underlying testosterone-mediated protection against aging and potential avenues for cardiac therapy during aging and heart failure.

MATERIALS AND METHODS

Cell culture and treatment

H9c2 rat cardiomyocyte cells (ATCC 30-2002, ATCC, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin and streptomycin at 37°C in a 5% carbon dioxide incubator. The cells were purchased from the American Type Collection Center (ATCC), a commercial cell bank, in December, 2019 and used directly without authentication test by us. The cells were exposed to 200 μM hydrogen peroxide (H_2O_2) (cat. no. 216763, Sigma, St. Louis, MO, USA) and 0.001-1 nM testosterone (cat. no. T1500, Milipore-Sigma, USA) alone or in combination for 24 h and harvested for subsequent analysis. Transfected cells were exposed to 200 μM H_2O_2 and 1 nM testosterone alone or in combination 24h after transfection for 24 h and harvested for subsequent analysis. All experiments were performed with mycoplasma-free cells.

Measurement of senescence-associated β-galactosidase (SA-β-gal) activity. SA-β-gal activity was measured as previously described [22]. Briefly, cells were washed in PBS, fixed in 2% formaldehyde/0.2% glutaraldehyde for 15 min at room temperature, rinsed and stained at 37 °C using reagents from a commercial kit (cat. no. 9860, Cell Signaling, USA) according to the supplier's instructions. 600 cells per treatment were examined and the percentages of SA-β-Gal positive cells were calculated.

Flow cytometry

H9c2 cells were seeded in the wells of 6-well plates at a density of 1×10^5 cells/well, cultured, then washed once with PBS, followed by the addition of cell penetrating probe 2,7-dichlorodihydro-fluorescein diacetate (DCFH-DA, cat. no. D6883, Milipore-Sigma, USA) to a final concentration of 10 μM and incubated at 37 °C for 20 min. The cells were rinsed three times to remove excessive probe and analyzed in a flow cytometer (FACSLyric Research System, BD Biosciences, USA). The results were analyzed using FlowJo software.

Western blotting analysis

Cells were washed with PBS, lysed in RIPA buffer (Vazyme, USA) plus protease inhibitor cocktail (Roche, USA) on ice for 15 min. The cell lysate was centrifuged and the supernatant was mixed with the loading buffer, boiled at 100°C for 5 min, and 50 μg proteins were separated on 12% SDS-PAGE after protein quantification. The proteins were then transferred to PVDF membranes (Invitrogen, USA) and blocked with 5% skim milk powder (BD Biosciences, USA) for 1 h at room temperature. The membranes were incubated with primary antibodies (mouse monoclonal antibodies against SIRT1, 1/1000, cat. no. MA5-27217, IGF1, cat. no. PA5-27207, 1/1000, $p16^{INK4a}$, 1/1000, cat. no. MA5-17142, ThermoFisher, USA) at 4 °C overnight, washed three times with TBST buffer, and reacted to the secondary antibody for 30 min at room temperature. The signal was detected using a ECL Detection kit (cat. no. GERPN2109, Sigma-Aldrich, USA).

Plasmid construction and cardiomyocyte transfection

Plasmids were constructed by Hauda Biotech, Wuhan, China. The silencing RNA sequences of IGF1 were (forward): ACCGGGCACCTGCAATAAAGATACACATC.

ATACTCGAGTATGATGTGTATCTTTATTGCAGGTGCTTTTTTGAATTC, (reverse): GA ATTCAAAAAAGCACCTGCAATAAAGATACACATCATACTCGAGTATGATGTGTATCTT TATTGCAGGTGCCCGGT; the silencing RNA sequences of SIRT1 were (forward): ACCGGG ATGCTGTGAAGTTACTGCTACTCGAGTAGCAGTAACTTCACAGCATCTTTTTTGAA.

TTC; (reverse): GAATTCAAAAAAGATGCTGTGAAGTTACTGCTACTCGAGTAG.

CAGTAACTTCACAGCATCCCGGT. For transfection, H9c2 cells in the exponential phase of growth were plated in the wells of six-well plates and cultured overnight. The cells were then transfected with overexpression vector (50 nM), siRNAs (50 nM), or their negative controls (50 nM) using Lipofectamine 2000 (Invitrogen, CA, USA) for 24 h according to the manufacturer's protocol. Transfected cells were then exposed to 200 μM HP for 24 h in medium containing 5% FBS and harvested to measure the expression of IGF1 or SIRT1 using real-time polymerase chain reaction (qRT-PCR) and Western blot analysis.

Quantitative real-time PCR (qRT-PCR). After total RNA was extracted using the TRIzol reagent (Qiagen, USA) and reversely transcribed into cDNA using random primers and purified using the QIAquick PCR extraction kit (cat. no. 28104, Qiagen, USA). Primers used in the qRT-PCR are listed in Table 1. PCR was performed in a 10 μL reaction volume containing 0.4 μL50 × Rox Reference Dye, 2 μL each primer, 2 μL cDNA and 3.6 μL nuclease-free water. The PCR program included heating at 95 °C for 10 min followed by 40 cycles of 95 °C for 10 s, 55 °C for 20 s and then 72 °C for 35 s. The Ct values were determined with three biological replicates with three technical replicates for each value. The expression level relative to the reference gene (GAPDH) was calculated using the $2^{-\Delta\Delta Ct}$ method [23].

Statistical analysis

Results are expressed as means ± SE. Statistical comparisons among multiple groups were analyzed by one-way ANOVA with the Tukey post hoc test using the GraphPad Prism 7.0 software. A p-value ≤ 0.05 was considered statistically significant.

Table 1. Primers used in qRT-PCR

Gene	Primer	Primer sequence
IGF	Forward	5'- CTCTTCAGTTCGTGTGTGGAGAC
	Reverse	5'- CAGCCTCCTTAGATCACAGCTC
SIRT1	Forward	5'- TAGACACGCTGGAACAGGTTGC
	Reverse	5'- CTCCTCGTACAGCTTCACAGTC
ACTA1	Forward	5'-AGGTCATCACCATCGGCAACGA
	Reverse	5'-GCTGTTGTAGGTGGTCTCGTGA
MYH7	Forward	5'-GGAGTTCACACGCCTCAAAGAG
	Reverse	5'-TCCTCAGCATCTGCCAGGTTGT
SERCA2	Forward	5'-GGACTTTGAAGGCGTGGATTGTG
	Reverse	5'-CTCAGCAAGGACTGGTTTTCGG
MYH6	Forward	5'-GCTGGAAGATGAGTGCTCAGAG
	Reverse	5'-CCAGCCATCTCCTCTGTTAGGT
GAPDH	Forward	5'-GTCTCCTCTGACTTCAACAGCG
	Reverse	5'-ACCACCCTGTTGCTGTAGCCAA

RESULTS

Testosterone protects cardiomyocytes against H_2O_2-induced aging

To induce cardiac aging, H_2O_2 was used to treat H9c2 cells. Analysis showed that the senescence measured using the percentage of SA-β-gal-positive cells and the levels of ROS and $p16^{INK4a}$ increased significantly after exposure to H_2O_2 (Fig. 1A, B and C, $P < 0.01$). However, aging was

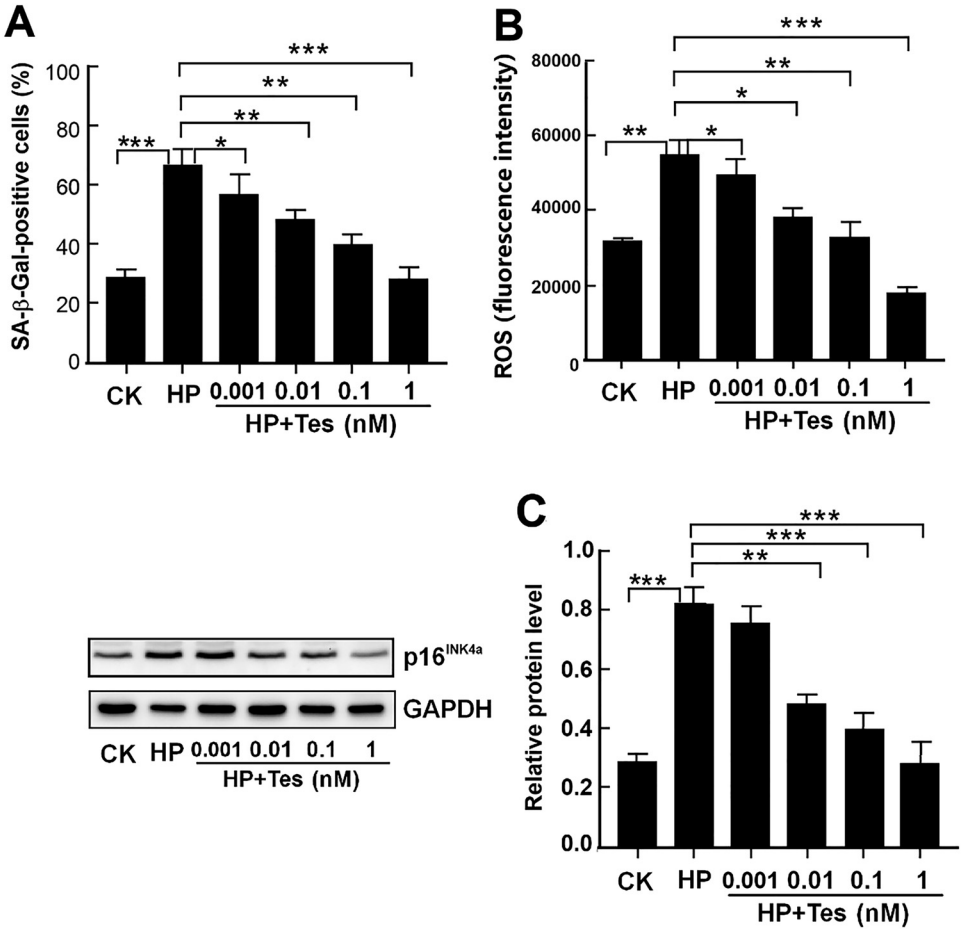

Fig. 1. Effect of testosterone (Tes) on hydrogen peroxide (HP)-induced senescence in cultured H9c2 cells. Cells were exposed to 200 μM HP and 0.001-1 nM Tes alone or in combination for 24 h and harvested for subsequent analysis. A. Percentage of SA-β-gal-positive cells; B. ROS level measured after DCFH-DA staining. C. Left panel: representative Western blots of p16 ^{INK4A}, right panel: protein level. CK, untreated H9c2 cells, HP, HP -treated cells, HP + Tes, cells exposed to HP and different concentrations of Tes. *, ** and *** denote $P < 0.05$, 0.01 or 0.001 between the values under the bars. All experiments were independently repeated three times

reduced when the cells were co-treated with testosterone, and the reduction increased as the concentration of testosterone was increased from 0.001 to 1 nM (Fig. 1A, B and C). Since 1 nM testosterone generated the greatest protection, this concentration was used in all subsequent experiments.

Testosterone upregulates IGF1 and SIRT1 expressions

To investigate whether IGF1 and SIRT1 are involved in H_2O_2-induced senescence, the mRNA and protein levels of IGF1 and SIRT1 were quantified. As illustrated in Fig. 2A and B, H_2O_2 treatment resulted in significant reductions in the levels of IGF1 and SIRT1 both at mRNA and protein levels as compared with the untreated cells ($P < 0.05$ or $P < 0.01$). However, these reductions were fully offset by co-treatment with 1 nM testosterone. On the other hand,

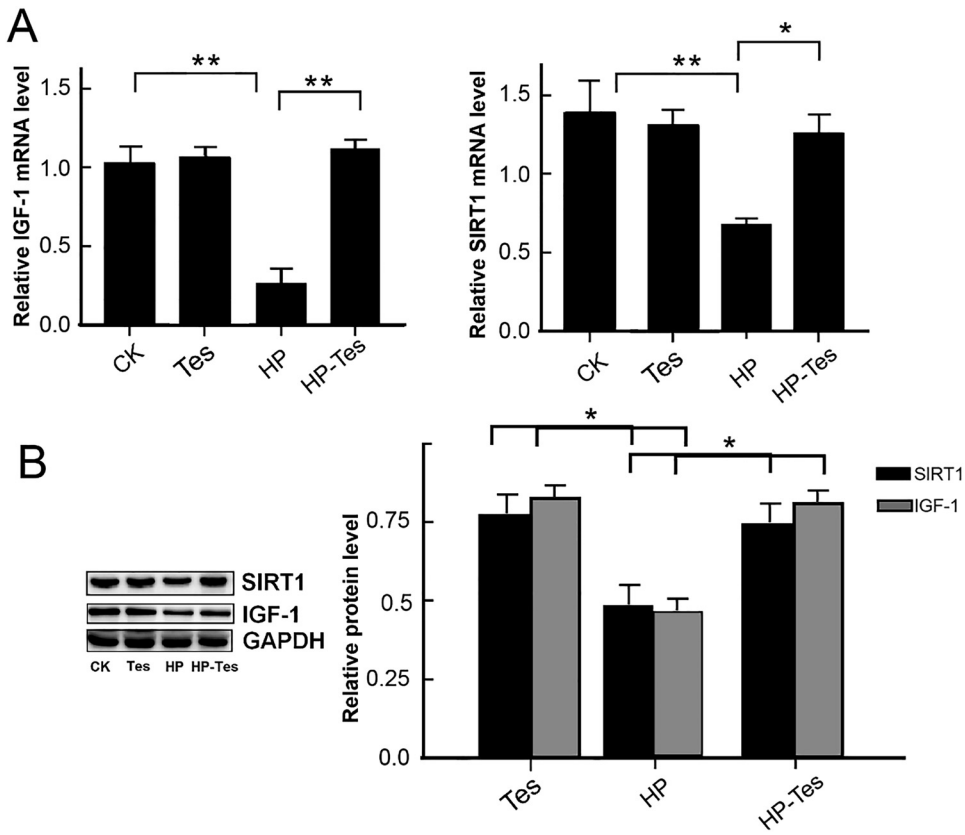

Fig. 2. Expression of IGF1 and SIRT1 in H9c2 cells after hydrogen peroxide (HP) and testosterone (Tes) treatment. Cells were exposed to 200 μM HP and 1 nM Tes alone or in combination for 24 h and harvested for subsequent analysis. A. Relative mRNA level; B. Left panel: representative Western blots, right panel: relative protein level. CK, nontreated cells, Tes, Tes-treated cells, HP+Tes, cells exposed to HP and 1 nM Testosterone. *, ** and *** denote $P < 0.05$, 0.01 or 0.001 between the values under the bars. All experiments were independently repeated three times

testosterone alone did not change the expression of IGF1 and SIRT1 in non-treated H9c2 cells (Fig. 2A and B). These findings demonstrated that IGF1 and SIRT1 signaling pathways are involved in the testosterone-mediated protection of H_2O_2-induced cardiomyocyte aging.

IGF1 is involved in cardiomyocyte protection and SIRT1 expression

We then transfected H9c2 cells with overexpressing and silencing constructs. After transfection, the mRNA and protein levels of IGF1 were up-regulated and down-regulated, respectively (Fig. 3A and B). We further examined whether IGF1 has a role in reducing the aging. Analysis showed that overexpression of IGF1 significantly reduced the percentage of SA-β-gal-positive cells and ROS level, whereas silencing of IGF1 significantly increased them (Fig. 4A and B). These data suggested that IGF1 is involved in protection of cardiomyocytes against senescence.

To elucidate the interaction between SIRT1 and IGF1, we investigated whether IGF1 affects the expression of SIRT1. Analysis showed that silencing of IGF1 significantly down-regulated SIRT1expression, whereas overexpression of IGF1 increased SIRT1expression, but the increase was not statistically significant (Fig. 4C), suggesting that the expression of SIRT1 is somewhat regulated by IGF1 in the cells.

SIRT1 is involved in cardiomyocyte protection

We next assessed the role of SIRT1 in testosterone-mediated protection of cardiomyocytes against senescence. After silencing or overexpressing SIRT1, the levels of SIRT1 were decreased or increased significantly at mRNA and protein levels, respectively (Fig. 5A and B). After overexpressing SIRT1, the percentage of SA-β-gal-positive cells and ROS level were significantly reduced in the H_2O_2-treated cells, whereas silencing SIRT1 significantly increased the percentage and ROS level (Fig. 6A and B).

To elucidate the interaction between SIRT1 and IGF1, we investigated whether SIRT1 affects the expression of IGF1. Analysis showed that overexpression and silencing of SIRT1

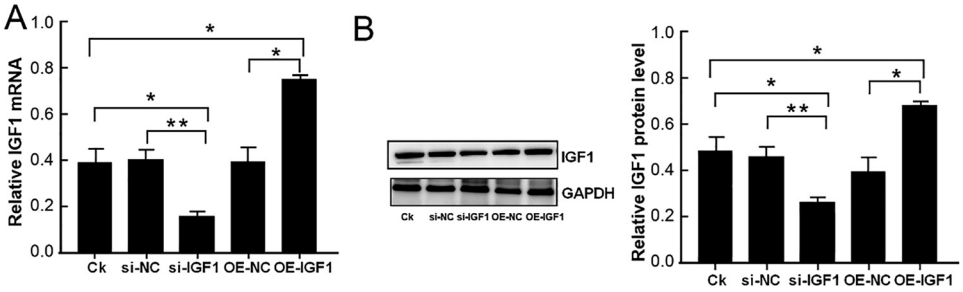

Fig. 3. IGF1 expression in hydrogen peroxide (HP)-treated H9c2 cells after transfection with overexpressing and silencing constructs. Cells were transfected and exposed to 200 μM HP for 24 h and harvested for subsequent analysis. A, left panel: relative mRNA levels, B, middle panel: representative Western blots and relative protein content. CK, non-transfected cells, si-NC, negative control vector for silencing, si-IGF1, IGF1 silencing vector. OE-NC, negative control vector for overexpression, OE-IGF1, IGF1 overexpression construct. *, ** and *** denote $P < 0.05$, 0.01 or 0.001 between the values under the bars. All experiments were independently repeated three times

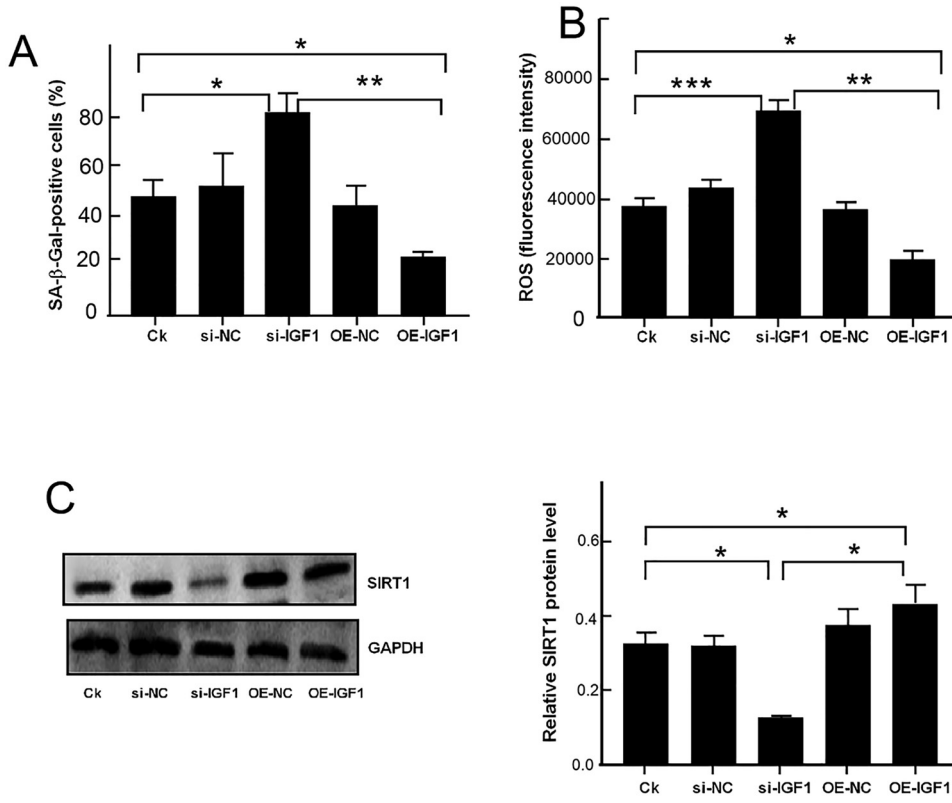

Fig. 4. Reduction of senescence and regulation of SIRT1 expression by IGF1 in hydrogen peroxide (HP)-treated cells. Cells were exposed to 200 μM HP for 24 h and harvested for subsequent analysis. A. Percentage of SA-β-gal-positive cells; B. ROS levels. C. Left panel: representative Western blots, right panel: relative protein levels. CK, non-transfected cells, si-NC, negative control vector for silencing, si-IGF1, IGF1 silencing vector. OE-NC, negative control vector for overexpression, OE-IGF1, IGF1 overexpression construct. *, ** and *** denote $P < 0.05$, 0.01 or 0.001 between the values under the bars. All experiments were independently repeated three times

significantly up-regulated or down-regulated IGF1expression, respectively (Fig. 6C), suggesting that the expression of IGF1 is regulated by SIRT1 in the cardiomyocyte cells.

IGF1 and SIRT1 pathways are involved in testosterone-mediated senescence protection

To further confirm that the IGF1 and SIRT1 pathways are involved in the protection of H9c2 cells from H_2O_2-induced aging by testosterone, IGF1 and SIRT1 knockdown H9c2 cells were treated with H_2O_2 and testosterone. Both SA-β-gal-positive cells and ROS level assessments showed that while senescence was induced after H_2O_2 treatment in the knockdown H9c2 cells with higher SA-β-gal-positive cells and ROS level as compared to non-treated knockdown cells, it was not attenuated by co-treatment with testosterone in both IGF1 and SIRT1 knockdown

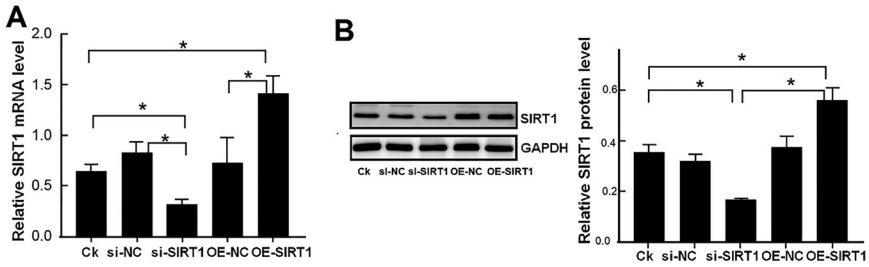

Fig. 5. SIRT1 expression in hydrogen peroxide (HP)-treated H9c2 cells after transfection with overexpressing and silencing constructs. Cells were transfected and exposed to 200 μM HP for 24 h and harvested for subsequent analysis. A. Relative mRNA levels, B. Left panel, representative Western blots, right panel, relative protein content. CK, non-transfected cells, si-NC, negative control vector for silencing, si-SIRT1, SIRT1 silencing vector, OE-NC, negative control vector for overexpression, OE-SIRT1, SIRT1 overexpressing construct. *, ** and *** denote $P < 0.05$, 0.01 or 0.001 between the values under the bars. All experiments were independently repeated three times

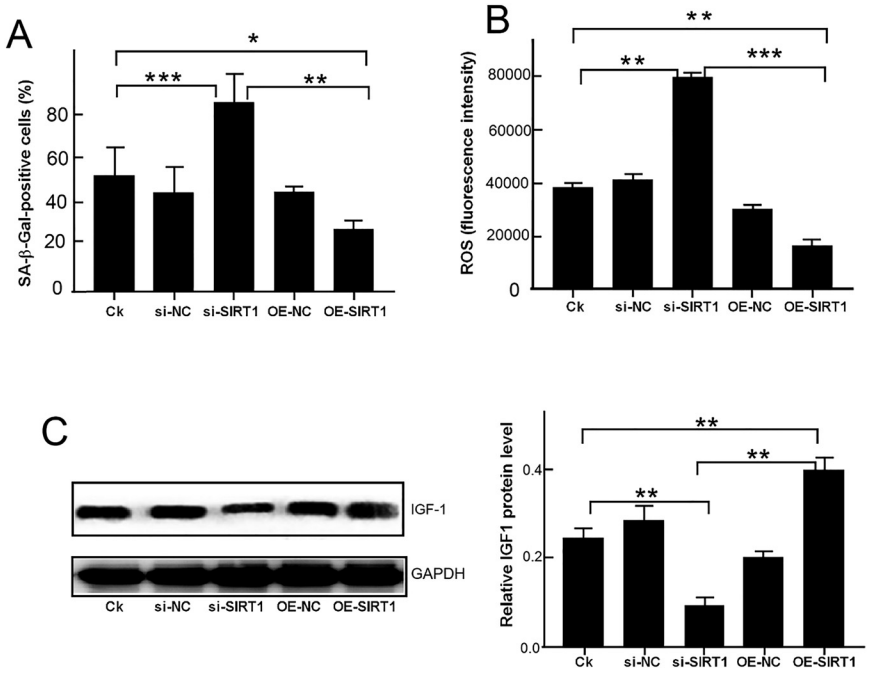

Fig. 6. Reduction of senescence and regulation of IGF1 expression by SIRT1 in hydrogen peroxide (HP)-treated cells. Cells were exposed to 200 μM HP for 24 h and harvested for subsequent analysis. A. Percentage of SA-β-gal-positive cells; B. ROS levels. C. Left panel: representative Western blots, right panel: relative protein levels. CK, non-transfected cells, si-NC, negative control vector for silencing, si-SIRT1, SIRT1 silencing vector. OE-NC, negative control vector for overexpression, OE- SIRT1, SIRT1 overexpression construct. *, ** and *** denote $P < 0.05$, 0.01 or 0.001 between the values under the bars. All experiments were independently repeated three times

H9c2 cells (Fig. 7A–D), suggesting that IGF1 and SIRT1 pathways are important for the testosterone-mediated protection.

IGF1 and SIRT1 pathways suppress the H_2O_2-induced fetal cardiac gene program

When myocardial cells age, myocardial stiffness increases and systolic and diastolic functions decrease, which is related to the change in the fetal cardiac gene program [24]. This process involves the upregulation of cardiac contractile protein isoforms such as α-myosin heavy chain 7 (MYH7) and α-skeletal actin (ACTA-1) as well as the downregulation of α-myosin heavy chain (MYH6) and sarco/endoplasmic reticulum calcium ATPase-2 (SERCA2) [25, 26]. Our analysis showed that H_2O_2-induced aging increased levels of ACTA1 and MYH7 transcripts when compared to untreated cells, and decreased levels of SERCA2 and MYH6

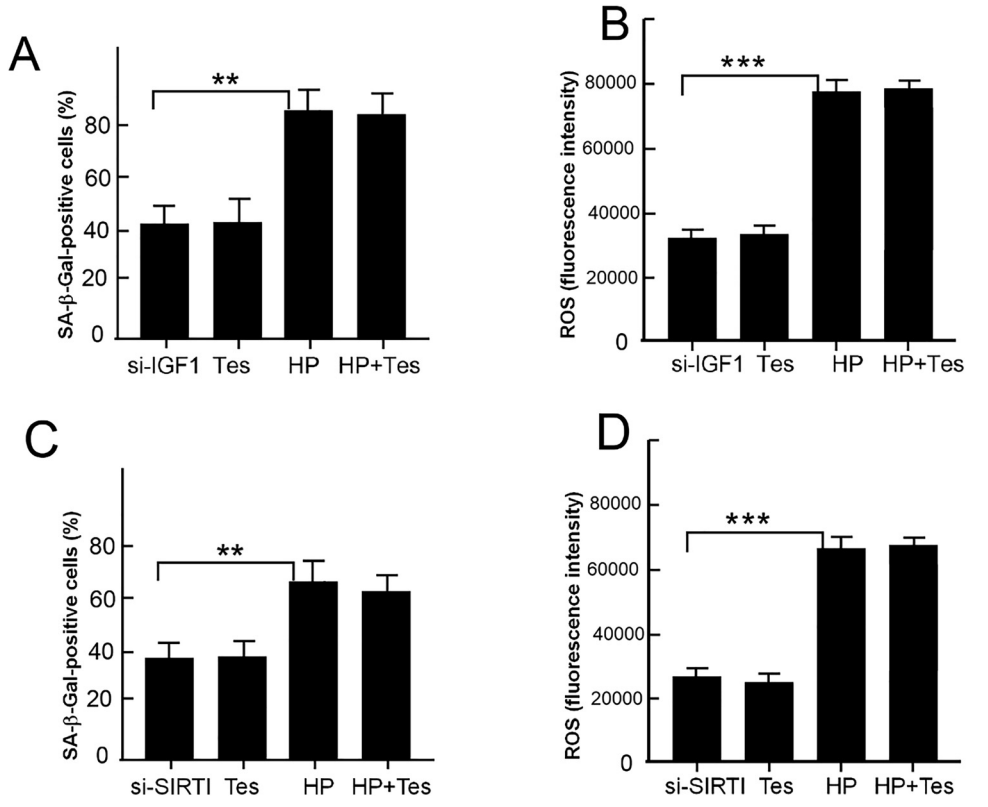

Fig. 7. Loss of protection against senescence by testosterone (Tes, 1 nM) after knockdown of IGF1 and SIRT1. Cells were exposed to 200 μM hydrogen peroxide (HP) for 24 h and harvested for subsequent analysis. A, C, Percentage of SA-β-gal-positive cells; B, D, ROS levels. si-IGF1, si-SIRT1, IGF1 and SIRT1 knockdown H9c2 cells, Tes, HP, HP+Tes, IGF1 and SIRT1 knockdown H9c2 cells treated with Tes and HP alone or in combination. ** and *** denote $P < 0.01$ or 0.001 between the values under the bars. All experiments were independently repeated three times

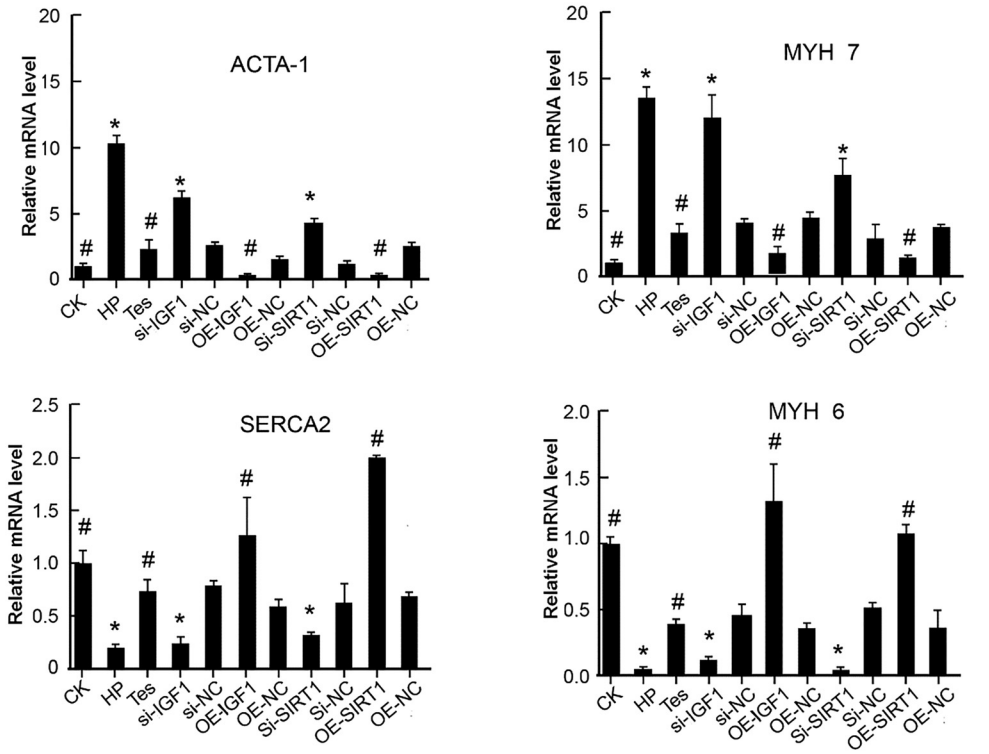

Fig. 8. mRNA levels of the genes in the fetal cardiac gene program after exposure of H9c2 cells to testosterone (Tes, 1 nM) and hydrogen peroxide (HP) or transfected with IGF1 or SIRT1 overexpressing and silencing vectors. Cells were exposed to 200 μM HP for 24 h or transfected for 48 h, and harvested for subsequent analysis. * and # denote $P < 0.05$ vs CK and HP, respectively. All experiments were independently repeated three times

transcripts. Exposure of the cells to testosterone increased ACTA1 and MYH7 expression and decreased SERCA2 and MYH6 levels as compared to H_2O_2-treated cells. When IGF1 or SIRT1 was silenced, similar changes were observed as after H_2O_2 treatment. On the other hand, overexpression of IGF1 or SIRT1 completely prevented the activation of ACTA1 and MYH7 in the fetal gene program caused by H_2O_2 (Fig. 8).

DISCUSSION

Oxidative stress and cellular aging are intertwined in the growth of cardiomyocytes, leading to the occurrence and progression of heart diseases. In this study, we investigated the possible molecular mechanism of testosterone-mediated protection of cardiomyocytes against aging. We found that testosterone can attenuate H_2O_2-induced aging in a dose-dependent manner. Furthermore, we showed that down- and up-regulation of IGF1 and SIRT1 have similar function as H_2O_2 and testosterone in the process. Subsequently we found that the expression of

SIRT1 and IGF is interactively regulated by IGF1 and SIRT1, and knockdown of IGF1 and SIRT1 eliminates the testosterone-mediated aging protection. Our results are consistent with others showing that SIRT1 has cardioprotective effects on oxidative stress-dependent cellular senescence by promoting autophagy and inhibiting apoptosis [27]. Finally, we demonstrated that testosterone and activated IGF1 and SIRT1 pathway could upregulate the expression of cardiomyocyte contraction/diastolic-associated proteins. Our results suggest that testosterone plays a protective role in cell senescence via IGF1 and SIRT1 signaling pathways.

H_2O_2 has been demonstrated to induce cell death by apoptosis and necrosis due to greater accumulation of several inflammatory cytokines [28] and formation of senescence-associated heterochromatin foci [29]. We found that after exposure to H_2O_2, cell aging and the ROS level were significantly increased and the expression level of $p16^{INK4a}$, a key signaling component of the senescence machinery [30, 31] was significantly upregulated, indicating that H_2O_2 had successfully induced senescence in the mouse H9c2 cells in vitro. The senescent cells were used as a cellular cardiac aging model to investigate the effect of testosterone.

After the age of 40, the levels of total testosterone and bioactive testosterone begin to decrease gradually and heart tissue starts to age [32, 33]. Large observational cohort studies have shown that testosterone replacement therapy (TRT) can improve cognitive and heart function, inhibit dyslipidemia, reduce insulin resistance and subsequently the incidence of heart failure in elderly men [34–36]. However, the mechanisms underlying the protective activity are not fully elucidated. It is currently believed that the anti-aging effects of testosterone are exerted through the androgen receptor-mediated pathway [37] and the Gas6/Axl signaling pathway [38]. However, the involvement of these protein mediators could change with age. For example, the vitamin K-dependent protein Gas6 decreases with age and is positively correlated with the testosterone level [39]. On the other hand, SIRT1 is one of the most extensively studied members of the sirtuin family and is implicated in metabolic control and mitochondrial biogenesis, DNA damage repair and intracellular oxidative stress response, which is considered to be another important pathway for testosterone-mediated in anti-cell-senescence. SIRT1 deacetylates a variety of DNA repair proteins such as Ku70, NBS1 and Werner Synaptic Helicase (WRN) to enhance their DNA repair activity and maintain gene integrity [40]. SIRT1 protects the cells from oxidative damage by inhibiting ROS production via suppressing NLRP3 inflammasome activation [41]. Resveratrol, the agonist of SIRT1, attenuates oxidative stress levels in cells and reduces mitochondrial ROS production by up-regulating MnSoD expression and increasing intracellular glutathione (GSH) levels [42]. In this study, we found that testosterone exerts anti-cell-aging effect by up-regulating SIRT1 levels, and this attenuation is also regulated by IGF1. Therefore, SIRT1 is required for the IGF1-dependent protection against the damage. In a previous study, IGF1 was found to have a number of physiological effects, leading to hypertrophy [43], vasodilation and prosurvival effects [44]. In our study, we did not observe any morphological changes of the treated cells, likely due to the short duration and the *in vivo* character of the experiment.

In H_2O_2-treated H9C2 cells, testosterone reduced the percentage of senescent cells and intracellular ROS levels and up-regulated both IGF1 and SIRT1 levels. On the other hand, testosterone had no impact on untreated cells in aging and IGF1 and SIRT1 levels, indicating that testosterone has anti-aging role only in the H_2O_2-treated cells. No morphological changes such as alteration of cell size of H9C2 cells is observed after H_2O_2-treatment. It is likely that such change could be concentration- and exposure time-dependent. At the concentration (200 μM)

and exposure time (24 h) used in this study, H_2O_2 may not be able to generate remarkable cell size change. Up- and down-regulation of IGF1 and SIRT1 generated similar effect on the senescence of H_2O_2-treated H9C2 cells as did H_2O_2 and testosterone, further confirming that the protection of cardiomyocytes by testosterone is mediated via the IGF1and SIRT1 signaling pathways. Previous studies have shown that IGF1 and SIRT1 impinge on the same signaling pathways to exert biological functions [45]. Enhancement of the IGF1 signaling pathway can effectively promote skeletal muscle regeneration, cell survival and renewal [46]. The central component of IGF signaling pathway includes the kinase Akt, which controls both protein synthesis, via the kinases mammalian target of rapamycin (mTOR) and glycogen synthase kinase 3β (GSK3β), and protein degradation, via the transcription factors of the FoxO family [47]. Overexpression of IGF1 can result in the phosphorylation of PDK1, SGK1, and JNK1, which might play an important role in the process of anti-cell-aging. It is likely that IGF1 triggers cytoplasmic phosphorylation events, whose consequent downstream signal transduction leads to elevated SIRT1 expression. The networks of transcriptional factors and coactivators involved in regulating SIRT1 activity in anti-cell-aging are quite intricate. SIRT1 also deacetylates a variety of aging- and apoptosis-related proteins such as p53, Smad7 and FOXO4 to protect cells from senescence and apoptosis induced by various harmful factors and cancers [48–50]. For example, Orimo et al. showed that the overexpression of SIRT1 significantly inhibits the p53 activity caused by high glucose [51]. Kume et al. found that SIRT1 promotes ubiquitin-proteasome degradation by deacetylating Lys60 and Lys70 sites of Smad7, inhibiting the apoptosis of mesangial cells induced by transforming growth factor p (TGF-p) [52]. In addition, the expression of IGF1 and SIRT1 appears to be mutually regulated, although the mechanism is unclear. Previously, IGF1 was shown to increase SirT1 expression through a c-Jun NH(2)-terminal protein kinase 1 (JNK1)-dependent signaling mechanism [18, 19].

Testosterone has been shown to be effective in clinical treatment of cardiovascular disease [53–55]. Our study demonstrates that the IGF1 and SIRT1 pathways are involved in the testosterone-mediated protection of cardiomyocytes against senescence, because in IGF1 and SIRT1 knockout cells testosterone did not show anti-aging activity. Therefore, these pathways may be further explored as therapeutic targets for cardiac aging. In addition, the expression of several fetal genes such as MYH7, ACTA-1 and MYH6 was shown to be influenced by the up- and down-regulations of the pathways, suggesting that these genes and the downstream cellular signaling pathways may also play a role in testosterone-mediated anti-aging activity and could be further explored to reveal the more detailed molecular mechanism underlying the protective effect of testosterone.

There are several limitations to our study. The rat H9C2 cardiomyocyte used in the study may not be able to fully mimic the characteristics of human cardiomyocytes and tissues, and it has not been investigated whether other cardio-protective genes such as adiponectin, UCP-1 and MT-2 were activated after testosterone treatment. The conclusions need to be further verified in animal and human studies.

CONCLUSION

Testosterone can attenuate cardiomyocyte aging induced by H_2O_2 and up-regulate SIRT1 and IGF1 in the senescent cardiomyocyte cells. The expression of IGF and SIRT1 is mutually related and the IGF1 and SIRT1 pathways are likely involved in the protection, because in the IGF1 and

SIRT1 knockout cells testosterone does not protect the cells from H_2O_2-induced aging. Therefore, these pathways may be a potential target to treat heart aging and heart failure.

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ABBREVIATIONS

SIRT1	NAD^+ -dependent deacetylase sirtuin 1
IGF	insulin-like growth factor-1
SA-β-gal	senescence-associated beta-galactosidase
ROS	reactive oxygen species
ACTA1	actin alpha 1
MYH7	myosin heavy chain 7
MYH6	myosin heavy chain 6
SERCA2	sarcoplasmic or endoplasmic reticulum calcium ATPase 2
DMEM	Dulbecco's modified eagle's medium
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
qRT-PCR	real-time polymerase chain reaction
FBS	fetal bovine serum
ANOVA	analysis of variance

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