Apurinic/apyrimidinic endonuclease/redox factor 1 (APE1) alleviates myocardial hypoxia-reoxygenation injury by inhibiting oxidative stress and ameliorating mitochondrial dysfunction

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Received October 25, 2017; Accepted February 16, 2018

DOI: 10.3892/etm.2019.7212

Abstract. Oxidative stress and mitochondrial dysfunction are considered to be activators of apoptosis and serve a pivotal role in the pathogenesis of myocardial ischemia-reperfusion (MI/R) injury. Apurinic/apyrimidinic endonuclease/redox factor 1 (APE1) is a multifunctional protein that processes the cellular response to DNA damage and oxidative stress. Little is known about the role of APE1 in the pathogenesis of MI/R injury. The aim of the present study was to investigate the effects of APE1 on hypoxia-reoxygenation (H/R)-induced H9c2 cardiomyocyte injury and the underlying mechanism responsible. It was demonstrated that H/R decreased cell viability and increased lactic dehydrogenase (LDH) release, as well as reducing APE1 expression in H9c2 cells. However, APE1 overexpression induced by transfection with APE1-expressing lentivirus significantly increased H9c2 cell viability, decreased LDH release, decreased apoptosis and reduced caspase-3 activity in H/R-treated H9c2 cells. APE1 overexpression ameliorated the H/R-induced increases in reactive oxygen species and NAPDH oxidase expression, as well as the decreases in superoxide dismutase activity and glutathione expression. Furthermore, APE1 overexpression increased mitochondrial membrane potential and ATP production, stabilized electron transport chain activity (as illustrated by increased NADH-ubiquinone oxidoreductase, succinate dehydrogenase, coenzyme Q-cytochrome c oxidoreductase and cytochrome c oxidase activities) and decreased the ratio of B-cell lymphoma 2-associated X protein/B-cell lymphoma 2 in H/R, improving mitochondrial dysfunction. In conclusion, the results of the present study suggest that APE1 alleviates H/R-induced injury in H9c2 cells by attenuating oxidative stress and ameliorating mitochondrial dysfunction. APE1 may therefore be used as an effective treatment for MI/R injury.

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Key words: apurinic/apyrimidinic endonuclease/redox factor 1, apoptosis, myocardial hypoxia-reoxygenation injury, mitochondrial dysfunction, oxidative stress

Introduction

Myocardial ischemia-reperfusion (MI/R) injury is one of the leading causes of morbidity and mortality worldwide, particularly in developed countries (1). Although a number of pharmacological agents have been developed for the treatment of myocardial disease, few effective strategies for MI/R injury prevention are available (2). It is therefore necessary to elucidate the underlying molecular mechanism associated with the development of MI/R injury in order to develop an effective treatment. Oxidative stress serves a critical role in the pathophysiology of MI/R injury (3) and reactive oxygen species (ROS) overproduction caused by I/R injury induces oxidative stress, leading to DNA injury and changes in protein kinase activation, which in turn trigger apoptosis, cardiac dysfunction and even heart failure (4-6). Mitochondrial dysfunction occurs in the early stage of ischemia and may result in mitochondrial calcium overload, mitochondrial membrane depolarization, mitochondrial permeability transition pore (mPTP) opening, the release of pro-apoptotic proteins and cytochrome c and even cardiomyocyte death (7,8). As such, molecules and compounds that are able to attenuate oxidative stress and improve mitochondrial dysfunction may be potential candidates for cardioprotective treatment following MI/R injury.

Apurinic/apyrimidinic endonuclease 1 (APE1) is a multifunctional protein that serves a vital role in the cellular response to DNA injury and oxidative stress (9). APE1 also serves a role in controlling cellular processes, including apoptosis, proliferation, inflammation and angiogenesis, and is present in survival pathways (10,11). A number of studies have reported that APE1 expression is associated with a number of pathological conditions, including cancer, neurodegenerative diseases and cardiovascular disease (9,11,12). Leak *et al* (13) revealed that decreased APE1 expression and endonuclease activity cause oxidative base lesions and apurinic/apyrimidinic sites, triggering ischemic cell death. The role of APE1 in the cardiovascular system (14,15), as well as cytoplasmic and mitochondrial localizations of this protein have also been reported (16,17). APE1 may be associated with decreased mitochondrial fragmentation and may improve mitochondrial function (18). Mitochondrial 8-oxoguanine glycosylase, which is able to remove 8-hydroxy-2'-deoxyguanosine to prevent further DNA damage, improves mitochondrial function and decreases apoptotic events in H9c2 cells under oxidative stress

conditions (19). Based on the results of the aforementioned studies, it was hypothesized that APE1 may serve a therapeutic role in MI/R injury, potentially via regulating oxidative stress and mitochondrial function.

The aim of the present study was to demonstrate the effects of APE1 on H9c2 cells with hypoxia/reoxygenation (H/R)-induced injury and to explore the underlying mechanisms. The results of the present study demonstrate that APE1 is able to reduce oxidative stress and maintain mitochondrial function in H/R conditions *in vitro*, effectively protecting cells from MI/R injury.

Materials and methods

Materials and regents. Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). An Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from BD Biosciences (San Jose, CA, USA). Rabbit polyclonal antibodies against APE1 (ALX-210-723-R100) and rabbit monoclonal antibodies against NAPDH oxide 2 (NOX2; ALX-804-196-T050) were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Rabbit polyclonal antibodies against B-cell lymphoma 2 (Bcl-2; ab59348) and rabbit monoclonal antibodies against Bcl-2-associated X protein (Bax; ab32503) were purchased from Abcam (Cambridge, UK). Rabbit monoclonal antibody against tubulin (2146) was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). A JC-1 Mitochondrial Membrane Potential Detection kit was supplied by Beyotime Institute of Biotechnology (Haimen, China). 2',7'-dichlorofluorescein acetyl acetate (DCFH-DA) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The detection kits for superoxide dismutase (SOD; cat. no. A001-1-1) and glutathione (GSH; cat. no. A005) were purchased from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China).

Cell culture and H/R injury model. The H9c2 cardiomyocyte line (rat embryonic cardiomyoblasts) was obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai Institute of Biotechnology, Shanghai, China). Cells were maintained in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin in a humidified atmosphere containing 5% CO_2 at 37˚C. In order to induce H/R injury, the cells were placed in an anaerobic chamber containing 2.5% O_2, 5% CO_2 and 92.5% N_2 at 37˚C for 3, 6, 12 or 24 h, followed by reoxygenation under normoxic conditions (95% air and 5% CO_2) at 37˚C for 6 h as previously described (20). Control cells were maintained under normal culture conditions as above.

Generation and transfection of lentiviruses expressing APE1. APE1 cDNA was first synthesized and constructed into a lentivirus expressing vector using a lentivirus expressing system (cat. no. K532000; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Human wild type APE1 cDNA was cloned into pcDNA3.1-HA vectors (Invitrogen; Thermo Fisher Scientific, Inc.). cDNAs were then cloned into the third generation lentivirus (LV) vector pBOB with XbaI and BamHI sites. LVs expressing APE1 (LV-APE1) or empty vector (LV-Scramble, as controls) amplification were prepared by transient transfection in HEK 293 T cells (cat. no. K1538 Thermo Fisher Scientific, Inc.). Following growth to 80-90% confluence, HEK 293T cells were transfected with recombinant LV-APE1 [virus preservation solution (0.5 μl)/DMEM (1 ml); MOI=5] or LV-Scramble adenovirus at 37˚C for 90 min. The virus solution was then removed and 10% DMEM was added to culture the HEK 293T cells at 37˚C. When cell morphology became rounded, with cells still demonstrating adherence to the wall, the supernatant and cells were collected. Following repeated freezing and thawing three times (37-70˚C) to lyse cells, the supernatant was centrifuged (1,006 x g) at 4˚C, for 5 min and the virus supernatant was collected and stored at -80˚C. Subsequently, the amplified virus (MOI=20) was transfected to H9c2 cells. The cells were treated with a reagent as indicated for further experiments after 24 h. The infection efficiency (95-100%) after 24 h was assessed using western blotting as described later.

MTT assessment of H9c2 cell viability. Cell viability was assessed using an MTT kit. Briefly, H9c2 cells were seeded into 96-well-plates at $2x10^5$ cells/well. Following H/R and LV-Scramble or LV-APE1 transfection, 100 μl of MTT solution (0.5 mg/ml) was added to each well incubated for an additional 4 h at 37˚C. Dimethyl sulfoxide (DMSO, 100 ml/well) was added to dissolve the formazan crystals. Absorbance at 570 nm was measured using an epoch microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The control group was considered to be 100% viable and results were expressed as a percentage of the control group.

Detection of lactate dehydrogenase (LDH) release. LDH levels in the cell supernatant were assessed using a commercially available kit (cat. no. A020-2) according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute). Briefly, cell supernatants were collected, mixed with reagents including matrix buffer, coenzyme 1,2,4-dinitrophenylhydrazine and NaOH solution from the LDH assay kit and incubated for 5 min at room temperature. Absorbance was detected at 450 nm using a microplate reader. Results were expressed as a percentage of the LDH level in the control group.

Measurement of intracellular ROS generation. Intracellular ROS generation was detected by flow cytometry following staining with cell-permeable fluorogenic probe DCFH-DA as previously described (21). Briefly, H9c2 cells ($1x10^5$ cells/well) were seeded into 6-well plates overnight and exposed to H/R for 12 h or transfected with LV-Scramble or LV-APE1 followed by H/R treatment. Cells were stained with the non-fluorescent DCFH-DA probe (10 μM) for 20 min at 37˚C and washed in PBS once. Cells were re-suspended in PBS (500 μl) and the intracellular accumulation of DCF was measured using an Olympus X51 fluorescence microscope (Olympus Corp., Tokyo, Japan, magnification 200x). The fluorescence intensity was analyzed using a flow cytometer. The fluorescence intensity in control group was arbitrarily assigned a value of 100% and the results were calculated as a percentage of the control group.

Measurement of mitochondrial membrane potential (MMP). Decrease in MMP, a marker for mitochondrial dysfunction, is one of the earliest events that result in apoptosis (22). In the

present study, MMP was measured using a JC-1 assay kit as previously described (23). Briefly, H9c2 cells were seeded into 6-well plates at a density of $1x10^5$ cells/well and treated as above. Cells were washed twice with PBS and incubated with JC-1 for 20 min at 37˚C. Carbonyl cyanide m-chlorophenylhydrazone, included in the JC-1 assay kit, was used as a positive control. The fluorescent signals were detected using confocal laser scanning microscopy (LSM 700; Carl Zeiss AG, Oberkochen, Germany) with 530 and 630 nm as red excitation and emission wavelengths, respectively, and 488 and 530 nm as the green excitation and emission wavelengths, respectively. Red fluorescence was attributable to a potential-dependent aggregation in mitochondria. Green fluorescence, reflecting the monomeric form of JC-1, entered into the cytosol after mitochondrial membrane depolarization.

Electron transport chain (ETC) activity chain complex activity and ATP production capacity. ETC activity, including the activity of NADH-ubiquinone oxidoreductase (complex I; cat. no. ab109721), succinate dehydrogenase (complex II; cat. no. ab109908), coenzyme Q-cytochrome c oxidoreductase (complex III; cat. no. ab109905) and cytochrome c oxidase (complex IV; cat. no. ab109911), were analyzed using commercial kits (Abcam) according to the manufacturer's protocol. To measure the complex I, II, III or IV activity, the detergent was added to cells to extract transmembrane proteins. Absorbance was measured on a Spetramax M5 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). ATP generation was detected using a CellTiter-Glo luminescent ATP assay kit (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol. The supernatant was collected and added to the substrate solution. Luminescence was measured using a luminometer (Thermo Fisher Scientific Inc.).

Flow cytometric analysis of apoptosis. Apoptosis was analyzed using an Annexin V-FITC apoptosis detection kit followed by flow cytometry according to the manufacturer's protocol. In brief, H9c2 cells were transfected with LV-Scramble or LV-APE1 followed by H/R treatment. At the last stage of the treatment, floating and attached cells were harvested and washed twice with cold PBS. The pellets were resuspended in 500 μl 1X binding buffer (provided in the kit) and incubated with Annexin V-FITC (5 μl) and propidium iodide (PI; 10 μl) for 5 min in the dark at room temperature. Apoptosis was then analyzed using a flow cytometer. The number of cells in early (Annexin V^+/PI^-) and late (Annexin V^+/PI^+) apoptosis was expressed as a percentage.

Measurement of SOD activity and GSH level. H9c2 cells were grown on 6-well plates and treated as above. Cells were harvested and cytosolic protein was extracted in RIPA lysis buffer (Beyotime Institute of Biotechnology; cat. no. P0013B). Protein concentration was quantified using a Bradford Protein Assay (cat. no. 5000001; Bio-Rad Laboratories, Inc., Hercules, CA, USA). SOD activity was measured using a total SOD assay kit with WST-8 according to the manufacturer's protocol. The GSH expression was detected using a GSH assay kit according to the manufacturer's protocol. The absorbance of each final solution was measured at 450 (SOD) and 340 nm (GSH). SOD activity was calculated according to the SOD standard control and expressed as U/mg protein. GSH expression was expressed as nmol/mg protein.

Western blot analysis. H9c2 cells were treated as described, collected and lysed in RIPA lysis buffer to measure the expressions of APE1, NOX2, Bax and Bcl-2 proteins. The protein concentration was determined using a protein assay kit according to the manufacturer's protocol (Bio-Rad Laboratories, Inc.). Equal amounts of protein (30 μg) were separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Following blocking with 5% nonfat milk for at room temperature 2 h, membranes were incubated with the primary antibodies against APE1 (1:1,000), NOX2 (1:1,000), Bax (1:2,000), Bcl-2 (1:2,000) or tubulin (1:2,000) overnight at 4˚C, followed by incubation with horseradish peroxidase-conjugated second antibodies (cat. no. 7076S; 1:5,000; Cell Signaling Technology, Inc.) at 37˚C for 1 h. Bands were visualized using an enhanced chemiluminescence system (Beyotime Institute of Biotechnology), scanned using a Bio-Rad gel imaging system (Bio-Rad Laboratories, Inc.) and analyzed using Quantity One software v4.62 (Bio-Rad laboratories, Inc.).

Statistical analysis. All experiments were repeated independently at least for three times. Data are expressed as the mean ± standard division and analyses were performed using one-way analysis of variance followed by the least significant difference (LSD) test using GraphPad Prism v.6 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

H/R treatment induces cytotoxicity and decreases APE1 expression in H9c2 cardiomyocytes. To assess the potential effect of APE1 on MI/R injury, APE1 expression in H9c2 cells treated with H/R was measured. MTT results revealed that viability was significantly reduced in cells exposed to 6, 12 or 14 h hypoxia followed by 6 h reoxygenation compared with the control (Fig. 1A). LDH release is a biomarker of cell injury (24) and it was observed that H/R treatment significantly increased LDH release in a time-dependent manner (Fig. 1B). The results of western blotting revealed that APE1 expression decreased with increased hypoxia duration, reaching the lowest level when cells were exposed to 12 h of hypoxia followed by 6 h of reoxygenation (Fig. 1C and D). Based on these results, 1 h hypoxia followed by 6 h reoxygenation was selected for use in subsequent experiments. These results suggest a potential role of APE1 in MI/R injury.

APE1 overexpression alleviates H/R-induced cytotoxicity and apoptosis in H9c2 cardiomyocytes. To further investigate the role of APE1 in H/R-induced injury in H9c2 cardiomyocytes, cells were transfected with LV-APE1 or LV-Scramble. The results of western blotting revealed that LV-APE1 transfection significantly increased the expression of APE1 protein compared with LV-Scramble transfection in the control and H/R treatment groups (Fig. 2A and B). Cell viability was also significantly increased in LV-APRE1 transfected H9c2 cells compared with LV-Scramble transfected H9c2 cells under

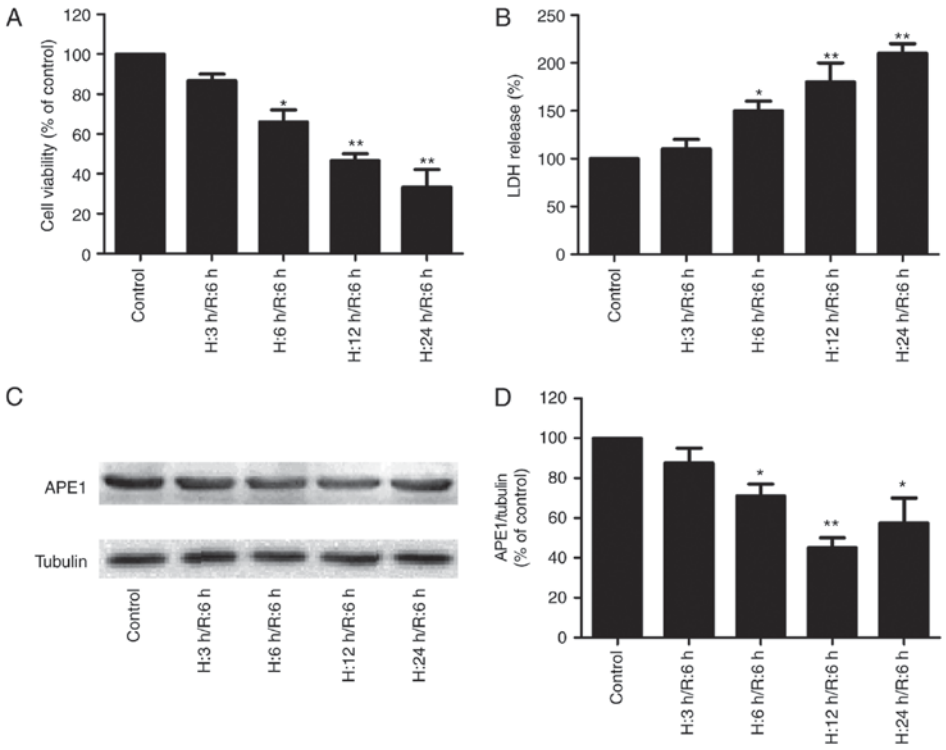

Figure 1. Effects of H/R on cell viability, LDH release and APE1 expression in H9c2 cells. H9c2 cells were exposed to hypoxia for 3, 6, 12 or 24 h and reoxygenated for 6 h. (A) Cell viability was detected using an MTT assay, (B) LDH release in the supernatants was measured using a kit and (C) the expression of APE1 protein was measured using western blotting and (D) quantified. Tubulin was used as a loading control. *P<0.05 and **P<0.01 vs. control group. H/R, hypoxia/reoxygenation; LDH, lactose dehydrogenase; APE1, apurinic/apyrimidinic endonuclease/redox factor 1.

H/R treatment, while LV-APE1 or LV-Scramble had no effect on cell viability in control group (Fig. 2C). In the untreatment group, transfection with LV-APE1 or LV-Scramble had no effect on LDH release, whereas LV-APE1 transfection in the H/R treatment group, significantly decreased LDH release compared with LV-Scramble-transfected cells, indicating that APE1 overexpression reverses H/R-induced H9c2 cell damage (Fig. 2D). The effect of APE1 on apoptosis in H/R-treated H9c2 cells was further investigated using flow cytometry (Fig. 2E). Annexin V-FITC/PI apoptosis detection results revealed that H/R treatment caused a significant increase in apoptosis compared with control cells in LV-Scramble transfection groups (Fig. 2F). However, transfection with LV-APE1 significantly reduced H/R-induced apoptosis compared with the LV-Scramble transfection group in H/R groups (Fig. 2F). No significant differences in cell viability were observed in control cells transfected with LV-APE1 or LV-Scramble. Furthermore, H/R treatment significantly increased the activity of caspase-3 compared with the control in the untreatment and LV-Scramble transfection groups, whereas LV-APE1 transfection significantly decreased the activity of caspase-3 compared with LV-Scramble transfection in the H/R treatment group (Fig. 2G). These results indicate that APE1 overexpression promotes proliferation, attenuates cytotoxicity and inhibits apoptosis in H9c2 cells, alleviating H/R injury.

APE1 overexpression alleviates H/R-induced oxidative stress in H9c2 cardiomyocytes. Oxidative stress serves an important role in the pathogenesis of MI/R injury (25). The effect of APE1 on oxidative stress in H/R conditions was investigated in the present study. In cells transfected with LV-Scramble, H/R treatment significantly increased intracellular ROS production compared with the control group (Fig. 3A and B), while LV-APE1 transfection reduced intracellular ROS levels compared with the LV-Scramble group. NOX2 enzymes serve a critical role in ROS production (26). The results of western blotting revealed that H/R treatment increased the expression of NOX2 protein in the LV-Scramble transfection group, whereas LV-APE1 transfection significantly decreased this upregulation (Fig. 3C and D). The effect of APE1 on SOD activity and GSH expression was also assessed. The results from SOD and GSH detection kits indicated that, under H/R conditions, LV-APE1 transfection significantly increased SOD activity (Fig. 3E) and GSH levels (Fig. 3F). In the control group, no significant differences in SOD activity and GSH expression were observed following transfection with LV-Scramble or LV-APE1. These results suggest that APE1 reverses H/R-induced oxidative stress by attenuating oxidative stress and enhancing antioxidant defense.

APE1 overexpression ameliorates H/R-induced mitochondrial dysfunction in H9c2 cardiomyocytes. Mitochondrial dysfunction is reflected in the structure, function and mitochondria number of cardiomyocytes (27). Mitochondrial dysfunction leads to diminished energy generation, loss of myocyte contractility, altered electrical properties and eventual cardiomyocyte death (27). A reduction in MMP is an early indicator of apoptosis induction. In the present

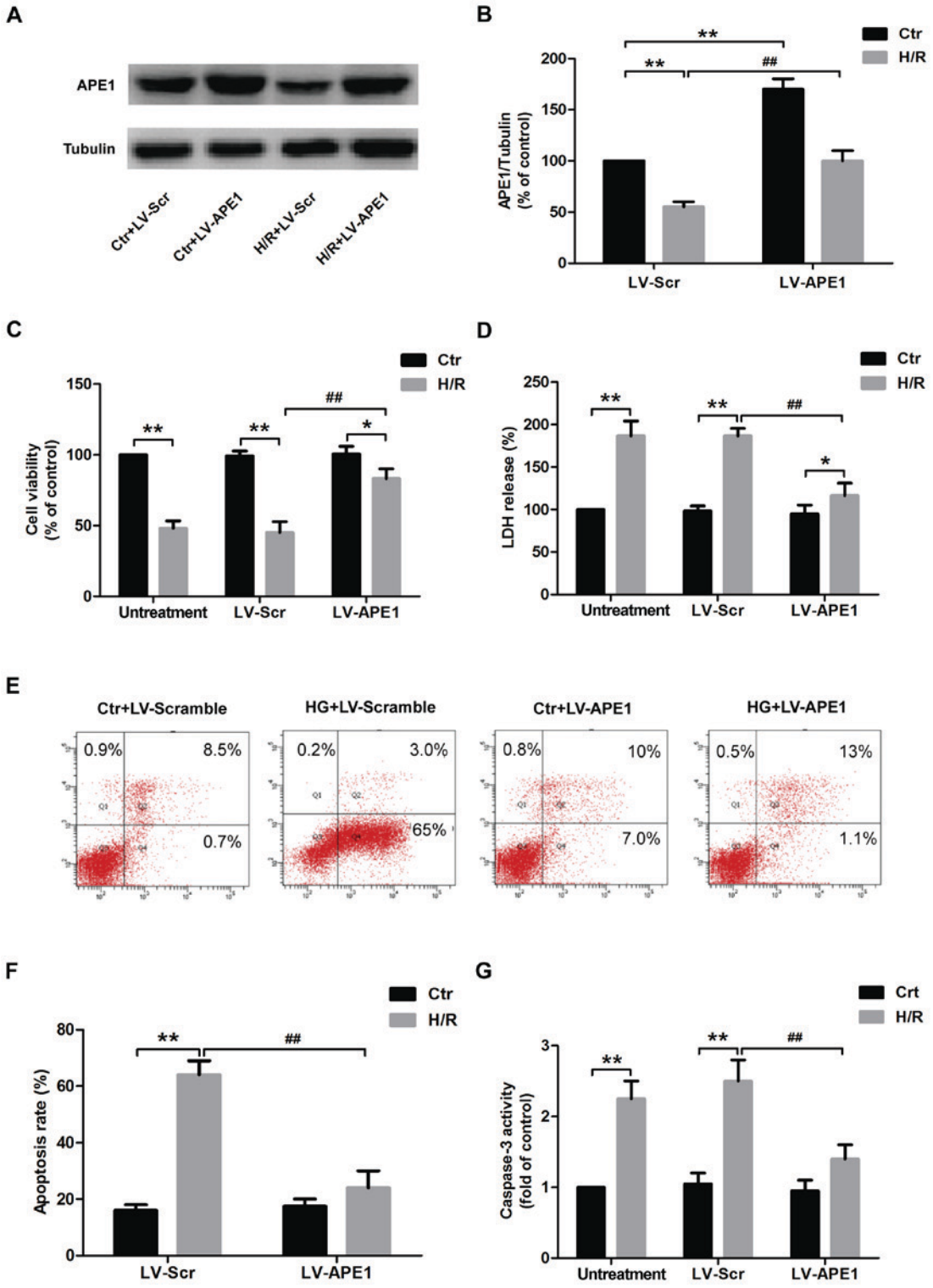

Figure 2. Effects of APE1 overexpression on cell injury and apoptosis in H/R-treated H9c2 cells. H9c2 cells were transfected with LV-APE1 or LV-Scr and exposed to H/R. (A) APE1 protein expression was measured using western blotting and (B) quantified. (C) Cell viability was detected using an MTT assay. (D) LDH release was measured using a kit. (E) Apoptosis was assessed by flow cytometry and (F) quantified. (G) Caspase-3 activity was detected using a Caspase-3 Colorimetric Assay kit. **P<0.01 vs. Ctr and $^{\#\#}$P<0.01 vs. H/R treatment alone. APE1, apurinic/apyrimidinic endonuclease/redox factor 1; H/R, 12 h hypoxia/6 h reoxygenation; LV, lentivirus; Scr, scramble; LDH, lactose dehydrogenase; Ctr, control.

study, JC-1 staining result reveled that green fluorescence was significantly increased in the LV-Scramble group and red fluorescence was significantly decreased in the H/R group compared with the control group (Fig. 4A). However, under H/R conditions, green fluorescence was significantly decreased and red fluorescence was significantly increased in the LV-APE1 group compared with the LV-Scramble group. These results indicate that APE1 reversed the H/R-induced downregulation of MMP in H9c2 cells. ATP generation is associated with the ETC including complex I-IV activities (22). The effect of APE1 on the activity of these enzymes was assessed and it was demonstrated that H/R treatment reduces

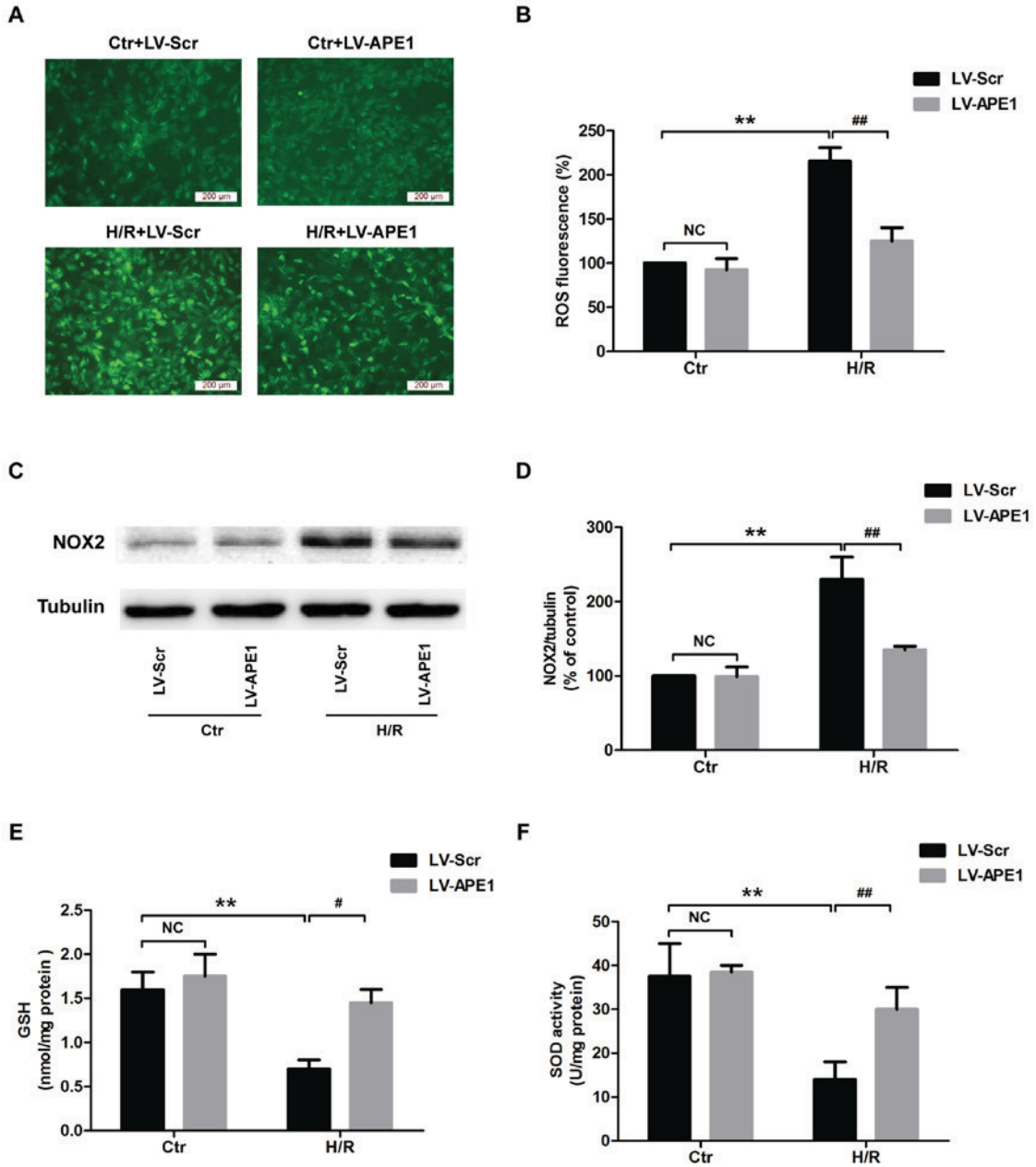

Figure 3. Effects of APE1 overexpression on oxidative stress in H/R-treated H9c2 cells. H9c2 cells were transfected with LV-APE1 or LV-Scr and subjected to H/R. (A) ROS production was measured using DCFH-DA staining (magnification, x200) and (B) quantified. (C) The expression of NOX2 protein was detected using western blotting and (D) quantified. (E) GSH expression and (F) SOD activity were measured using kits. $^{\#}$P<0.05, **P<0.01 and $^{\#\#}$P<0.01, as indicated. APE1, apurinic/apyrimidinic endonuclease/redox factor 1; H/R, 12 h hypoxia/6 h reoxygenation; LV, lentivirus; Scr, scramble; ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorofluorescein acetyl acetate; NOX2, NAPDH oxide 2; GSH, glutathione; SOD, superoxide dismutase; Ctr, control.

the activities of complex I (Fig. 4B), complex II (Fig. 4C), complex III (Fig. 4D) and complex IV (Fig. 4E) in LV-Scramble-transfected H9c2 cells, while these changes were blocked by LV-APE1 transfection under H/R condition. Furthermore, in the LV-Scramble-transfected H9c2 cells, ATP generation was reduced in the H/R treatment groups compared with control group, while in H/R-treated H9c2 cells (Fig. 4F). ATP generation was increased in the LV-APE1 transfection group compared with the LV-Scramble transfection group (Fig. 4G), suggesting that APE1 reduces the H/R-induced downregulation of mitochondrial ATP production. Finally, it was demonstrated that LV-APE1 transfection significantly ameliorated H/R-induced increase in the Bax/Bcl-2 ratio in H9c2 cells (Fig. 4H). These results suggest that APE1 improves mitochondrial dysfunction in H/R-treated H9c2 cells.

Discussion

I/R injury within the myocardium is widely accepted as a primary contributor for the development of ischemic cardiovascular diseases (28). During this process, H/R-induced injury to myocardial cells triggers a number of mechanisms to alleviate cellular damage caused by oxidative stress (29) and mitochondrial dysfunction (30). In the present study, it was demonstrated that H/R decreases cell viability, increases LDH release, apoptosis and oxidative stress and causes mitochondrial dysfunction in H9c2 cardiomyocytes.

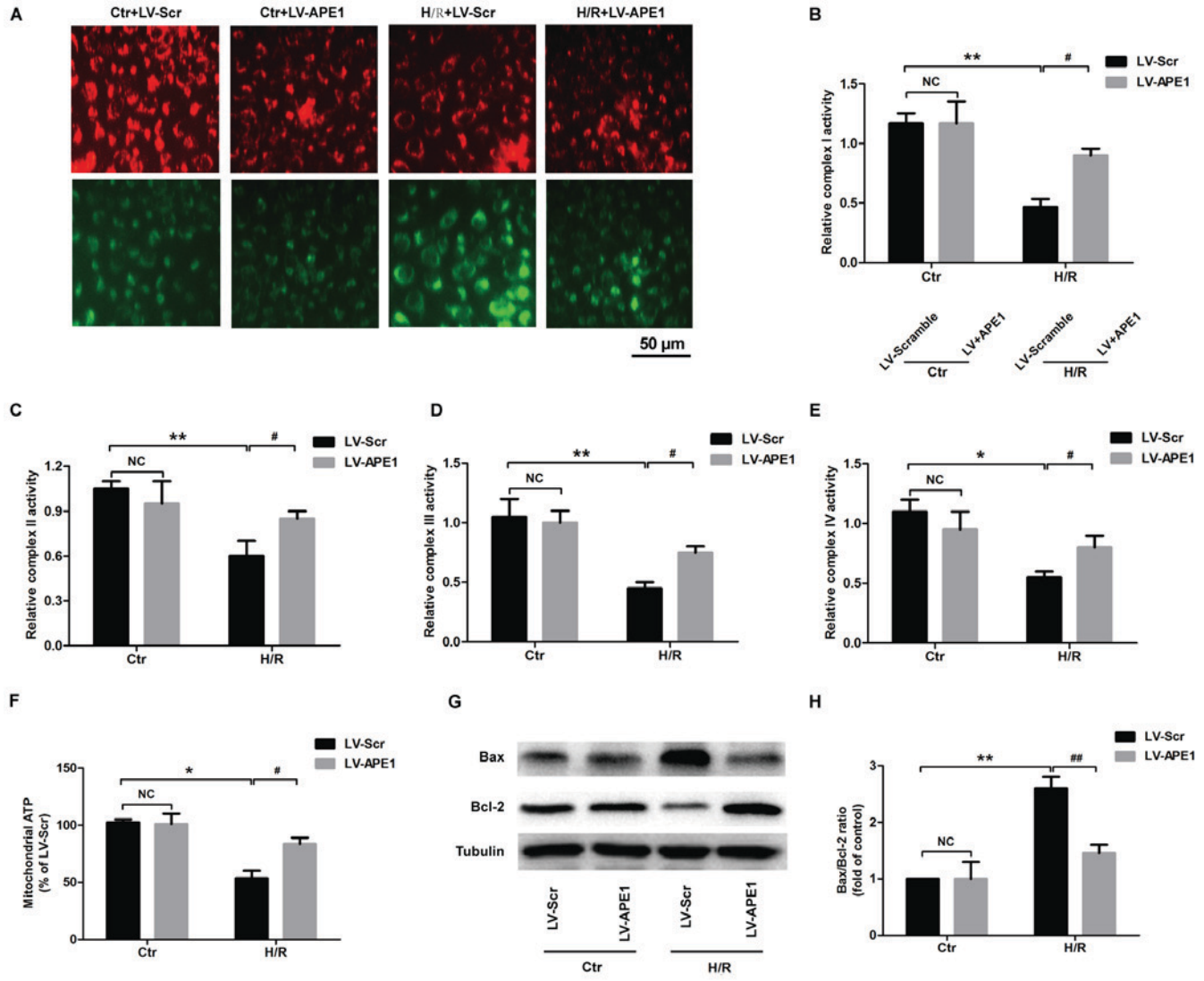

Figure 4. Effects of APE1 overexpression on mitochondrial function in H/R-treated H9c2 cells. H9c2 cells were transfected with LV-APE1 or LV-Scr and subjected to H/R. (A) The mitochondrial membrane potential was detected using JC-1 staining. (B) Complex I, (C) complex II, (D) complex III and (E) complex IV activities were detected using microplate assay kits. (F) ATP production was assessed using the CellTiter-Glo luminescent ATP assay kit. (G) Bax/Bcl-2 ratio was measured using western blotting and (H) quantified. *P<0.05, $^{\#}$P<0.05, **P<0.01, and $^{\#\#}$P<0.01, as indicated. APE1, apurinic/apyrimidinic endonuclease/redox factor 1; H/R, 12 h hypoxia/6 h reoxygenation; LV, lentivirus; Scr, scramble; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; Ctr, control.

APE1 is a multifunctional protein that serves important roles in DNA repair and redox regulation, as well as anti-oxidant and anti-apoptosis activities (9). While APE1 downregulation is consistent with the DNA repair failure coinciding with apoptotic cell death, APE1 upregulation may repair the cells after an injury (31). Previous studies have reported that the multifunctional enzyme APE1 is associated with the progression of a number of human diseases, including cancer, cardiovascular disease and neurodegenerative diseases, making it a promising focus of research into the treatment and management of human diseases (10,11). Jin *et al* (15) reported that serum APE1 levels were higher in s patients with coronary artery disease compared with control patient, which may be a result of protective endogenous APE1 release. It has also been reported that APE1 activation is required to protect cells from oxidative injuries (32,33). The results of the present study indicate that H/R treatment significantly decreases the expression of APE1 protein, suggesting that APE1 downregulation may serve a role in the development of MI/R injury. Considering that APE1 is associated with cardiovascular disease, apoptosis, oxidative stress and I/R injury, the lack of APE1 in H/R-induced myocardial injury may be expected. Further investigation revealed that APE1 overexpression significantly increased cell viability, reduced LDH release, decreased apoptosis and reduced caspase-3 activity, protecting cells against H/R treatment-induced injury and apoptosis. These results are consistent with previous reports that APE1 exhibits a cytoprotective activity in normal endothelial cells (34) and that APE1 overexpression inhibits hypoxia-induced endothelial cell apoptosis (35). These results indicate that APE1 overexpression attenuates H/R-induced injury to H9c2 cardiomyoblasts, providing a potential novel strategy for the treatment of MI/R injury.

MI/R causes an increase in oxidative stress during reperfusion, resulting in further cardiomyocyte apoptosis and mitochondrial dysfunction (2,22). A number of reports have demonstrated that APE1 reduces intracellular ROS

production (36,37). This is consistent with the results of the present study, in which H/R treatment significantly increased ROS generation. However, APE1 overexpression remarkably decreased the production of ROS in H/R-treated H9c2 cells. NOX is a family of proteins that produces ROS when activated; NOX2 is the main source of cytoplasmic ROS generation and serves a vital role in the pathogenesis of MI/R injury (38). In the present study, it was demonstrated that H/R treatment obviously increased the expression of NOX2 in H9c2 cells, while this effect was reversed by APE1 overexpression. In addition, the role of SOD and GSH in the maintenance of cellular redox homeostasis has been reported (39). GSH depletion activates apoptotic signaling, resulting in eventual cell death (40). The results of the present study demonstrated that H/R treatment reduces the activity of SOD and GSH expression in H9c2 cells. However, these effects were blocked by APE1 overexpression. Taken together, these results suggest that APE1 attenuates oxidative stress and enhances the antioxidant defense, thereby reducing apoptosis and providing a cardioprotective effect in MI/R injury.

MI/R causes excessive oxidative stress during reperfusion (41), resulting in cardiomyocyte apoptosis and mitochondrial dysfunction (2). These events trigger MMP dissipation, as well as consequent mPTP opening and mitochondrial ETC complex activity deletion, ultimately resulting in myocardial injury (22). In addition, it has been reported that APE1 localizes to the mitochondria in response to oxidative stress (42,43). Notably, mitochondrial targeting of a truncated APE1 form leads to increased survival in human umbilical vein endothelial cells treated under H_2O_2 conditions (44). Siddiqui *et al* (45) demonstrated that APE1 silencing in Huntington's disease cells resulted in exacerbated mitochondrial dysfunction, suggesting that APE1 is critical for mitochondrial function. Similarly, APE1 overexpression in the present study increased MMP and ETC complex activities (complex I-IV) as well as ATP production, alleviating H/R-induced mitochondrial dysfunction. It has previously been reported that the main regulators of mitochondrial apoptosis pathway are the Bcl-2 family proteins (46). Bax translocation causes a loss of ATP content, mitochondrial swelling and rupture of the outer mitochondrial membrane (47,48), further increasing the release of ROS and cytochrome c, leading to apoptosis. The anti-apoptotic members of the Bcl-2 protein family, including Bcl-2, are able to promote cell survival (49). In the present study, APE1 overexpression decreased the expression of Bax and increased the expression of Bcl-2 compared with the H/R alone group. These results suggest that modulation of the Bcl-2 family may serve a role in the mechanism by which APE1 improves mitochondrial dysfunction in H/R injury. The scope of the present study did not include investigating the effect of H/R injury on APE1 activity or APE1's downstream target for regulating apoptosis. Future studies should be expanded to include these experiments.

In conclusion, the results of the present study demonstrate that APE1 overexpression alleviates H/R-induced damage in H9c2 cardiomyocytes. The protective role of APE1 may be achieved via attenuating oxidative stress and suppressing mitochondrial damage, which provides an insight into the biological functions of APE1 in MI/R injury.

Acknowledgements

Not applicable.

Funding

Funding information is not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JH analyzed and wrote the manuscript. HD and FL participated in experiments and data analysis. J-CL and X-CY conducted the statistical analysis to the data. WC made substantial contributions to the conception and design of the study and given final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

Not applicable.

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