FOXO1 silence aggravates oxidative stress-promoted apoptosis in cardiomyocytes by reducing autophagy

Yuzhen Ning1,2, Zhiliang Li1 and Zhihong Qiu3

1Vasculocardiology Department, Zhujiang Hospital, Southern Medical University, Guangzhou, 510280, China
2Department of Internal Medicine, the Affiliated Hospital of Inner Mongolia Medical University, Hohhot, 010059, China
3Department of Respiratory Medicine, Hebei Medical University Affiliated North China Petroleum Bureau General Hospital, Renqiu, 062552, China

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ABSTRACT — Mechanisms underlining oxidative stress-induced injury to cardiomyocytes during myocardial infarction (MI) or acute ischemia/reperfusion (I/R) are not well recognized. Forkhead box O (FOXO) transcription factors have been defined as critical mediators of oxidative stress resistance in multiple cell types, but their cardioprotective functions have not been reported previously. In the present study, we investigated the promotion to FOXO1 by the treatment with hydrogen peroxide (H2O2) during the H2O2-induced apoptosis in cardiomyocyte H9c2 cells. We then silenced FOXO1 with FOXO1-specific siRNA, and re-evaluated the H2O2-induced apoptosis. In addition, we also examined the H2O2-induced autophagy and the autophagy induction post FOXO1 silence. Results demonstrated that H2O2 induced a significantly high level of apoptosis in H9c2 cells. Interestingly, the FOXO1 in both mRNA and protein levels were not significantly regulated, however, the phosphorylated form of FOXO1 was significantly promoted in the H2O2-treated H9c2 cells. On the other hand, post the significant knockout of FOXO1 with the transfection with FOXO1-specific siRNA, the apoptosis induction was more significant in H9c2 cells subjected to H2O2. In addition, we found a significantly higher level of autophagy induction in the H2O2-treated H9c2 cells. However, the autophagy was markedly reduced by the knockout of FOXO1. In summary, these data support the critical role for FOXO1 in promoting cardiomyocytes against oxidative stress probably through inducing autophagy.

Key words: Cardiomyocytes, Oxidative stress, FOXO1, Apoptosis, Autophagy

INTRODUCTION

Myocardial ischemia/reperfusion (I/R) injury is on the top list of mortality causes worldwide (Murphy and Steenbergen, 2008). Cardiac oxidative injury during I/R causes damage to cardiomyocytes or even results in cell death followed by such physiopathological processes (Murphy and Steenbergen, 2008) as fibrosis, hypertrophy, and ventricular chamber dilation, and ultimately leads to heart failure (Finkel and Holbrook, 2000; Vanden Hoek et al., 1997; Becker et al., 1999). Overloaded reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide and hydroxyl radicals in oxidative stress deregulate the stress-signaling pathways, and can compromise cell viability and trigger apoptosis (Lee et al., 2009; Chandra et al., 2000), by inducing damage to DNA, protein and lipids (Martindale and Holbrook, 2002). On the other hand, there are multiple markers exerting protective roles against the ROS-mediated cardiac injury, including AMP-activated protein kinase (AMPK), sirtuins (Sirts) (Alcendor et al., 2007; Wang et al., 2009) and the activated phosphoinositide 3-kinase/AKT (PI3K/AKT) signaling (Chen et al., 2013). The moderate Sirt1 expression induces resistance to oxidative stress and apoptosis in heart (Alcendor et al., 2007). It has been found that AMPK deficiency significantly increases MI/R injury in an AMPK-defective mouse model (Wang et al., 2009). Therefore, it is promising to identify anti-oxidative stress pathway for preventing the progression of heart disease.

Forkhead box O (FOXO) transcription factors such as FOXO1 and FOXO3 regulate cell proliferation, metabolism, and aging in a variety of cell types (Huang and Tindall, 2007), including cardiomyocytes (Evans-Anderson et al., 2008; Sengupta et al., 2009). FOXO1- or FOXO3-
deficient mice are embryonic lethal due to impaired vasculogenesis or develop cardiac hypertrophy as adults (Castrillon et al., 2003; Hosaka et al., 2004; Ni et al., 2006). In particular, FOXO1 is crucial for sustaining cardiomyocyte metabolism and cell survival (Puthanveetil et al., 2013), and mediates the oxidative stress response by interacting with Hippo-YAP signaling (Shao et al., 2014). Also, multiple signaling pathways or markers have been found to be implicated in the anti-oxidative stress. SIRT1 activation induced by resveratrol posed marked effect on the FOXO1-associated apoptotic signaling in heart. (Sin et al., 2014). Activation of AMPK inhibits cardiomyocyte hypertrophy by modulating the FOXO1/MuRF1 signaling pathway in vitro (Chen et al., 2010). Moreover, FOXO1 is the most well-recognized member of the FOXO family, functioning as a key mediator of autophagy (Zhang et al., 2013), and mediates the oxidative stress response by promoting autophagy. The present study suggests that FOXO1 plays a key protective role in the anti-oxidative stress response by mediating autophagy.

**MATERIALS AND METHODS**

**Cell culture and treatment**

Human H9c2 cardiomyocytes were purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA), and were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Rockville, MD, USA), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) for normal culture, or supplemented with 2% FBS for maintaining cell at 37°C. For H₂O₂ treatment, H9c2 cells with more than 95% confluence were subjected to DMEM combined with 2% FBS, and were assayed for the knockout efficiency post another inoculation of 24 hr, or were subjected to other treatment.

**Apoptosis assay and caspase 3 activity assay**

The H₂O₂-induced apoptosis of H9c2 cells was assayed with an Annexin V-FITC apoptosis detection kit (Roche Diagnostics, IN, USA). In brief, 3 × 10^5 H9c2 cells post treatment were successively stained with Annexin V-FITC and propidium iodide, and then cells were detected by a FACScan flow cytometer (Bio-Rad, Hercules, CA, USA). Induced cell apoptosis is presented as percent apoptotic cells to total cells. Caspase 3 activity was assayed by the SensoLyte Homogeneous AMC Caspase-3/7 Assay Kit (AnaSpec, Fremont, CA, USA) in the H9c2 cells post the various treatments; cells were lysed in each group, and the cell lysate was added to the assay plate containing the Ac-DEVD-AMC substrate solution, and plates were incubated at 37°C in the dark for 30 min. Then the AMC fluorescence was assayed with an excitation/emission of 342/441 nm using a spectrophotometer (Bio-Rad).

**Western blotting analysis**

H9C2 cells were scratched and were homogenized in an ice-cold Cell Lysis Buffer (Cell Signaling Technology Inc., Danvers, MA, USA), and the lysate was centrifuged at 12,000 × g for 30 min at 4°C. Then the cellular supernatant was collected and was stored at -80°C before use. Protein samples were separated with 10-12% SDS-PAGE gel and were transferred to a PVDF membrane (Millipore, Bedford, MA, USA). Post the blockage of non-specific binding sites, with 2% BSA (Ameresco, Fremont, CA, USA) in the H9c2 cells post the treatment were successively stained with Annexin V-FITC against LC3II/I (Abcam), or against β-actin with an ice-cold Cell Lysis Buffer (Cell Signaling Technology Inc.), against LC3II/I (Abcam), or against β-actin (Sinobioc, Beijing, China). The membrane was then washed for three times with Tris-Buffered Saline and Tween 20 (TBST), and then was incubated with Horseradish Peroxidase (HRP)-linked secondary anti-rabbit antibody for another 1 hr at room temperature. The specific binding band was scanned and quantified according to the band density by Image J software.

**Real-time quantitative PCR**

Total mRNA from H9c2 cells was extracted using the Total RNA Isolation Kit (Ambion, Austin, TX, USA). The cDNA of FOXO1 or β-actin was reverse-transcribed from each sample with the Superscript First-Strand Syn-

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thesis System (Gibco BRL, Grand Island, NY, USA) and with the FOXO1-, or β-actin-specific reverse primer. The mRNA level of FOXO1 was examined by real-time quantitative RT-PCR with the TaqMan system based on a LightCycle 2.0 (Roche, Mannheim, Germany). The primer sequences are available upon request. The amplification was performed with the following course: 45°C, 2 min and 10 min at 95°C; and 40 cycles of 94°C, 10 sec, 60°C, 30 sec. The mRNA level of FOXO1 is presented as a relative value to β-actin with the 2(-Delta Delta C(T)) Method (Livak and Schmittgen, 2001).

Quantitative GFP-LC3 analysis and electron microscopy
Green fluorescence protein (GFP)-LC3 reporter (Sinobio) was used to quantify the H$_2$O$_2$-promoted autophagic vesicles in H9c2 cells. H9c2 cells with more than 85% confluency were transfected with the GFP-LC3 reporter plasmid with Lipofectamine 2000 (Invitrogen). At 6 hr later, the cells were updated with DMEM supplemented with 2% FBS. For the H$_2$O$_2$ treatment, cells were subjected to 200 μM H$_2$O$_2$ for 2 hr. For the FOXO1 silence, cells were transfected with FOXO1-specific siRNA (50 nM) or scrambler oligonucleotides as control (50 nM). After another 24-hr inoculation, the GFP-positive vesicles were visualized and counted under fluorescence microscopy.

Statistical evaluations
Quantitative data are presented as mean ± S.E. Student’s t test was performed to evaluate the difference between two groups on the cell apoptosis, protein levels, AMC intensity, mRNA level, or GFP-LC3 dot number. A p value of 0.05 or less was considered to be significant.

RESULTS

Hydrogen peroxide induces apoptosis in cardiomyocyte H9c2 cells
To investigate the oxidative injury to cardiomyocytes, we treated H9c2 cells with hydrogen peroxide (H$_2$O$_2$), and then examined the apoptosis induction in the treated cells. It was found that H$_2$O$_2$ induced significantly higher levels of apoptotic H9c2 cells (9.73 ± 1.18 vs 4.25 ± 0.52 for 24 hr post treatment (H.P.T.), 17.78 ± 1.80 vs 6.16 ± 0.75 for 48 hr post treatment (H.P.T.), p < 0.01 respectively) (Fig. 1A). We then analyzed the apoptosis-associated markers such as caspase 3 and its substitute, Poly ADP ribose polymerase (PARP) with western blotting (Fig. 1B). It was demonstrated that more procaspase 3 were cleaved into active caspase (Fig. 1C) at both 24 and 48 hr post treatment (p < 0.01 respectively). Also, more PARP were lyzed by the active caspase 3 in the H$_2$O$_2$-treated H9c2 cells (p < 0.01 respectively for either 24 or 48 H.P.T., Fig. 1D). In addition, a fluorescence-based assay for caspase 3 activity confirmed the promotion to higher caspase 3 by the H$_2$O$_2$ treatment (p < 0.01 respectively for either 24 or 48 H.P.T., Fig. 1E). Taken together, these results confirm the apoptosis induction by H$_2$O$_2$ treatment in cardiomyocyte H9c2 cells.

FOXO1 silence aggravates the H$_2$O$_2$-induced apoptosis in H9c2 cells
To investigate the role of FOXOs in the oxidative stress damage or anti-oxidative stress progress in H9c2 cells, we then silenced the FOXO1 in H9c2 cells, and then re-evaluated the apoptosis induction in the H$_2$O$_2$-treated H9c2 cells. FOXO1-specific siRNA was utilized to silence FOXO1. As shown in Fig. 3A, transfection with 25 or 50 nM siRNA-FOXO1 markedly downregulated the mRNA level of FOXO1, causing a 55% (for 25 nM, p < 0.01) or 75% (for 75 nM, p < 0.001) reduction. However, the apoptosis induction by the treatment with 200 μM H$_2$O$_2$ was significantly aggravated by the transfection with 25 or 50 nM siRNA-FOXO1 (Fig. 3B, p < 0.05 respectively). Next, the levels of activated caspase 3 and lyzed PARP in the H$_2$O$_2$-treated and siRNA-FOXO1-transfected H9c2 cells were also analyzed with western blotting. As shown respectively in Figs. 3D and
The cleaved caspase 3 and lyzed PARP were upregulated to higher levels (p < 0.05 or p < 0.01). The assay for caspase 3 activity reconfirmed the promotion to caspase 3 activity by the FOXO1 silence in the H_{2}O_{2}-treated H9c2 cells (p < 0.05 for either 25 or 50 nM). Therefore, we confirmed the deterioration by the FOXO1 silence to the H_{2}O_{2}-promoted apoptosis in H9c2 cells.

Fig. 1. Hydrogen peroxide induces apoptosis in cardiomyocyte H9c2 cells. A: Apoptotic H9c2 cells induced by the treatment with 200 μM H_{2}O_{2} for 24 or 48 hr; B: Western blot analysis of cleaved caspase 3 (active form) and the lyzed PARP, which was catalyzed by the active caspase 3 in H9c2 cells treated with 200 μM H_{2}O_{2} for 24 or 48 hr; C and D: Relative level of activated caspase 3 (Cleaved CASP 3) (C) or of lyzed PARP (D) to β-actin in the H_{2}O_{2}-treated or in the control H9c2 cells; E: Caspase 3 activity, evaluated by AMC fluorescence in the H_{2}O_{2}-treated H9c2 cells. Experiments were repeated independently in triplicate. **p < 0.01.
OXIDATIVE STRESS: INDUCTION OF AUTOPHAGY IN CARDIOMYOCYTES

**FOXO1 silence reduces the H₂O₂-induced autophagy in H9c2 cells**

Oxidative stress has been shown to induce autophagy in cardiomyocytes (Essick et al., 2013). In order to deduce the role of FOXO1 in the oxidative stress-induced autophagy in cardiomyocytes, we examined the autophagy induction in the H₂O₂-treated H9c2 cells, and then evaluated the regulation of FOXO1 in such autophagy induction. As indicated in Fig. 4A (Column 2 vs Column 1), the treatment with 200 μM significantly induced autophagic vesicle formation (GFP-positive dots) (Fig. 4B, p < 0.01). Western blotting (Fig. 4C) demonstrated that the conversion of the I subunit to II unit of microtubule-associated proteins 1A/1B light chain 3A (LC3) was upregulated by the H₂O₂ treatment; the ratio of LC3II to LC3I was 0.36 ± 0.042 in the H₂O₂-treated H9c2 cells, significantly higher than 0.065 ± 0.008 in the control H9c2 cells (Fig. 4D, p < 0.01). Then we re-evaluated the autophagy induction in the H₂O₂-treated H9c2 cells, post the transfection with 50 nM siRNA-FOXO1. Interestingly, the H₂O₂-induced autophagic vesicles were significantly reduced by the siRNA-FOXO1 transfection, compared to the siRNA-Con transfection (p < 0.05, Fig. 4A). And the conversion of LC3I to LC3II was also markedly reduced by the siRNA-FOXO1 transfection (Column 3 vs Column 2 in Fig. 4D, p < 0.05). Therefore, we confirmed the reduction of H₂O₂-induced autophagy in H9c2 cells by the FOXO1 silence with FOXO1-specific siRNA trans-
Fig. 3. FOXO1 silence with siRNA aggravated the H$_2$O$_2$-induced apoptosis in H9c2 cells. A: Relative mRNA level of FOXO1 in the H9c2 cells which were transfected with siRNA-FOXO1 or siRNA-Con with a concentration of 25 or 50 nM; B: Influence of the siRNA-FOXO1 or siRNA-Con transfection with a concentration of 25 or 50 nM on the H$_2$O$_2$-induced apoptosis; C: Western blot analysis of active caspase 3 (cleaved form) and the lyzed PARP in H9C2 cells treated with 200 μM H$_2$O$_2$ and were transfected with 25 or 50 nM siRNA-FOXO1 or siRNA-Con; D and E: Relative level of cleaved caspase 3 (D) or lyzed PARP (E) in the H$_2$O$_2$-treated and siRNA-transfected H9C2 cells; F: AMC fluorescence assay for caspase 3 activity in the H$_2$O$_2$-treated and siRNA-transfected H9C2 cells. All results were repeated independently in triplicate. * p < 0.05, ** p < 0.01, or *** p < 0.001.
FOXO1 regulates OS-promoted autophagy and apoptosis in cardiomyocytes

**DISCUSSION**

It has been well recognized that overproduced ROS in oxidative stress reduce cell viability and induce apoptosis (Lee et al., 2009; Chandra et al., 2000). In the present study, we confirmed the apoptosis induction by hydrogen peroxide in cardiomyocyte H9c2 cells, significantly higher level of apoptotic H9c2 cells, higher levels of activated caspase 3 (cleaved caspase 3) and more lysed PARP.
ly, we found that the phosphorylated form of FOXO1 was
of FOXO1 in both mRNA and protein levels. Interest-
more lyzed PARP. Thus, we con
el of caspase 3 activity, with more cleaved caspase 3 and
more oxidative stress (Kops et al., 2002; Chiribau et al., 2008). Moreover, FOXOs are also regulated by ROS, and hydro-
gen peroxide treatment also regulated the acetylation or
decetylation of FOXO proteins (Brunet et al., 2004; van
der Horst et al., 2004). The present study did not find sig-
ificant regulation by the H2O2 treatment of the expression
of FOXO1 in both mRNA and protein levels. Interest-
ly, we found that the phosphorylated form of FOXO1 was
markedly promoted by the H2O2 treatment in H9c2 cells.

Posttranslational modification of FOXOs by phos-
phorylation or acetylation is well-defined for modulating
gene expression (van der Horst and Burgering, 2007),
FOXO1 activity is repressed through the phosphoryla-
tion of FOXO1, leading to FOXO1 transfer into the cyto-
plasm (Brunet et al., 1999). FOXO1 phosphoryla-
tion on the cardiomyocytes has not
been addressed. The present study clearly demonstrated
that the silence by siRNA transfection markedly aggravat-
ed the H2O2-induced apoptosis, by promoting higher lev-
els of caspase 3 activity, with more cleaved caspase 3 and
more lyzed PARP. Thus, we confirmed the protective role
of FOXO1 against the H2O2-induced apoptosis in cardio-
ymocytes.

Recent evidence has revealed that FOXO1 promotes
receptorization (Sengupta et al., 2011; Zhao et al., 2010). FOXO1
induces the expression of the RAS-related GTP-

binding protein RAB7A in mouse cardiomyocytes, and
mediates the fusion of mature autophagic vesicles with
lysosomes (Harilaran et al., 2010). The chemical inhibi-
tion of FOXO1 via the PI3K/Akt/FOXO1 axis results from
defective autophagy in human glioblastoma stem cells.
Therefore, the regulatory role of FOXO1 in autophagy
might be important mechanism for the anti-apoptosis
effect against oxidative stress in cardiomyocytes.

In the present study, we found a markedly-upregulated
autophagy in cardiomyocytes by the H2O2 treatment. And
what’s more, the FOXO1 silence significantly inhibit-
ed the H2O2-induced autophagy in H9c2 cardiomyocytes,
by reducing the formation of autophagic vesicles and by
downregulating the conversion of LC3I to LC3II. Given
the differential regulation by the FOXO1 silence on the
H2O2-induced apoptosis and autophagy in H9c2 cardio-
ymocytes, we speculated that FOXO1 should exert a key
mediatory role between apoptosis and autophagy in cardi-
omyocytes subjected to H2O2 treatment.

In conclusion, the current study confirmed the induc-
tion of apoptosis and autophagy in H9c2 cardiomyocyte
cells by H2O2 treatment, along with the marked upregu-
lation of FOXO1. In addition, FOXO1 silence aggravat-
ed the H2O2-induced apoptosis, but reduced the H2O2-
induced autophagy. This implies the key mediatory role
between apoptosis and autophagy in cardiomyocytes
under oxidative stress.

Conflict of interest---- The authors declare that there is
no conflict of interest.

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