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APEX1 protects against oxidative damage-induced cardiomyocyte apoptosis

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Abstract: Apurine/pyrimidine-free endonuclease 1 (APEX1) is a multifunctional enzyme that contributes to oxidization-mediated DNA-cleaved base excision repair and redox activation of transcription factors. However, the role of APEX1 during cardiomyocyte oxidative stress injury is not completely understood. In the present study, whether APEX1 protects oxidative damage-induced cardiomyocytes was investigated. mRNA and protein expression levels of APEX1 were downregulated in the mouse model of cardiac ischemia-reperfusion injury. Furthermore, the expression of APEX1 in hydrogen peroxide (H_2O_2)-treated neonatal mice cardiomyocytes was also decreased. APEX1 knockdown aggravated H_2O_2-treated cardiomyocyte apoptosis indexes. By contrast, APEX1 overexpression reversed H_2O_2-induced oxidative damage, as demonstrated by decreased caspase 3 and Bax expression levels. Moreover, homeobox A5 upregulated APEX1. The results of the present study indicated that APEX1 displayed protective effects against oxidative damage, suggesting that APEX1 may serve as a unique protective strategy for cardiac ischemia-reperfusion injury.

Introduction

It has been suggested that oxidative stress serves as a second messenger in a number of physiological and pathological processes, including cardiac ischemia-reperfusion (I/R) injury, diabetes, inflammation, atherosclerosis, and Alzheimer's disease (Yodoi *et al.*, 2001; Martindale and Holbrook, 2002; Calabrese *et al.*, 2005). In physiological functions, the redox state of the cell is determined by the precise balance between reactive oxygen species (ROS) levels and endogenous antioxidant levels. However, intracellular ROS excessively accumulate during oxidative stress, which induces endogenous DNA damage, leading to cell apoptosis (Levonen *et al.*, 2014; Espinosa-Diez *et al.*, 2015; Di Marzo *et al.*, 2018).

Apurine/pyrimidine-free endonuclease 1 (APEX1) consists of a large, conserved apurinic or apyrimidinic (AP) endonuclease domain and a unique N-terminal ref1 domain (Li and Wilson, 2014; Whitaker and Freudenthal, 2018). ROS can produce AP sites in DNA (Laev *et al.*, 2017). APEX1 recognizes the AP site and cleaves the phosphodiester backbone 5' to the lesion, resulting in DNA single-strand breaks (Dyrkheeva *et al.*, 2016; Kuznetsova *et al.*, 2018; Kladova *et al.*, 2018). APEX1 not only directly participates in the DNA repair process but also regulates transcription by activating/modulating important transcription factors. For example, APEX1 activates/regulates critical transcription factors, including AP-1, early growth response 1, hypoxia-inducible factor 1 subunit-α, p53, and NF-κβ to influence biological events such as the stress response and inflammation (Schindl *et al.*, 2001; Ando *et al.*, 2008; Luo *et al.*, 2008; Fantini *et al.*, 2008; Poletto *et al.*, 2016). However, few studies have detected the role of APEX1 in cardiac I/R injury.

The aim of the present study was to investigate the effects of APEX1 on cardiomyocyte oxidative stress injury. The results indicated that APEX1 expression was reduced by oxidative stress, and APEX1 effectively inhibited hydrogen peroxide (H_2O_2)-induced cell death by negatively regulating Bax and caspase 3.

Materials and Methods

Cell cultures and treatments of cardiomyocytes

Cardiomyocytes were obtained as previously described (Vidyasekar *et al.*, 2015). Briefly, the hearts of 1–2 days old

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neonatal C57BL/6 mice were dissected, and the ventricles were minced and dissociated using 0.125% trypsin. Cells were cultured in gelatin-coated dishes with DMEM/F12 (Thermo Fisher Scientific Inc. catalog no. 11320033) supplemented with 10% FBS (Thermo Fisher Scientific Inc. catalog no. 10099133C). After 1.5 h, non-adherent cells were collected, and 10^6 cells were seeded into one well of collagen-coated silicon-based 6-well culture plates. The culture medium was changed to serum-free DMEM/F12 at 48 h. After 24 h, cardiomyocytes were treated with 10^{-4} M H_2O_2 (Matsuda *et al*., 2016).

Ischemia-reperfusion model

The animals used in the I/R model were twenty SPF-level C57BL/6 male mice, 12 weeks old and weighing 25–30 g. They were purchased from GemPharmatech Co., Ltd., China. They were housed at 24 ± 2°C and 12 h–12 h dark–light cycles. Mice were randomly divided into sham group and I/R group. Both groups were anesthetized by intraperitoneal injection of a mixture of methylthiazine (10 mg/kg) and ketamine (150 mg/kg), tracheal intubation and ventilation (type 7025, Harvard Apparatus, March-Hugstetten, Germany). The left coronary artery was ligated for 30 min and perfused for 24 h to induce ischemia-reperfusion injury in the I/R group. All protocols were approved by the guidelines of the Animal Care and Use Committee of Fudan University and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

RNA interference

The three selected mouse APEX1-targeted shRNA sequences were obtained from BLOCK-iT™ RNAi Designer, Thermo Fisher Scientific, Inc.: shAPEX1-1, GCAAATCTGCCACACTCAAGA; shAPEX1-2, TCGGTATTCCAGTCTTACCAG; and shAPEX1-3, ATTGAGATCCCCACATAGCAC. The shRNA sequences were cloned into pLKO vector, and lentiviral particles were generated using 293T cells. pLKO.1-GFP lentiviral particles were used as a control.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from cultured cardiomyocytes using TRIzol® reagent. Total RNA was reverse transcribed into cDNA using the ReverTra Ace-α RT-PCR kit (Toyobo Life Science), according to the manufacturer's protocol. Subsequently, qPCR was performed using the Bio-Rad IQ5 multicolor detection system (Bio-Rad Laboratories, Inc.) with Power SYBR Green PCR Master Mix (Takara Bio, Inc.) (2 min at 95°C for enzyme activation followed by 40 cycles of 15 s at 95°C and 30 s at 60°C for the amplification step). The mRNA expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method and normalized to the internal reference gene β-actin (Livak and Schmittgen, 2001).

Western blot analysis

Western blotting was performed according to standard protocols. Briefly, total protein was extracted from cardiomyocytes using RIPA buffer (Beyotime Institute of Biotechnology) and quantified using the bicinchoninic acid assay (Thermo Fisher Scientific Inc.). Proteins were separated via SDS-PAGE and transferred to PVDF membranes. Subsequently, the membranes were incubated with APEX1 (1:10000, catalog no. ab92744, Abcam), caspase 3 (1:1000, catalog no. 9664, Cell Signaling Technology), Bax (1:1000, catalog no. 2772, Cell Signaling Technology), HOXA5 (1:1000, catalog no. ab140636, Abcam). After washing three times, blotted membranes were then incubated with horseradish peroxidase-conjugated rabbit secondary antibody (1:5000, catalog no. KC-RB-035, Kang-Chen Biotechnology, Shanghai, China). β-actin (1:5000, catalog no. ab20272, Abcam) was used as the internal control.

TUNEL assay

The TUNEL assay was performed using the One-Step TUNEL Apoptosis assay kit (Beyotime Institute of Biotechnology). Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Subsequently, cells were incubated with TUNEL test solution for 60 min and observed using a fluorescence microscope.

Statistical analysis

Data are presented as the mean ± SEM. Comparisons among multiple groups were analyzed using one-way ANOVA followed by the LSD *post hoc* test. Comparisons between two groups were analyzed using the Student's *t*-test. $P < 0.05$ was considered to indicate a statistically significant difference. All experiments were repeated at least three times.

Results

APEX1 is downregulated during heart I/R. To elucidate the role of APEX1 during heart I/R, a cardiac I/R model was established, and subsequently, ventricles were obtained. Compared with the control group, I/R significantly reduced APEX1 mRNA expression levels (Fig. 1A). In addition, the western blotting results indicated that I/R downregulated APEX1 protein expression (Fig. 1B). The results suggested that APEX1 served a role in I/R.

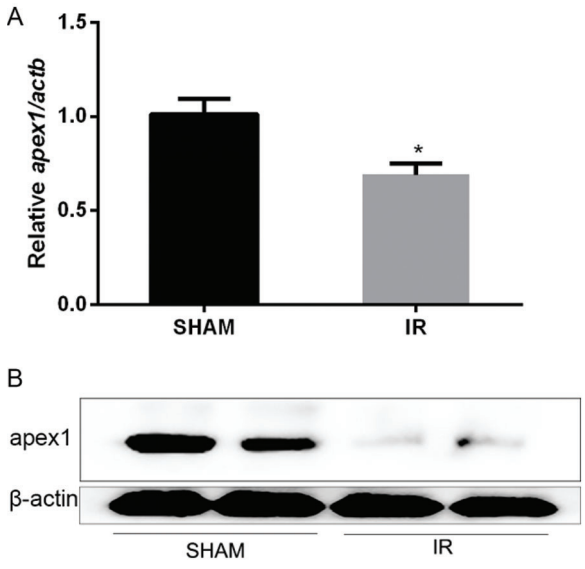

FIGURE 1. Relative expression of APEX1 in a mouse model of cardiac ischemia-reperfusion. APEX1 (A) mRNA and (B) protein expression levels. *$P < 0.05$ *vs.* control. APEX1, apurine/pyrimidine-free endonuclease 1.

APEX1 is downregulated in H_2O_2-treated cardiomyocytes. To investigate whether APEX1 contributed to the productive role against I/R in cardiomyocytes, cardiomyocytes were treated with H_2O_2. RT-qPCR and western blotting results suggested that H_2O_2 reduced APEX1 expression levels compared with the control group (Fig. 2). The results indicated that APEX1 displayed a protective role in cardiomyocytes.

Lentivirus-mediated APEX1 RNA interference vector construction. To investigate the protective role of APEX1 in cardiomyocytes, three shRNA sequences of APEX1 were selected to test their interference efficiency. The shRNA sequences were cloned into pLKO vector, and lentiviral particles were produced using the 293T cell line. RT-qPCR and western blotting were performed to detect the interference efficiency of the three candidate sequences. The RT-qPCR results indicated that the three candidate shRNAs knocked down APEX1 expression. APEX1 expression was reduced by 48%, 73%, and 30% by shAPEX1-1, shAPEX1-2, and shAPEX1-3, respectively (Fig. 3A). In addition, similar results were obtained by western blotting (Fig. 3B); therefore, shaAPEX1-2 was used for subsequent experiments.

APEX1 is associated with cell apoptosis in H_2O_2-treated cardiomyocytes. Subsequently, the protective effect of APEX1 against oxidative stress-induced damage of cardiomyocytes was investigated. H_2O_2 significantly increased Bax and caspase 3 expression levels compared with the control group. Additionally, APEX1 knockdown further enhanced the expression levels of Bax and caspase 3 (Fig. 4A). A similar increase in expression was observed using the TUNEL assay. Compared with the control group, H_2O_2 significantly induced TUNEL signaling, and APEX1 knockdown further increased the TUNEL signal (Fig. 4B). However, APEX1 overexpression reversed H_2O_2-induced Bax and caspase 3 expressions (Fig. 4C).

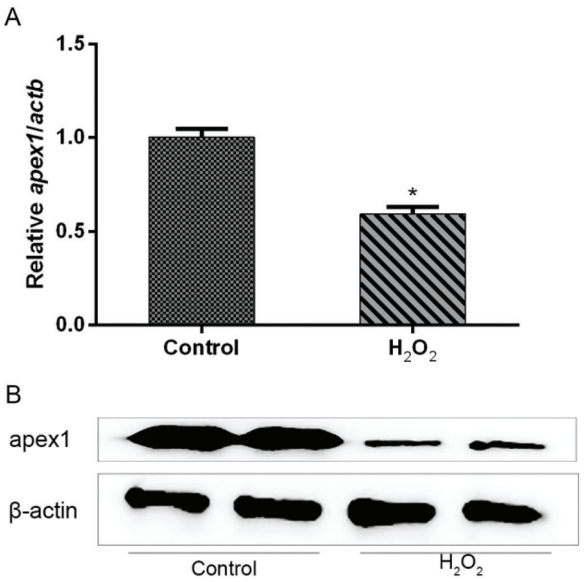

FIGURE 2. Relative expression of APEX1 in H_2O_2-treated cardiomyocytes. APEX1 (A) mRNA and (B) protein expression levels in cardiomyocytes following H_2O_2 (100 μM) treatment for 24 h. *$P < 0.05$ *vs.* control. APEX1, apurine/pyrimidine-free endonuclease 1.

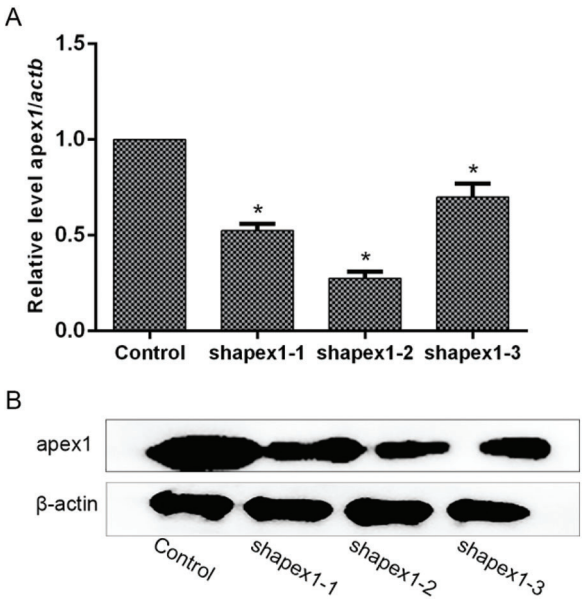

FIGURE 3. Candidates of shRNA-mediated APEX1 downregulation in cardiomyocytes. Transfection efficiency was determined by (A) reverse transcription-quantitative PCR and (B) western blotting. *$P < 0.05$ *vs.* Control. shRNA, short hairpin RNA; APEX1, apurine/pyrimidine-free endonuclease 1.

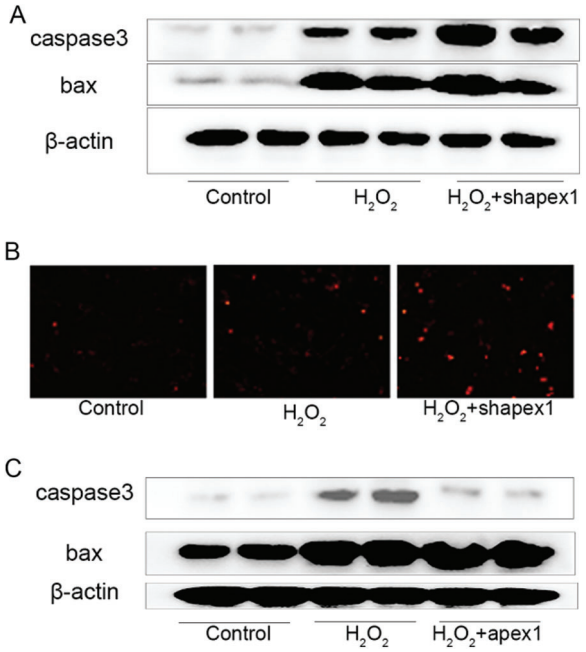

FIGURE 4. Role of APEX1 in H_2O_2. Caspase 3 and Bax protein expression levels were determined by western blotting following APEX1 knockdown in cardiomyocytes treated with H_2O_2 (100 μM) for 24 h. (B) The TUNEL assay was performed to assess the role of APEX1 in cardiomyocytes treated with H_2O_2 (100 μM) for 24 h. (C) Caspase 3 and Bax protein expression levels were determined by western blotting following APEX1 overexpression in cardiomyocytes treated with H_2O_2 (100 μM) for 24 h. APEX1, apurine/pyrimidine-free endonuclease 1.

The results suggested that APEX1 suppressed H_2O_2-induced cardiomyocyte apoptosis.

Homeobox A5 (HOXA5) is upstream of APEX1. Oxidative stress-induced cardiomyocyte apoptosis downregulated APEX1 expression. To identify which factors regulated APEX1

expression, the expression of several related transcription factors was knocked down using siRNAs. The results indicated that the HOXA5-targeted siRNA significantly decreased APEX1 expression levels (Fig. 5A). In addition, HOXA5 was downregulated in I/R injury (Fig. 5B). The results suggested that APEX1 was downstream of HOXA5.

Discussion

Although reperfusion therapy is a potential therapeutic strategy for ischemic heart disease, as it reduces myocardial damage and improves clinical outcomes, reperfusion after ischemia-induced ROS production can lead to DNA damage, restrict myocardial repair and result in cardiac dysfunction (Görge *et al.*, 1991). Recent studies have demonstrated that APEX1 serves an important role in the regulation of oxidative DNA damage in ischemic injury. In the present study, the two main findings may aid in understanding the protective effects of APEX1 against oxidative damage. First, APEX1 expression was reduced in the mouse model of cardiac ischemia-reperfusion *in vivo* and *in vitro*. Second, APEX1 protected against H_2O_2-induced oxidative damage. Therefore, the present study suggested a promising therapeutic strategy for cardiac ischemia-reperfusion injury.

APEX1 is a vital multifunctional protein that displays a pleiotropic role in controlling cellular oxidative stress and promoting genomic stability (Li and Wilson, 2014). APEX1, the main apurinic/apyrimidinic endonuclease in eukaryotic cells, serves a central role in the DNA base excision repair pathway, repairing uracil, alkylation, oxidation, and abasic sites, as well as DNA single-strand breaks (Dyrkheeva *et al.*, 2016).

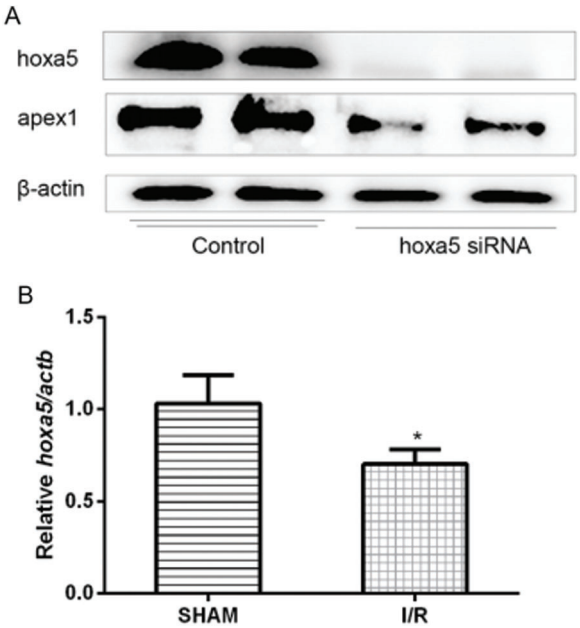

FIGURE 5. HOXA5 is upstream of APEX1. (A) APEX1 and HOXA5 protein expression levels were determined by western blotting in siRNA-transfected cardiomyocytes. (B) HOXA5 mRNA expression levels in a mouse model of I/R were determined by reverse transcription-quantitative PCR. *$P < 0.05$ *vs.* control. HOXA5, homeobox A5; APEX1, apurine/pyrimidine-free endonuclease 1; siRNA, small interfering RNA; I/R, ischemia-reperfusion.

APEX1 has an important role in myocardial disease. Jeon *et al.* (2004) reported that heterozygous APEX1 mice displayed impaired endothelium-dependent vasodilation, reduced blood nitric oxide levels, and hypertension. Martinet *et al.* (2002) demonstrated that APEX1 expression was increased in human carotid atherosclerotic plaques. Furthermore, Jin *et al.* (2017) reported that serum APEX1 could be used to identify myocardial injury in viral myocarditis without an endomyocardial biopsy. However, the relationship between APEX1 and I/R has not been previously reported. The results of the present study suggested that APEX1 inhibited apoptosis during I/R, which indicated that APEX1 might serve as a potential therapeutic target for cardiac ischemia-reperfusion injury.

Conclusions

Our study is the first description of APEX1's role in the heart. APEX1 expression was reduced by oxidative stress, which was regulated by HOXA5. APEX1 effectively inhibited hydrogen peroxide-induced cell death by negatively regulating Bax and caspase 3. The results of the present study indicated that APEX1 displayed protective effects against oxidative damage, suggesting that APEX1 may serve as a unique protective strategy for cardiac ischemia-reperfusion injury.

Availability of Data and Materials: The datasets used during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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