Sulforaphane prevents rat cardiomyocytes from hypoxia/reoxygenation injury in vitro via activating SIRT1 and subsequently inhibiting ER stress

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Aim: Sulforaphane (SFN), a natural dietary isothiocyanate, is found to exert beneficial effects for cardiovascular diseases. This study aimed to investigate the mechanisms underlying the protective effects of SFN in a model of myocardial hypoxia/reoxygenation (H/R) injury in vitro.

Methods: Cultured neonatal rat cardiomyocytes pretreated with SFN were subjected to 3-h hypoxia followed by 3-h reoxygenation. Cell viability and apoptosis were detected. Caspase-3 activity and mitochondrial membrane potential (ΔΨm) was measured. The expression of ER stress-related apoptotic proteins were analyzed with Western blot analyses. Silent information regulator 1 (SIRT1) activity was determined with SIRT1 deacetylase fluorometric assay kit.

Results: SFN (0.1–5 μmol/L) dose-dependently improved the viability of cardiomyocytes, diminished apoptotic cells and suppressed caspase-3 activity. Meanwhile, SFN significantly alleviated the damage of ΔΨm and decreased the expression of ER stress-related apoptosis proteins (GRP78, CHOP and caspase-12), elevating the expression of SIRT1 and Bcl-2/Bax ratio in the cardiomyocytes. Co-treatment of the cardiomyocytes with the SIRT1-specific inhibitor Ex-527 (1 μmol/L) blocked the SFN-induced cardioprotective effects.

Conclusion: SFN prevents cardiomyocytes from H/R injury in vitro most likely via activating SIRT1 pathway and subsequently inhibiting the ER stress-dependent apoptosis.

Keywords: sulforaphane; cardioprotection; cardiomyocytes; hypoxia/reoxygenation; apoptosis; ER stress; SIRT1 signaling; Ex-527
pathway plays an essential role in cell survival and cardioprotection against H/R injury\cite{12}. It has been shown that SIRT1 is implicated in the changes of cardiomyocyte apoptosis and infarct size. Inhibition of SIRT1 by pharmacological or genetic interference elicited cardiac cell apoptosis and increased infarct size during I/R injury\cite{13,14}. Both ER stress and SIRT1 signaling pathway are now recognized to play an essential function in deciding cardiac cell life and death. More recently, studies showed that a close relationship may exist between ER and SIRT1\cite{15}. However, little is known between them during I/R injury.

The aims of the present study were to determine the protective effects of SFN on cardiomyocytes exposed to H/R injury and to elucidate the mechanism of SFN protecting cardiomyocytes against ER stress-induced apoptosis by SIRT1 signaling pathway during H/R injury.

Materials and methods
Reagents
SFN was obtained from Sigma-Aldrich (St Louis, MO, USA) and diluted in 10% dimethyl sulfoxide (DMSO) (Cell Signaling Technology, Inc, Beverly, MA, USA). The final concentration of DMSO never exceeded 0.1% in either control or treated cells. Specific SIRT1 inhibitor Ex-527 was also obtained from Selleck Chemicals (Houston, USA). Fetal bovine serum and DMEM were purchased from Gibco Co Ltd (USA). The lactate dehydrogenase (LDH) commercial kit was obtained from Biovision (Mountain View, CA, USA). Bax (1:1000), Bcl-2 (1:500) and SIRT (1:1000) antibodies were purchased from Cell Signaling Technology Inc (Beverly, MA, USA).

Primary culture of neonatal rat cardiomyocytes and simulated ischemia reperfusion in vitro
Cardiomyocytes were isolated from 1 to 2-d-old Sprague-Dawley rats as previously described\cite{19}. Sprague-Dawley rats were obtained from the Laboratory Animal Center of Guangdong Province (Guangzhou, China). All animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health (NIH Publication No 85–23, revised 1996). All investigations were approved by the Bioethics Committee of Southern Medical University, Guangzhou, China. The isolated cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin, and then maintained in 5% CO2 incubator at 37 °C. After 72 h, cultured cardiomyocytes were used in subsequent experiments. To simulate the ischemia reperfusion model, cardiomyocytes were plated in a hypoxia chamber (5% CO2 and 95% N2) for 3 h at 37 °C, followed by reoxygenation in a normoxia chamber (5% CO2 and 95% O2) for 3 h at 37 °C in DMEM with 10% FBS.

The experimental design was divided into four steps
Step 1: After 72 h, cultured cardiomyocytes were randomly divided into five groups: 1) control group: cardiomyocytes were maintained in normoxic condition without any treatment; 2) H3/R3 group: Hypoxia 3 h, following Reoxygenation 3 h; 3) H3/R6 group: Hypoxia 3 h, following Reoxygenation 6 h; 4) H3/R9 group: Hypoxia 3 h, following Reoxygenation 9 h; 5) H3/R12 group: Hypoxia 3 h, following Reoxygenation 12 h.

Step 2: When the reoxygenation duration of experimental model was determined, cardiomyocytes were randomly divided into six groups to be exposed to the optional concentration: control group, H/R group and SFN groups (0.1, 0.5, 1 and 5 μmol/L).

Step 3: When ideal concentration of SFN was screened out, cardiomyocytes were randomly divided into four groups: 1) control group: cardiomyocytes were treated with PBS and incubated in normoxic condition. 2) SFN group: cardiomyocytes were treated with 5 μmol/L SFN and incubated in normoxic condition. 3) H/R group: as above design, cardiomyocytes were subjected to hypoxia for 3 h, followed by reoxygenation for 3 h. 4) SFN+H/R group: cardiomyocytes pretreated with 5 μmol/L SFN were exposed to 3 h of hypoxia followed by 3 h reoxygenation. SFN was pretreated 1 h before hypoxia.

Step 4: To explore the role of SIRT1 in myocardial protection with SFN pretreatment during H/R injury, cardiomyocytes were randomly divided into six groups (Figure 1): 1) control group; 2) Ex-527 (E) group; 3) H/R group; 4) Ex-527 (E)+H/R group; 5) SFN+H/R group; 6) SFN (5 μmol/L)+Ex-527 (E)+H/R group. Ex-527 was pretreated 1 h before hypoxia.

The concentration of Ex-527 is 1 μmol/L.

Cell viability analysis
Cell viability was determined by the MTS Cell Proliferation Assay kit as previously described\cite{17,18}. Cells were seeded at a density of 4×10^4 cells/well in a flat-bottomed 96-well plates. After the treatment, 20 μL MTS solution was added to each well and incubated for 3 h at 37 °C. The absorbance measurement was detected at 490 nm and used to calculate the relative ratio of cell viability.

LDH measurement
Lactate dehydrogenase (LDH) leakage is usually monitored to evaluate the extent of cell injury. After experimental treatment, 100 μL of culture medium was taken to assess LDH leakage is usually monitored to evaluate the extent of cell injury. After experimental treatment, 100 μL of culture medium was taken to assess LDH leakage is usually monitored to evaluate the extent of cell injury. After experimental treatment, 100 μL of culture medium was taken to assay cell injury. After experimental treatment, 100 μL of culture medium was taken to assay cell injury. After experimental treatment, 100 μL of culture medium was taken to assay cell injury. After experimental treatment, 100 μL of culture medium was taken to assay cell injury. After experimental treatment, 100 μL of culture medium was taken to

Detection of apoptotic cells
Evaluation of apoptosis was performed with a commercially available cell death detection kit to find DNA strand breaks using the terminal deoxynucleotidyl transferase-mediated dUDP nickend labeling (TUNEL) reagent according to the manufacturer’s protocol (Promega). Cells that showed positive TUNEL staining in the nuclei were identified as apoptotic. To further visualize fragmented nuclei, Hoechst 33258 staining was used to identify the morphological features of apoptosis as previously described\cite{19}. Cardiomyocytes were...
Nuclear proteins. Cardiomyocytes were incubated with fluorescent
Experimental protocol of step 4. Control and Ex-527 (E) group:
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The resulting fluorescence was measured at 340 nm excitation
Extraction kit according to the manufacturer’s instructions.
metric Assay kit as previously described
Sirt 1 activity was determined with SIRT1 Deacetylase Fluoro
SIRT1 activity analysis
The absorbance at 405 nm of the released pNA was monitored
caspase-3 assay kit (BioVision Inc, Milpitas Blvd, Calif,
Caspase-3 activity was measured by using a commercial
Measurement of caspase-3 activity
fixed with 4% paraformaldehyde (pH 7.4) for 1 h at room
temperature and washed three times in PBS. Cells were then
stained with Hoechst 33258 for 15 min and washed in PBS
times. Apoptotic cells with nuclear staining of Hoechst
33258 contained characters of apoptosis, condensed chromatin
and nuclear shrinkage. The percentage of apoptosis cells were
counted from each optical field as the apoptotic ratio in com-
parison to control group.
Determination of mitochondrial membrane potential (∆Ψm)
Mitochondrial membrane potential (MMP) was measured
with a cationic dye of 5,5′,6,6′-tetrachloro 1,1′,3,3′-tetraethyl-
benzimidazolcarboxyanaia iodide (JC-1) as previously
described[39]. Cardiomyocytes were incubated with fluorescent
probe JC-1 for 30 min at 37°C. After incubation, the cells were
washed in PBS for three times. Fluorescence was imaged at a
488 nm excitation wavelength, and emission signals were cap-
tured at 590 and 530 nm for red and green channels with con-
focal microscopy, separately, and the JC-1 ratio always refers
to the red/green ratio.
Measurement of caspase-3 activity
Caspase-3 activity was measured by using a commercial-
ized caspase-3 assay kit (BioVision Inc, Milpitas Blvd, Calif,
USA). Caspase-3 activity was expressed as optical density.
The absorbance at 405 nm of the released pNA was monitored
with a spectrophotometer. The relative activity of enzyme is
evaluated through the rate of absorption value in treatment
group and normal group. We suppose the activity in normal
group is 1.
SIRT1 activity analysis
Sirt 1 activity was determined with SIRT1 Deacetylase Fluoro-
metric Assay kit as previously described[39]. Nuclear proteins
were extracted using the Nuclear and Cytoplasmic Protein
Extraction kit according to the manufacturer’s instructions.
The resulting fluorescence was measured at 340 nm excitation
and 440 nm emission wavelengths with a fluorescent micro-
plate reader.
Western blotting analysis
Cardiomyocytes were washed by ice PBS for three times. Cell
lysates were centrifuged at 12 000 r/min at 4°C for 15 min, and
the supernatant was collected. The samples were denatured
by SDS sample buffer and in boiling water for 10 min, exposed
to 10% SDS-PAGE, and then electroblotted onto nitrocellulose
membranes. The membranes were blocked by 5% non-fat
milk for 2 h at room temperature. The membranes were incu-
bated overnight at 4°C with different primary antibodies. The
membranes were rinsed with TBS-T and incubated with the
secondary HRP-conjugated antibodies. GAPDH antibody was
used as the loading control. The data analysis were used with
Image-Pro Plus through measuring the densities of immuno-
reactive bands.
Statistical analysis
All of the values were expressed as the mean±SD and statis-
tically analyzed with SPSS 19.0. One-way ANOVA was
performed to test significance of biochemical data of different
groups. A difference of P<0.05 was considered to be statisti-
cally significant. For each assessment, at least three indepen-
dent experiments were performed.
Results
SFN alleviated H/R injury
To explore the optional reoxygenation time, cell viability of
different time durations was measured by MTS. As shown in
Figure 2A, cell viability significantly declined in a time-
dependent manner (P<0.05). The release of LDH was used
as an index of cardiomyocyte injury. LDH activity increased
with prolonged duration of reoxygenation (Figure 2B). Taken
together, to conduct our experiment with a relatively ideal
cell viability and to provide an accurate result, we decided the
optional duration for mimicking I/R is hypoxia 3 h and reoxy-
genation 3 h.
We next determined the effect of SFN on cardiomyocytes’
viability during H/R. The results demonstrated that H/R
caused a significant decline in cell viability compared with the
control group (P<0.05). Pretreatment with SFN (0.1, 0.5, 1, and
5 μmol/L) was found to increase cell viability significantly in a
dose-dependent manner. The cell viability went to the peak at
a concentration of 5 μmol/L (P<0.05 vs H/R group, Figure 2C
and 2D). Therefore, 5 μmol/L SFN was used in treated group
for subsequent experiments.
SFN attenuated H/R-elicited apoptosis in cultured neonatal rat
cardiomyocytes
As shown in Figure 3, representative photomicrographs of
TUNEL assay results depicted apoptosis of H/R-induced
cardiomyocytes. TUNEL staining showed that the apop-
totic cells increased remarkably in the I/R group com-
pared with the control group and SFN decreased apoptotic
cells obviously (P<0.05, Figure 3A and 3B). Caspase-3 has
been known as a pivotal protein in the final pathway of apoptosis\cite{22}. To further characterize the inhibitory effect of SFN on myocardial cell apoptosis, we examined whether SFN could suppress caspase-3 activity. In our present study, the activity of caspase-3 was significantly greater in H/R group than that in control group (P<0.05). However, SFN could decrease the activity of caspase-3 which was induced by H/R injury (P<0.05, Figure 3C). Therefore, SFN could attenuate H/R-induced apoptosis in cultured neonatal rat cardiomyocytes.

SFN preserved the cardiomyocytes against H/R injury partly through the attenuation of ER stress-induced apoptosis activation

We then moved to investigate the underlying mechanism of the protective effects offered by SFN. As shown in Figure 4A and 4B, in accordance with ER stress-dependent apoptosis activation, H/R remarkably suppressed Bcl-2/Bax ratio compared with control group (P<0.05). However, SFN pretreatment could elevate the ratio of Bcl-2/Bax (P<0.05).

As ER stress was often implicated in the activation of apoptosis during H/R injury, we next examined whether SFN preserved cardiomyocytes by modulating ER stress-dependent apoptosis. The levels of ER stress protein markers GRP78, CHOP and cleaved caspase-12 in H/R group were higher than those in H/R group (P<0.05), but lower in SFN and SFN+H/R groups compared with those in H/R group (Figure 4A, 4C, 4D and 4E, P<0.05).

SFN reversed the decrease in mitochondrial membrane potential (ΔΨm) and cell viability of neonatal rat cardiomyocytes after H/R injury

Mitochondria plays an essential function in cell apoptosis during H/R injury. The decline of ΔΨm was regarded as an early event in the apoptotic cascade\cite{23}. The ΔΨm and cell viability were decreased after H/R injury. SFN reversed the decrease in ΔΨm and cell viability of neonatal rat cardiomyocytes after H/R injury (P<0.05). Specific SIRT1 inhibitor Ex-527 blocked the effect of SFN (P<0.05, Figure 5).

SFN pretreatment increased the expression of SIRT1 protein

To investigate whether SFN could activate SIRT1 signal pathway, we detected the protein expression of SIRT1 by Western blotting. The expression of SIRT1 increased significantly in SFN pretreated group compared with the H/R group (P<0.05, Figure 4A, 4F). Specific SIRT1 inhibitor Ex-527 could block the effect (P<0.05, Figure 6A and 6F). However, no significant difference in SIRT1 expression was detectable between control group and H/R group (P>0.05, Figure 6A and F). Meanwhile, SFN significantly elevated the ratio of Bcl-2/Bax in cardiomyocytes exposed to H/R injury (P<0.05). Compared with
the H/R+SFN group, the expression of SIRT1 and the ratio of Bcl-2/Bax were markedly decreased in H/R+SFN+Ex-527 group (P<0.05, Figure 6A and 6F). Thus, SIRT1 pathway was involved in the anti-apoptotic effects of SFN on neonatal rat cardiomyocytes.

SIRT1 pathway was involved in modulating ER stress-induced apoptosis activation

To explore whether SIRT1 was involved in the cardioprotective effect of SFN against ER stress, we used SIRT1-specific inhibitor Ex-527 to block SIRT1 pathway. SFN pretreatment decreased the up-regulation of GRP78, CHOP and cleaved caspase-12 expression levels in neonatal rat cardiomyocytes after H/R injury (P<0.05). Ex-527 partially abolished the cardioprotective effects of SFN against ER stress-induced apoptosis after H/R injury (P<0.05, Figure 6A, 6C-6E).

SFN pretreatment increased SIRT1 activity to attenuate the H/R injury

To further verify our results, we detected the SIRT1 activity in different groups. We found that H/R inhibited the SIRT1 activity compared with control group (P<0.05). SFN pretreatment elevated the downregulation of SIRT1 activity of neonatal rat cardiomyocytes exposed to H/R injury (P<0.05). Ex-527 partially blocked the effect of SFN (P<0.05, Figure 7).

Discussion

In the present study, we demonstrated SFN could alleviate H/R injury by suppressing ER stress-dependent apoptosis in primary neonatal rat cardiomyocyte. In addition, we further demonstrated that the cardioprotective effects of SFN depended on the activation of SIRT1 signaling pathway, which was shown to suppress ER stress and cardiomyocytes apoptosis in cardiomyocytes exposed to H/R.

SFN, an isothiocyanate that is present in cruciferous vegetables, has been shown to exert beneficial preventive effect for cardiovascular diseases[9, 24, 25]. However, the mechanism still remains unclear. Mounting evidence indicates that apoptosis results in post-ischemic cardiomyocyte death[26]. In a previous study, SFN could protect cardiomyocytes against oxidative damage[27]. Our present study found SFN had anti-apoptotic effects in primary neonatal rat cardiomyocytes undergoing H/R.
increased cell viability and reduced LDH release in cardiomyocytes subjected to H/R. Anti-apoptotic effect with TUNEL staining assay indicated that SFN might exert a protective effect against apoptosis undergoing H/R. Caspase-3 is one of the key executioners of mammalian cell apoptosis. In this study, SFN also significantly suppressed caspase-3 activity. In short, these data indicated that SFN may have a beneficial effect on cardiomyocytes subjected to H/R.

We investigated the effect of SFN on ER stress in cardiomyocytes undergoing H/R injury. The ER is an important membranous organelles in all eukaryotic cells, which is extremely sensitive to the stimulation of ischemia and hypoxia.[28] Studies have shown that ER stress is a pivotal pathophysiological mechanism of myocardial ischemia reperfusion (I/R) injury.[29-31]

In addition, some studies demonstrated that SFN reduces ER stress in different models of cell injury. SFN reduces ER stress in type 1 diabetic mouse model accompanying with a reduction of testicular apoptotic cell death.[32] Similar effects were also observed in human hepatocytes.[33] Consistent with previous findings, we found that the expression levels of ER stress-associated apoptosis proteins, including GRP78, CHOP and cleaved caspase-12, were upregulated in cardiomyocytes exposed to H/R. Interestingly, pretreatment with SFN decreased the expression levels of GRP78, CHOP and cleaved caspase-12. Meanwhile, the results were in agreement with changes of Bcl-2/Bax ratio. Therefore, the results indicated that SFN partially protected cardiomyocytes against H/R injury via mitigating ER stress-dependent apoptosis.

Figure 4. Sulforaphane (SFN) inhibited ER stress-induced apoptosis and elevated the expression of SIRT1 in cardiomyocytes after H/R injury. (A) The expression of ER stress-related apoptotic proteins were measured by Western blotting. (B–F) ER stress-related apoptosis proteins and SIRT1 were quantified in cardiomyocytes after H/R injury. Data are expressed as Mean±SD of three independent experiments. *P<0.05 vs control; #P<0.05 vs H/R.
The anti-apoptotic effects of the SIRT1 have been demonstrated by several studies in myocardial I/R injury. Previous studies have verified that some drugs such as curcumin, melatonin and sildenafil can protect cardiomyocyte against I/R injury by modulating SIRT1 signaling\cite{13, 34, 35}. In the present study, we paid attention to the effect of SIRT1 pathways in SFN-elicited protection. First of all, we detected the protein expression of SIRT1 by Western blotting. The expression of SIRT1 increased significantly in SFN pretreated group compared with the H/R group. Specific SIRT1 inhibitor Ex-527 could block the effect. However, there was no significant difference in SIRT1 expression between control group and H/R group. To further verify our results, we detected the SIRT1 activity and found that SFN elevated SIRT1 activity and Ex-527 decreased SIRT1 activity to abrogate the effect of SFN. Moreover, H/R decreased the SIRT1 activity compared with control group. Consistent with our findings, Liu et al. demonstrated that exogenous NAD$^+$ supplementation restored SIRT1 activity without changing SIRT1 expression under the H/R condition in H9c2 cardiac myoblasts\cite{21}. Some other studies reported that myocardial I/R insult decreased SIRT1 expression\cite{36}. It may be resulted from different experimental conditions and model making time\cite{37}. Our results showed that SFN pretreatment remarkably elevated the SIRT1 activity compared with the H/R group. Ex-527, the inhibitor of SIRT1, blocked the cardioprotective effect of SFN against apoptotic cell death during H/R injury, including reducing cell viability and increasing apoptotic cardiomyocytes by measuring mitochondrial membrane potential. Consistent with previous studies, our study also showed that SIRT1 activation played an essential function in modulating antiapoptotic signals by upregulating the ratio of Bcl-2/Bax. In this study, our results indicated that SIRT1 activation and ER stress were closely related during H/R injury-elicited apoptotic process. SFN pretreatment elevated the expression of SIRT1, which was associated with the ER stress. The function of the SIRT1 pathway in the mediation of cardioprotective effect of SFN against H/R-elicited ER stress was further verified by the SIRT1 inhibitor Ex-527. Inhibition of SIRT1 activity contributed to upregulation of ER stress protein markers GRP78, CHOP and Cleaved caspase-12. Moreover, the cardioprotective effect of SFN was suppressed. Therefore, these results manifested that SFN can mitigate ER stress-induced cardiomyocyte apoptosis by activating of SIRT1 signal pathway during H/R injury. However, we should acknowledge that there are still some limitations in the present study. First, our experiment was limited in vitro, which requires further study in vivo. In addition, the underlying mechanisms by which SFN mitigated ER stress remains

Figure 5. Effects of sulforaphane (SFN) on mitochondrial membrane potential and cell viability in primary neonatal rat cardiomyocytes undergoing H/R and SIRT1 specific inhibitor Ex-527 (E) blocked the effects of SFN. (A, B) Mitochondrial membrane potential was detected by JC-1 staining in cardiomyocytes. (C) Cardiomyocyte viability was assessed with MTS assay. Data are expressed as Mean±SD of five independent experiments. *P<0.05 vs control; ^P<0.05 vs H/R; ^P<0.05 vs H/R+SFN.
poorly defined and needs further study.

In conclusion, the study demonstrated that SFN can protect cardiomyocytes against H/R injury and the underlying mechanisms of SFN-mediated cardioprotection may be dependent on the activation of SIRT1 signaling pathway, which alleviated ER stress-induced apoptosis. Our findings provides a preclinical evidence of the cardioprotective effect of SFN in H/R injury. Due to its efficacy, we suggest that SFN could be considered as a potential pharmacological approach to limit myocardial ischemic reperfusion damage.

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Author contribution
Ai-hua CHEN and Li-zi WANG designed the research; Yun-peng LI, Shu-lin WANG, Bei LIU and Lu TANG performed the research; Xian-bao WANG, Rong-ren KUANG, Cong ZHAO and Xue-ming CAO contributed new analytical tools and reagents; Yun-peng LI, Xu-dong SONG, Xiang WU and Ping-zhen YANG analyzed data; Yun-peng LI wrote the paper.

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Figure 6. SIRT1 specific inhibitor Ex-527 (E) abolished the protective effects of SFN against ER stress-induced apoptosis in cardiomyocytes after H/R injury. (A) The expression of ER stress-related apoptotic proteins under different conditions. (B–F) ER stress-related apoptosis proteins and SIRT1 were quantified in cardiomyocytes after H/R injury. Data are expressed as Mean±SD of five independent experiments. \( ^bP<0.05 \) vs control; \( ^eP<0.05 \) vs H/R; \( ^hP<0.05 \) vs H/R+SFN.
Figure 7. SIRT1 activity under different conditions. (A) SFN could activate SIRT1 activity to attenuate H/R injury; (B) Ex-527 (E) decline SIRT1 activity to abolish the cardioprotective effect of SFN. Data are expressed as Mean±SD of five independent experiments. *P<0.05 vs control; **P<0.05 vs H/R; ***P<0.05 vs H/R+SFN.

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