PEP-1-CAT protects hypoxia/reoxygenation-induced cardiomyocyte apoptosis through multiple sigaling pathways

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Abstract

Background: Catalase (CAT) breaks down H2O2 into H2O and O2 to protects cells from oxidative damage. However, its translational potential is limited because exogenous CAT cannot enter living cells automatically. This study is aimed to investigate if PEP-1-CAT fusion protein can effectively protect cardiomyocytes from oxidative stress due to hypoxia/reoxygenation (H/R)-induced injury.

Methods: H9c2 cardomyocytes were pretreated with catalase (CAT) or PEP-1-CAT fusion protein followed by culturing in a hypoxia and re-oxygenation condition. Cell apoptosis were measured by Annexin V and PI double staining and Flow cytometry. Intracellular superoxide anion level was determined, and mitochondrial membrane potential was measured. Expression of apoptosis-related proteins including Bcl-2, Bax, Caspase-3, PARP, p38 and phospho-p38 was analyzed by western blotting.

Results: PEP-1-CAT protected H9c2 from H/R-induced morphological alteration and reduced the release of lactate dehydrogenase (LDH) and malondialdehyde content. Superoxide anion production was also decreased. In addition, PEP-1-CAT inhibited H9c2 apoptosis and blocked the expression of apoptosis stimulator Bax while increased the expression of Bcl-2, leading to an increased mitochondrial membrane potential. Mechanistically, PEP-1-CAT inhibited p38 MAPK while activating PI3K/Akt and Erk1/2 signaling pathways, resulting in blockade of Bcl2/Bax/mitochondrial apoptotic pathway.

Conclusion: Our study has revealed a novel mechanism by which PEP-1-CAT protects cardiomyocyte from H/R-induced injury. PEP-1-CAT blocks Bcl2/Bax/mitochondrial apoptotic pathway by inhibiting p38 MAPK while activating PI3K/Akt and Erk1/2 signaling pathways.

Keywords: Cell-penetrating peptide, PEP-1, Catalase, Cardiomyocyte, Apoptosis, MAPK

Introduction

Myocardial ischemia and reperfusion generate a large amount of reactive oxygen species (ROS) in cardiomyocytes subject to injury. ROS assaults intracellular organelles, cell membranes, and biological macromolecules including nucleic acid, protein, and lipid, resulting in oxidative stress and cell apoptosis [1,2]. Catalase (CAT) is one of essential enzymes metabolizing oxygen free radical via breakdown of H2O2 into H2O and O2, and thus protects cells from oxidative damage. However, exogenous CAT does not enter living cells automatically because of its poor permeability and cell membrane selectivity. Its translational value in protecting cells from oxidative stress damage, therefore, is very limited.

A great deal of efforts have been made to deliver full-length proteins into mammalian cells. Morris Group has designed a new type of PEP-1 peptide carrier (KETWWETWWTEWSQPKKKRKV) that enables the entering of large proteins into living cells [3]. In fact, several laboratories have successfully delivered full-length PEP-1 fusion proteins into cultured cells and nervous system by using this PEP-1 peptide carrier, including EGFP, β-Gal, antibodies, cyclophilin A, and human copper chaperone for Cu, Zn-SOD1 and CAT [4-7].
previous studies have demonstrated that PEP-1-CAT fusion proteins can be transduced into myocardium and protect against myocardial injury induced by ischemia-reperfusion in rats [8].

Cardiomyocyte apoptosis is an inevitable process during myocardial ischemia-reperfusion-induced injury [9]. We have previously reported an anti-apoptotic effect of PEP-1-CAT on H9c2 cardiomyocytes [10]. However, detailed mechanisms underlying the effect of PEP-1-CAT on H/R-induced H9c2 remain unknown. In the present study, we used the hypoxia-reoxygenation (H/R)-induced apoptosis model to investigate the mechanisms underlying the anti-apoptotic effect of PEP-1-CAT in H9c2 cells. H/R is a classic in vitro model mimicking myocardial ischemia-reperfusion injury in vivo. We found that PEP-1-CAT protected H9c2 from H/R-induced injury through blocking p38 MAPK activity and activating PI3K/Akt and Erk1/2 activity, which resulted in a blockade of Bax/Bcl-2/mitochondria apoptotic pathway and thus a reduction of cardiomyocyte apoptosis.

Materials and methods

Generation of biologically active PEP-1-CAT fusion protein

PEP-1-CAT fusion protein was isolated and purified as described by our laboratory previously [11]. Briefly, two prokaryotic expression plasmids for CAT and PEP-1-CAT were constructed using TA-cloning method. Both recombinant proteins were tagged with six histidine residues (His-tag) at the amino terminus. The two proteins were expressed and purified separately as described [11].

Cell culture

H9c2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) with 5 g/L glucose supplemented with 15% (v/v) fetal bovine serum (FBS, Hangzhou sijiqing Biological Engineering Materials Co., Ltd., China). Cells were routinely grown to subconfluency (>90% by visual estimate) in 75 cm² flasks at 37°C in a humidified atmosphere with 5% CO₂ prior to passage and seeding for experiments. To observe the morphological alteration, H9c2 cells were grown on cover slips and observed using a microscope (Nikon, Japan). To examine the aberrant nuclei in apoptotic cells, H9c2 cells were stained with 4,6-Diamidino-2-phenylindole (DAPI), and the nuclei were observed using a fluorescent microscope.

Immunocytochemistry staining

H9c2 cells were grown to confluence in a 24-well plate and treated with purified PEP-1-CAT (2 μM) or CAT (2 μM). 6 h later, cells were washed twice with 1 × PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Immunocytochemistry staining was performed by using rabbit anti-Hisprobe (diluted 1:200) (Santa Cruz Biotechnology, USA) and mouse anti-Troponin T antibodies (diluted 1:200) (Santa Cruz Biotechnology, USA). Cells were then incubated with tetra-ethyl rhodamine isothiocyanate (TRITC)-conjugated rat anti-rabbit Ig G (diluted 1:250) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig G (diluted 1:250) at 25°C for 1 h. After washing for 3 times with PBS, cells were incubated with DAPI (Sigma, USA) for 10 min. The immunostained cells were observed with a fluorescent microscope (Nikon, Japan).

Hypoxia-reoxygenation of H9c2 Cells

H9c2 cells were pretreated with or without PEP-1-CAT (2 μM) in low serum media (2% FBS) for 6 h followed by culturing in a low-oxygen condition (95% N₂ + 5% CO₂) for 21 h in a humidified hypoxia chamber (Stem Cell Technology, USA). After hypoxia incubation, the medium were replaced, and the cells were exposed to normal-oxygen condition (95% air + 5% CO₂) for reoxygenation for 6 h [12]. Control cells were cultured in normoxic conditions. The supernatant and cells were collected separately for further analysis.

Measurement of lactate dehydrogenase (LDH) and malondialdehyde (MDA) levels

H9c2 cells were treated with PEP-1-CAT, harvested and lysed as previously described LDH release and MDA content were measured using commercial kits (JianCheng Bioengineering Institute, China).

Superoxide anion production in H9c2

H9c2 cells were grown to confluence in a 24-well plate followed by H/R with CAT or PEP-1-CAT treatment. Cells were then split and cultured on cover slips and incubated with DHE (5 mM) (Beyotime Institute of Biotechnology) at 37°C for 30 min. The DHE staining detecting superoxide anion production was observed using a fluorescent microscope (Nikon, Japan) or quantified by Flow Cytometry.

Annexin V and PI binding assay

Annexin V and PI fluorescein staining kit (Bender MedSystems, Austria) were utilized to measure H9c2 cell apoptosis by following the manufacturer’s instruction. Briefly, 1 × 10⁶ cells were suspended in 200 μl 1 × binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). Cells were then incubated with Annexin V (1:20) for 3 min followed by incubation with propidium iodide (PI, 1 mg/ml) for 15 min. Apoptosis rate was evaluated by Flow Cytometry.

Measurement of mitochondrial membrane potential

Mitochondrial transmembrane potential was assessed using a sensitive fluorescent dye, a lipophilic cationic probe JC-1 (Invitrogen, USA). H9c2 cells were grown on
cover slips and incubated with 5 mM JC-1 dye (Molecular
Lar Probe) at 37°C for 15 min. Cells were then washed
with PBS for three times and analyzed immediately with
a fluorescent microscope. Red emission indicates mem-
brane potential-dependent JC-1 aggregates in mitochon-
dria. Green fluorescence reflects the monomeric form of
JC-1 appearing in cytoplasm after mitochondrial mem-
brane depolarization.

Quantitative reverse transcription polymerase chain
reaction (qPCR)
Total RNA from H9c2 cells was extracted using TRIZOL Reagent (Invitrogen). RNA concentration was determined
by UV spectrophotometry. qRT-PCR was performed using
Thunderbird SYBR Master Mix (TOYOBO, Japan). Pri-
mer sequences were: Bcl-2: 5′-CGA CTT TGC AGA
GAT GTC CA-GA3′ (forward), 5′-ATG CCG GTT CAG
GTA CTC AG-3′ (reverse); Bax: 5′- CTG CAG AGG
ATG ATT GCT GA-3′ (forward), 5′- GAT CAG CTC
GGC AAC TTT AG-3′ (reverse); β-actin: 5′-GTC CAC
CGC AAA TGC TTC TA-3′ (forward), 5′-TGC TGT
CAG CCT CAC GT TCG CA3′ (reverse). qPCR was
performed on a Real-time PCR Detection System (Slan,
Hongshi) with the followi ng cycles: 95°C for 1 min,
45 s for 40 cycles. β
s followed by 95°C for 15 s, 58°C for 15 s, and 72°C for
internal control.

Western blot analysis
Western blot was carried out to detect protein expres-
sion using following primary antibodies: rabbit anti-Bax
(Santa Cruz Biotechnology), mouse anti-Bcl-2 (Santa
Cruz Biotechnology), rabbit anti-Caspase-3 (Santa Cruz
Biotechnology), rabbit anti-PARP-1 (Santa Cruz Biotechn-
ology), rabbit anti-phospho-p38 MAPK (cell signaling
technology), and rabbit anti-p38 MAPK (cell signaling
technology). The protein expression levels were visual-
ized using enhanced chemiluminescence method.

Statistical analysis
All data are expressed as means ± SEM unless indicated
otherwise. Differences among groups were determined
by ANOVA. Differences between groups were deter-
mined by Student’s t-test with P < 0.05 considered statis-
tically significant.

Results
PEP-1-CAT restored H/R-altered H9c2 cell morphology
and decreased LDH and MDA levels
PEP-1-CAT fusion proteins were successfully trans-
duced into H9c2 cells as shown by immunocytochem-
istry staining (Additional file 1: Figure S1). CAT fusion
proteins, however, were unable to be transduced into the
cells (Additional file 1: Figure S1). H/R altered H9c2 cell
morphology. H/R treatment changed the spindle-shaped
and well-organized morphology to a shrink, round and
distorted morphology. PEP-1-CAT transduction, how-
ever, almost restored the spindle-shaped morphology
seen in the untreated cells (Figure 1A).

LDH release is an indicator of cellular injury. Com-
pared to untreated cells, LDH levels were markedly in-
creased by H/R injury. CAT transduction decreased
LDH release. PEP-1-CAT transduction, however, had a
greater impact on LDH levels compared to the CAT
transduction (Figure 1B). MDA reflects cardiomyocyte
oxidative damage. H/R treatment strikingly increased
the MDA level, but PEP-1-CAT significantly decreased
the MDA level (Figure 1C).

PEP-1-CAT had a greater effect on superoxide anion
production than CAT
H/R treatment significantly increased superoxide anion
production in H9c2 cells compared to the untreated
group. CAT transduction slightly reduced superoxide
anion level. PEP-1-CAT transduction, however, signifi-
cantly inhibited the level of superoxide anion. These re-
results demonstrated that PEP-1-CAT had a much
stronger effect than CAT on removing superoxide anion
from the injured cells (Figure 2).

PEP-1-CAT attenuated H/R-induced H9c2 cell apoptosis
Comparing to the control group, significantly more cells
underwent apoptosis as shown by the bright DAPI stain-
ing in H/R group. H/R treatment condensed the nuclei of
H9c2 cells, an indicator of apoptosis. PEP-1-CAT trans-
duction, however, restored H9c2 nuclei to the normal
morphology (Figure 3A). Quantitative analysis using Flow
Cytometry confirmed that PEP-1-CAT significantly
inhibited H/R-induced apoptosis (Figure 3B-C). PolyADP-
rbose polymerase-1 (PARP-1) is known to be involved in
DNA damage while caspase-3 is known to regulate cell
apoptosis. To determine whether PEP-1-CAT affects H/R-
induced PARP and caspase-3 cleavage, we treated cells
with H/R in the presence and absence of PEP-1-CAT and
analyzed their cleavages using anti-PARP-1 and Caspase-3
antibodies. As shown in Figure 3D, H/R induced PARP
and caspase-3 cleavage in H9c2 cells but the effects were
inhibited by PEP-1-CAT, further demonstrating that PEP-
1-CAT suppressed H/R-induced apoptosis.

PEP-1-CAT regulated the expression of apoptosis-related
proteins
To investigate the mechanism whereby PEP-1-CAT at-
tenuates H/R-induced H9c2 apoptosis, we examined the
expression of Bcl-2 and Bax. Both qRT-PCR and Western
blot analyses showed that Bcl-2 expression was mark-
edly increased in PEP-1-CAT-pretreated cells compared
to the H/R or CAT-treated group. As expected, Bax
expression was markedly decreased by PEP-1-CAT (Figure 4A-D), suggesting that PEP-1-CAT prevented H9c2 cells from apoptosis by increasing Bcl-2 while inhibiting Bax expression.

**PEP-1-CAT restored H/R-blocked mitochondrial membrane potential**

Untreated cells exhibited bright-staining mitochondria that emitted red fluorescence. H/R treatment caused the formation of monomeric JC-1, indicative of loss of membrane potential. PEP-1-CAT pretreatment, however, blocked the HR-induced formation of JC-1 monomers (Figure 5A-B), suggesting PEP-1-CAT can restore H/R-induced loss of mitochondrial membrane potential.

**PEP-1-CAT inhibited H/R-induced H9c2 apoptosis through regulating multiple signaling pathways**

Previous studies have shown that apoptosis is mediated by multiple signaling pathways or protein factors including PI3K/Akt, p38 and Erk1/2 MAPK, etc. [13,14]. To determine which pathways are involved in PEP-1-CAT-mediated protection of H/R-injured H9c2 cells, we treated H9c2 with specific inhibitors for each individual pathways. We found that PI3K/Akt and Erk1/2 signaling pathways were essential for mediating PEP-1-CAT inhibition of H/R-induced apoptosis because PI3K/Akt inhibitor wortmannin, PI3K siRNA, Erk1/2 inhibitor PD98059, or Erk1 siRNA blocked PEP-1-CAT-induced decrease of H9c2 apoptosis (Figure 6A-B). p38 MAPK appeared to be also important for PEP-1-CAT function. Although p38 MAPK inhibitor did not reverse PEP-1-CAT-mediated decrease of H9c2 apoptosis (Figure 6A-B), PEP-1-CAT transduction inhibited p38 phosphorylation (Figure 7), suggesting that PEP-1-CAT blocks p38 signaling. These results demonstrated that PEP-1-CAT attenuated p38 signaling while enhancing PI3K and Erk1/2 MAPK signaling.
Discussion

Myocardial apoptosis is a significant pathophysiological event in myocardial ischemia-reperfusion injury [9]. It is widely acknowledged that intervention of myocardial apoptosis is a very important approach to the prevention of myocardial ischemia-reperfusion injury [15]. Reperfusion causes myocardium to produce a large amount of ROS including superoxide anion (O$_2$–), hydroxyl radical (OH$^-$), and hydrogen peroxide (H$_2$O$_2$), etc [16]. CAT, one of the most important enzymes, can protect cells from oxidative damage. But its potential to be used to protect myocardium from H/R-induced injury is hindered by the poor permeability and the selectivity of cell membrane. By fusing CAT with a PEP-1 peptide, we were able to efficiently transduce PEP-1-CAT into H9c2 cells and protect myocardium from H/R-induced injury [10].

![Figure 2](image2.png)

**Figure 2** PEP-1-CAT inhibited H/R-induced superoxide anion production. (A) Superoxide anion production was observed using a fluorescent microscope. (B) Superoxide anion production was quantified by Flow Cytometry (n = 6). *P<0.01 vs control (CTL); &P<0.05 vs H/R; #P<0.05 vs H/R or H/R + CAT (CAT); n = 4.

![Figure 3](image3.png)

**Figure 3** PEP-1-CAT inhibited H/R-induced H9c2 cell apoptosis. H9c2 cells were pretreated with CAT or PEP-1-CAT for 6 h and placed into normoxic environment for 27 h or into hypoxia chamber for 21 h followed by 6 h reoxygenation. (A) Cell apoptosis was detected by DAPI staining. (B C) H/R-induced H9c2 apoptosis rate was quantified by Flow Cytometry. *P<0.01 vs control (CTL); &P<0.05 vs H/R; #P<0.01 vs H/R or H/R + CAT (CAT); n = 5. (D) PARP and caspase-3 protein expression was detected by western blot.
Figure 4 PEP-1-CAT regulated Bcl-2 and Bax expression. (A) Bcl2 and Bax mRNA expression was detected by qRT-PCR and normalized to β-actin. *P<0.01 vs control (CTL); &P<0.05 vs H/R; #P<0.05 vs H/R or H/R + CAT (CAT); n = 3. (B) Bcl2 and Bax protein expression was detected by western blot. (C) Normalization of Bcl2 and Bax expression to α-Tubulin. *P<0.01 vs control (CTL); &P<0.05 vs H/R; #P<0.05 vs H/R or H/R + CAT (CAT); n = 4. (D) Bcl2/Bax ratio. *P<0.01 vs control (CTL); &P<0.05 vs H/R; #P<0.05 vs H/R or H/R + CAT (CAT); n = 4.

Figure 5 PEP-1-CAT restored H/R-induced reduction of mitochondrial membrane potential. (A) Mitochondrial transmembrane potential was assessed by the lipophilic cationic probe JC-1. Red signal indicates JC-1 aggregates in mitochondria. Green signal shows cytosolic JC-1 monomers indicative of the loss of mitochondrial membrane potential. (B) Quantitative analysis of membrane potential in (A). *P < 0.05 vs control (CTL); &P < 0.05 vs H/R; #P < 0.05 vs H/R or H/R + CAT (CAT) group; n = 3.
The present study advanced our previous finding by identifying novel mechanisms underlying PEP-1-CAT function in protecting cardiomyocytes. We have found that PEP-1-CAT protects H/R-induced injury of H9c2 cells by restoring H/R-induced alteration of H9c2 morphology, inhibiting H/R-induced production of ROS, and blocking LDH release and MDA production, the two indicators for hypoxia-reoxygenation injury \cite{17,18}.

ROS causes damages to intracellular macromolecules such as DNA breakage and lipid membrane peroxidation, leading to cell apoptosis \cite{19}. Our data demonstrate that PEP-1-CAT blocks H/R-induced H9c2 apoptosis by regulating mitochondria-related apoptotic pathways. Recent

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\caption{PEP-1-CAT inhibited H/R-induced H9c2 apoptosis via p38, PI3K and Erk1/2 MAPK signaling pathways. (A) H9c2 apoptosis was assessed by Flow Cytometry. (B) Quantification of H9c2 apoptosis rate. \#P<0.01 vs CTL; $P<0.01$ vs H/R group; @P<0.01 vs H/R or PEP-1-CAT-treated group; @P<0.05 vs PEP-1-CAT-treated group; &P<0.05 vs H/R or PEP-1-CAT-treated group; n = 5.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\linewidth]{figure7.png}
\caption{PEP-1-CAT inhibited p38 phosphorylation. (A) Western blot analysis showing PEP-1-CAT inhibited p38 phosphorylation. (B) Quantitative analysis of p38 phosphorylation by normalizing to total p38. \$P<0.01$ vs CTL; \$P<0.05$ vs H/R group; \$P<0.01$ vs PEP-1-CAT-treated group; n = 4.}
\end{figure}
studies have shown that H/R injury induces mitochondria to produce a high level of ROS [20,21]. Excessive ROS damages mitochondria, opens its permeability transition pore (PTP) and thus induces mitochondrial permeability transition (MPT), leading to mitochondrial depolarization and outer membrane rupture, which causes cell apoptosis or death [22,23]. Our studies indicate that H/R induces a decreased mitochondrial membrane potential, suggesting an impairment of mitochondria function. PEP-1-CAT transduction, however, restores mitochondrial membrane potential. These data demonstrate that PEP-1-CAT protects H9c2 cells from H/R-induced apoptosis by maintaining mitochondria membrane integrity and function of cardiomyocytes. Moreover, previous studies indicate that Bcl-2 family is upregulated during the opening of PTP [24]. Our results demonstrate that PEP-1-CAT regulates the expression of Bcl-2 family. PEP-1-CAT significantly increases Bcl-2 while decreasing Bax protein levels that are altered by H/R injury.

PEP-1-CAT prevents cardiomyocyte from H/R-induced injury by regulating multiple signaling pathways. Although a number of signaling pathways are involved in H/R-induced myocardial injury and apoptosis, PEP-1-CAT protects cardiomyocytes through down-regulation of p38 MAPK and activation of PI3K and Erk1/2 signaling pathways. PEP-1-CAT transduction inhibits p38 MAPK phosphorylation, suggesting that p38 MAPK mediates, at least in part, the function of PEP-1-CAT. Blockade of PI3K and Erk1/2 signaling significantly attenuates PEP-1-CAT-mediated reduction of H9c2 apoptosis, indicating that PI3K and Erk1/2 signaling pathways are essential for PEP-1-CAT activity in protecting cardiomyocytes.

In summary, PEP-1-CAT transduction efficiently protects cardiomyocyte from H/R-induced apoptosis by blocking ROS production in mitochondria, which maintains mitochondria membrane integrity and inhibits the activation of Bcl2/Bax apoptotic pathway. Moreover, PEP-1-CAT blocks cardiomyocyte apoptosis by blocking p38 MAPK while activating PI3K and Erk1/2 MAPK signaling pathways. How these signaling pathways interact with each other in mediating PEP-1-CAT function will be an interesting subject for future study. Nevertheless, our study provides novel information and rationale for developing PEP-1-CAT as a therapeutic agent for treating or preventing myocardial ischemia-reperfusion injury.

Additional file

**Additional file 1: Figure S1.** Transduction of PEP-1-CAT into H9c2 cells.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LZ and SW designed and performed the experiments, collected the data and analyzed the results. JNW and JMT participated in the experimental design and interpretation of the results. LYG made fusion protein and evaluated the apoptosis by Flow Cytometry. FZ carried out Western blot. XK performed part of the in vitro experiments. JYY and YZH carried out the immunassays. SYC assisted with writing the manuscript. All the authors have read and approved the final manuscript.

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