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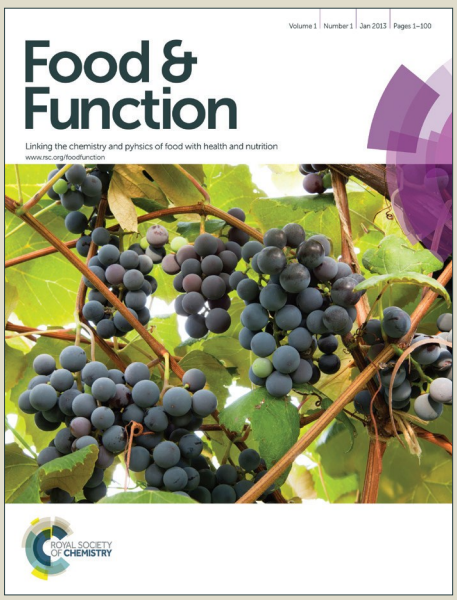

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Naringin protects against anoxia/reoxygenation-induced apoptosis in H9c2 cells via the Nrf2 signaling pathway

Chen RC[1], Sun GB[1*], Wang J[2], Zhang HJ[3] and Sun XB[1*]

[1]*Key Laboratory of Bioactive Substances and Resources Utilization of Chinese Herbal Medicine, Ministry of Education, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, P. R. China,* [2]*Research Center of Life Science and Environment Science, Harbin University of Commerce, Harbin, P. R. China,* [3]*Department of Pharmacology, Heilongjiang University of Chinese Medicine, Harbin, Heilongjiang, P. R. China*

[*]Correspondence: Xiaobo Sun and Guibo Sun, Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Sciences and Peking Union Medical College, No. 151, Malianwa North Road, Haidian District, Beijing, 100193, P. R. China. Tel: +86-10-62898496. Fax: +86-10-62898496. E-mail: sunxiaoboyzs@163.com.cn (XB Sun) and sunguibo@126.com.cn (GB Sun)

Abstract

Naringin (Nar) is a major and active flavanone glycoside derivative of several citrus species. The antioxidant properties of Nar have an important function in its cardioprotective effects in various models. However, the effects of Nar on Nrf2 activation and expressions of its downstream genes in myocardial cells have yet to be elucidated. This study was designed to investigate the protective effects of Nar against anoxia/reoxygenation (A/R)-induced injury in H9c2 cells and determine its effects on the activity of Nrf2 and the expression of phase II antioxidant enzymes. H9c2 cells were pretreated with Nar for 6 h before exposure to A/R. A/R treatment severely injured the H9c2 cells, which was accompanied by apoptosis. Nar also suppressed the A/R-induced mitochondrial membrane depolarization and caspase-3 activation. Nar pretreatment significantly reduced the apoptotic rate by enhancing the endogenous anti-oxidative activity of superoxide dismutase, glutathione peroxidase, and catalase, thereby inhibiting intracellular reactive oxygen species generation. Moreover, the presence of Nar alone in H9c2 cells increased the nuclear translocation of Nrf2 in a dose- and time-dependent manner, as well as consistently increased the protein levels of hemeoxygenase (HO-1) and glutamate cysteine ligase (GCLC). Nar increased the phosphorylation of ERK1/2, PKCδ, and AKT. However, the Nar-mediated Nrf2 activation and

cardioprotection were abolished through the genetic silencing of Nrf2 by siRNA and partially inhibited by specific inhibitors of ERK1/2, PKCδ, and AKT. Therefore, Nar provided cardioprotection by inducing the phosphorylation of ERK1/2, PKCδ, and AKT, which subsequently activated Nrf2 and its down-stream genes.

Keywords oxidative stress, apoptosis, Nrf2, phase II antioxidant enzymes, H9c2 cells

1. Introduction

Acute myocardial infarction (AMI) is a severe cardiovascular disease that remains a key contributor to death and disability worldwide [1]. The common therapeutic strategy for AMI in clinical practice is coronary reperfusion. However, sudden reperfusion to the ischemic myocardium may cause cell apoptosis and death, which ultimately leads to myocardial injury. This phenomenon is called myocardial ischemia/reperfusion (I/R) injury, which is inevitable and limits myocardial salvage [2]. A number of studies have indicated that myocardial apoptosis is initiated shortly after the onset of ischemia; the process becomes evidently enhanced during reperfusion, which is strongly associated with heart failure [3]. Therefore, inhibition of cardiomyocyte apoptosis is important to improve heart function and attenuate cardiac injury associated with reperfusion [4]. Previous studies have revealed that oxidative stress serves an important function in I/R-induced cardiomyocyte apoptosis [5]. Reactive oxygen species (ROS) generation has been observed during I/R exposure in cultured cells and cardiac tissues of mice [6-8]. ROS is a major factor generated at reperfusion that activates multiple molecular cascades of apoptosis [9,10]. Both in vitro and in vivo experiments showed that administration of antioxidants effectively protects against oxidative cardiovascular disorders [11,12].

Nrf2 is a member of the basic leucine zipper transcription factor family; this transcription factor is responsible for upregulating transcriptional antioxidant proteins and phase II detoxifying enzymes [13]. Under normal conditions, Kelch-like ECH-associated protein 1 (Keap1) is an ubiquitin ligase actin-binding protein that binds with Nrf2 in the cytoplasm and facilitates its ubiquitination and degradation [14,15]. The cysteine residues on Keap1 are oxidized when exposed to oxidants; this modification promotes the release of Nrf2 from the Keap1/Nrf2 complex and its translocation into

the nucleus [16]. After nuclear import, Nrf2 binds to antioxidant-response elements (AREs) to regulate the expression of its target genes, such as γ-glutamylcysteine synthetase (GCS), hemeoxygenase 1 (HO-1), glutathione S-transferase A1/2, NAD(P)H quinone reductase (NQO1), sestrin2 (Sesn2), and glutamate-L-cysteine ligase catalytic subunit (GCLC); these genes constitute a defense system against oxidative stress by scavenging free radicals [17]. Moreover, several studies have suggested that extracellular signal-regulated kinase (ERK1/2) [18], 5′ AMP-activated protein kinase (AMPK) [19], phosphatidylinositol-3-kinase (PI3K/AKT), and protein kinase C (PKC) [20] may have a central function in the activation of Nrf2. More recently, Nrf2 signaling has been demonstrated to be involved in the I/R-induced apoptosis of cardiomyocytes and cardiac tissues of mice [21].

Flavonoids are a group of polyphenolic compounds that are common components of the human diet. These compounds ubiquitously occur in foods of plant origin, such as vegetables, fruits, tea, and wine [22]. Naringin (Nar) is a major and active flavanone glycoside derivative of grape fruit and other related citrus species [23]. Studies have shown that Nar has multiple pharmacological properties, such as its hypolipidemic [24], anti-cancer [25], anti-inflammatory [23], cardioprotective [26,27], and anti-mutagenic [28] effects. Numerous reports have documented the direct free radical scavenger activity and indirect antioxidant properties of Nar, which serve an important function in its cardioprotective effects in various models [26,29,30]. However, the molecular mechanisms that underlie the protective effects of Nar against anoxia/reoxygenation (A/R)-induced cardiac insult remain poorly defined. Therefore, we tested the hypothesis that Nar could protect H9c2 cells against A/R-induced injury by inhibiting the activation of Nrf2 signaling.

2. Materials and methods

2.1. Cell culture and treatment

Rat embryonic cardiomyoblast-derived H9c2 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, USA) supplemented with 10% fetal calf serum at 37 ℃ with CO_2 incubation. In this study, we used oxygen and glucose deprivation (OGD) followed by

reperfusion to simulate myocardial A/R injury in vitro. H9c2 cells were incubated with glucose-free DMEM and exposed to a hypoxic environment of 95% N2 and 5% CO2 in airtight gas chambers at 37 ℃ for 4 h (CoyLaboratory, USA). Cells were removed from the gas chamber, and the OGD solution was replaced with normal culture medium for 24 h (recovery period) in a CO_2 incubator at 37 ℃. The cells were then used in subsequent experiments. In all experiments, cells were plated at an appropriate density according to the experimental design and used under 70% to 80% confluent. H9c2 cells were randomly divided into five groups: (1) the control group without any treatment; (2) the A/R group (model group), which was cultured under OGD for 4 h and then under recovery conditions for 24 h; and (3 to 5) the three A/R + Nar groups, which were pretreated with Nar at concentrations of 10, 20, or 40 μg/ml, respectively, for 6 h before OGD. Nar was purchased from Shanghai Winherb Medical S & T Development (>99% purity; Shanghai, China). In some experiments, the cells were exposed to PD98059 (40 μM), which is an inhibitor of ERK1/2 phosphorylation, LY294002 (10 μM), which is an inhibitor of AKT phosphorylation, or rottlerin (30 μM), which is an inhibitor of PKCα phosphorylation, for 30 min before Nar pretreatment. These inhibitors were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA).

2.2 Cell viability assay

Cell viability was determined by the MTT assay. H9c2 cells were seeded at a density of 1×10^4 cells/well in 96-well plates. After the different treatments, viable cells were stained with MTT (5 mg/ml) at 37 ℃ for 4 h. The medium was removed, and DMSO (150 ml) was added to each well to dissolve the formazan crystals. The optical density (OD) was determined at 570 nm with a microplate reader (Infinite M200 PRO; Switzerland). The survival ratio of H9c2 cells was expressed as a percentage of the control. In addition, the morphological changes in the H9c2 cells were observed under a microscope (Leica; Heidelberg, Germany).

2.3 LDH activity assay

H9c2 cells were cultured in 6-well plates at 3×10^5 cells/well. After treatment, the supernatant was used to measure the level of LDH release with an LDH assay kit, according to the manufacturer's

instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.4 Hoechst 33342/PI staining assay

H9c2 cells were cultured on cover slips in 24-well plates for 24 h. After treatment, the cells were incubated with 5 mg/ml of Hoechst 33342 at 37 ℃ for 15 min and washed twice with phosphate-buffered saline (PBS). The cells were then incubated with the PI working solution (20 μg/ml) for 1 min in the dark at room temperature and then immediately observed via fluorescence microscopy (Leica; Heidelberg, Germany).

2.5 Caspase-3 and caspase-9 activity assays

The caspase-3 and caspase-9 activity was measured using fluorometric assay kits (BioVision, USA) according to the respective instructions from the manufacturer. Briefly, different groups of H9c2 cardiomyocytes were lysed in the lysis buffer for 10 min on ice. Subsequently, 50 μl of the 2 × reaction buffer containing 10 mM dithiothreitol was added to each sample, and 5 μl of the substrate was added (1 mM DEVD-AFC or LEHD-AFC for caspase-3 or caspase-9, respectively). After incubation at 37 ℃ for 2 h, the samples were read on a Fluoroskan Ascent FL fluorometer (Thermo Fisher Scientific, USA) at 400 nm excitation and 505 nm emission wavelengths.

2.6. JC-1 staining assay

JC-1 (Invitrogen, USA) was used to determine the mitochondrial transmembrane potential. Briefly, H9c2 cells were cultured on cover slips in 24-well plates for 24 h. After treatment, cells were incubated with 1 μM JC-1 in the dark for 15 min and then washed twice with PBS. The cells labeled with JC-1 were observed by fluorescence microscopy (Leica; Germany). Moreover, flow cytometry was used to quantify changes in the mitochondrial membrane potential. The cells were suspended in warm medium at approximately 1×10^6 cells/ml. After staining with JC-1 and washing twice with PBS, the cells were analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ) using 488 nm excitation and green or orange-red emission wavelengths.

2.7. ROS staining assay

The production of intracellular ROS was monitored with a total ROS detection kit according to the manufacturer's instructions (Invitrogen, California). Briefly, H9c2 cells were cultured on cover slips in 24-well plates for 24 h. After treatment, cells were washed with the washing buffer and then incubated with ROS detection solution at 37 ℃ in the dark for 30 min and then visualized by fluorescence microscopy (Leica, Germany). We also harvested and suspended the cells in the washing buffer at approximately 1×10^6 cells/ml. After staining with the ROS detection solution, the cells were analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ).

2.8. Measurement of MDA levels and the activity of SOD, CAT and GSH-Px

H9c2 cells were cultured in 6-well plates at 3×105 cells/well. After treatment, the supernatant and the cells were collected to measure the MDA levels, as well as the SOD, CAT, and GSH-Px activity with the corresponding detection kit according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.9. Immunofluorescence Assay

Immunofluorescence was used to determine the cellular Nrf2 translocation. The cells were cultured on cover slips in 24-well plates for 24 h. After treatment, the cells were fixed with 4% paraformaldehyde for 30 min, washed twice with PBS, incubated in 0.1％Triton X-100 for 2 min on ice, and washed twice with PBS. The cells were then incubated with the antibodies against Nrf2 at a 1:50 dilution. Subsequently, the cells were washed and incubated for 2 h at room temperature with the FITC-conjugated anti-rabbit IgG at a 1:100 dilution. Cover slips were mounted on slides, and images of the labeled cells were visualized using fluorescence microscopy (Leica; Germany).

2.10. siRNA transient transfection

For siRNA inhibition studies, the cells were seeded into 6-well plates, cultured for 24 h, and transfected with Nrf2-specific siRNA (40 nM) or nonspecific siRNA using the Lipofectamine 2000 reagent according to the manufacturer's instructions. Transfection was conducted for 48 h. Subsequently, the cells were exposed to A/R injury after incubation with or without pretreatment

with Nar. Nonspecific siRNA was used to determine the efficiency of Nrf2-specific siRNA transfection.

2.11. Preparation of protein lysates

Cultured H9c2 cells were harvested, washed with PBS, and lysed with the cell lysis buffer containing 1% phenylmethylsulfonyl fluoride (PMSF). The lysate was centrifuged at 12000×g at 4℃ for 10 min to remove the insoluble materials. The supernatant containing the total protein extract was saved.

For nuclear Nrf2 detection, the nuclear protein fraction was extracted according to the manufacturer's protocol (Beyotime Institute of Biotechnology, Shanghai, China). After treatment, the cells were harvested and then washed with 1 × wash buffer. The cells were centrifuged at 1000×g for 5 min at room temperature, and the supernatant was discarded. The cells were lysed with the cytoplasmic protein extraction reagent for 30 min at 4℃ and then centrifuged at 12000×g for 10 min at 4℃. The supernatant (cytoplasmic protein extract) was carefully removed. The precipitate, which contained the cell nuclei, was washed twice with the washing buffer. The nuclei were lysed in the nuclear protein extraction reagent for 30 min at 4℃. The lysates were centrifuged at 12000×g for 10 min at 4℃. The supernatant, which contained nuclear protein, was saved. The protein concentration was determined using a BCA kit (Pierce Corporation, Rockford, USA) and stored at -80 ℃ until further use.

2.12. Western blot analysis

Equal amounts of protein fractions were separated by 12% SDS-PAGE and then transferred onto nitrocellulose membranes (Millipore Corporation, USA) in a Tris-glycine buffer at 100 V for 55 min. The membranes were blocked with 5% (w/v) non-fat milk powder in Tris buffer containing 0.1% (v/v) Tween-20 (TBST) by incubation for 2 h at room temperature. Subsequently, the respective membrane was incubated overnight at 4 ℃ with the following primary antibodies (Santa Cruz, CA): β-actin (1:2000); p-Akt1/2/3 (Ser 473; 1:500); Akt1/2/3 (1:500); p-ERK1/2 (Thr 202/Tyr 204; 1:500); ERK1/2 (1:500); p-PKCδ (Thr 507; 1:500); PKCδ (1:500); Nrf2 (1:500); lamin A (1:500); HO-1 (1:500); GCLC (1:500); caspase-3 (1:500); and caspase-9 (1:500). The

membranes were washed thrice with TBST, incubated with the respective secondary antibodies for 2 h at room temperature, and again washed thrice with TBST. Protein blots were developed using an enhanced chemiluminescence solution. Protein expression levels were visualized with the Image Lab software (Bio-Rad, USA).

2.13. Statistical analysis

Results from at least three independent experiments were expressed as mean±SE. Statistical comparisons between different groups were measured using Student's *t*-test or ANOVA with the Prism 5.00 software. Statistical significance was considered at $p<0.05$.

3 Results

3.1. Effects of Nar on cell viability in H9c2 cells

To evaluate the effect of A/R on the cell viability of cardiomyocytes, H9c2 cells were treated with different concentrations (10, 20, and 40 μg/ml) of Nar for 6 h. Subsequently, the cells were cultured under OGD for 4 h and then under recovery conditions for 24 h. As shown in Figure 2B, the cell viability decreased to 68.62% after A/R exposure, but its value increased to 74.54%, 83.36%, and 92.53% after pretreatment with 10, 20, and 40 μg/ml of Nar, respectively. Nar treatment alone had no effect on cell viability (Figure 2A). LDH may leak from cells after plasma membrane disruption and, thus, can be used as an indicator of cell injury. A/R treatment caused a significant increase in the LDH release of H9c2 cells, whereas pre-incubation with Nar markedly ameliorated the increased LDH release (Figure 2C). In addition, we also compared the changes in the H9c2 cell morphology of various experimental groups. As shown in Figure 2D, the cells of the control group appeared to have completely packed membranes, regular cell morphology, and round nuclei. By contrast, the cells in the A/R-treated group had incompletely packed membranes and shrinking cell nuclei. Nar pretreatment ameliorated these morphological changes in a dose-dependent manner.

3.2. Effects of Nar on cell death/apoptosis

Hoechst 33342/PI staining was used to distinguish the normal cells from the death or apoptotic cells. Hoechst 33342 stained all cells (live and dead), whereas PI-positive cells with condensed blue nuclei were regarded as dead/dying cells. Merged images of viable (blue) and apoptotic cells (pink) are shown in Figure 3A. A/R increased apoptotic rate in H9c2 cells, which was significantly alleviated by pretreatment with different concentrations of Nar (Figure 3B).

3.3. Effects of Nar on caspase-3 and caspase-9 activity

Caspase enzymes are major factors in the apoptotic cascade and serve key functions for the initiation of apoptosis in diverse biological processes. Our results showed that caspase-3 and caspase-9 activity significantly increased in the A/R group as compared with the control group. Pretreatment with Nar inhibited the activity of caspase enzymes in a dose-dependent manner (Figures 4A and 4B). Western blot analysis further confirmed the inhibition effects of Nar on caspase enzymes (Figures 4C and 4D).

3.4. Effects of Nar on mitochondrial membrane potential (ΔΨm)

The mitochondrion is a key organelle related to cell energy supply. Decreased mitochondrial membrane potential (ΔΨm) causes membrane depolarization and triggers a cascade of mitochondrion-dependent apoptosis. As shown in Figure 5A, mitochondria in normal H9c2 cells emitted a red fluorescence after JC-1staining. A/R exposure increased the amount of green fluorescence, which indicated the depolarization of the mitochondrial membrane potential. By contrast, pretreatment with Nar maintained the mitochondrial membrane potential. This effect of Nar was further confirmed by flow cytometry (Figure 5C).

3.5. Effects of Nar on ROS production

A fluorescence assay and quantitative flow cytometry approaches (Figure 5B) were used to determine the level of intracellular ROS, which exhibited green fluorescence under the microscope. We observed that A/R treatment significantly increased the intracellular ROS level in H9c2 cells, whereas pretreatment with Nar reduced the A/R-induced ROS production in a dose-dependent manner (Figure 5D).

3.6. Effects of Nar on MDA levels and the activity of SOD, CAT and GSH-PX

To measure the endogenous antioxidant capacity of H9c2 cells, we measured their MDA levels, as well as the SOD, CAT, and GSH-PX activity. MDA is a degradation product of membrane lipid oxidation, which is one of the primary events in oxidative damage. H9c2 cells exposed to A/R showed a significant increase in MDA levels, which was ameliorated by pretreatment with Nar. Moreover, A/R exposure induced a significant decrease in the activity of certain endogenous anti-oxidative enzymes, namely, SOD, CAT, and GSH-Px. However, pretreatment with Nar effectively increased activity of SOD, CAT, and GSH-Px in a dose-dependent manner. These results suggest that Nar may reduce oxidative stress injury by enhancing the endogenous anti-oxidant capacity of cells.

3.7. Effects of Nar on the expression of Nrf2 and its target genes

Western blot analysis was used to measure the expression of Nrf2 and its target genes in H9c2 cells. As shown in Figure 7A, A/R induced a moderate increase in the nuclear Nrf2 expression of H9c2 cells. Compared with A/R-treated group, Nar pretreatment could significantly increase the nuclear Nrf2 expression in H9c2 cells. We then examined the protein levels of GCL and HO-1, which are well-known target genes of Nrf2. In accordance with the change in Nrf2, the protein expression of GCLC and HO-1 was moderately increased with A/R exposure and significantly increased in Nar and A/R co-treated H9c2 cells (Figure 7A). More importantly, treatment with Nar alone could induce a significant increase in the nuclear Nrf2 translocation and the protein expression of GCLC and HO-1 in a dose-dependent manner (Figure 7B). The immunofluorescence assay further confirmed that Nar (40 μg/ml) induced the nuclear translocation of Nrf2 as compared with the control group (Figure 7C).

3.8. Effects of Nrf2-siRNA transfection on nuclear Nrf2 translocation and its target genes in H9c2 cells

As shown in Figure 8A, Nrf2-siRNA transfection significantly inhibited the Nrf2 translocation induced by A/R or Nar, thereby indicating that Nrf2 signaling in H9c2 cells can be efficiently knocked down by siRNA. We then examined the expression of the antioxidant proteins GCLC and

HO-1 in H9c2 cells transfected with Nrf2-siRNA. The Nar-induced up-regulation of these antioxidant proteins was significantly inhibited by Nrf2-siRNA transfection in H9c2 cells (Figure 8B). Therefore, the Nar-induced up-regulation of GCLC and HO-1 is Nrf2-dependent.

3.9. Nrf2-siRNA transfection abolished the protective effect of Nar on A/R-induced injury in H9c2 cells

As shown in Figure 8C, A/R induced a significant decrease in cell viability. Nrf2-siRNA transfection had no influence on the cell viability but increased the sensitivity of cells to A/R. Moreover, Nrf2-siRNA transfection eliminated the Nar-induced attenuation of A/R-induced cell death in H9c2. A/R exposure significantly increased the caspase-3 activity. The inhibition of Nrf2 signaling caused a greater increase of caspase-3 activity (Figure 8D). Notably, the protective effect of Nar on A/R-induced apoptosis was lost in H9c2 cells transfected with Nrf2-siRNA. These results indicated that Nrf2 signaling serves an important function in the protective effect of Nar on A/R-induced apoptosis in H9c2 cells.

3.10. Effects of Nrf2-siRNA transfection on ROS production in H9c2 cells

As shown in Figure 9, A/R increased the ROS levels in H9c2 cells as compared with the control group. Exposure of H9c2 transfected with Nrf2-siRNA to A/R resulted in a significantly greater ROS generation. These results indicated that Nrf2 signaling played an important role in preventing the A/R-induced oxidative stress in H9c2 cells. Pretreatment with Nar significantly diminished the A/R-induced increase of ROS production in H9c2 cells. However, Nar had no effect on the A/R-induced ROS generation in H9c2 cells transfected with Nrf2-siRNA. These results suggested that the Nar-induced antioxidant response in A/R-exposed H9c2 cells is mediated by the Nrf2 signaling pathway.

3.11. Involvement of AKT, ERK1/2, and PKCδ in the nuclear translocation of Nrf2

We determined the time dependence of nuclear Nrf2 translocation in response to Nar (40 μg/ml). Nuclear Nrf2 levels increased at 0.25 h and reached the highest levels at 3 h after Nar treatment (Figure 10A). Subsequently, we examined the protein expression of total and phosphorylated

(active form) ERK1/2, PKCδ, and AKT at different time points to identify the signaling pathway responsible for Nrf2 activation. As shown in Figure 10B, Nar significantly increased the phosphorylation of ERK1/2, PKCδ, and AKT. However, Nar had no effects on the total levels of ERK1/2, PKCδ, and AKT. In addition, the chemical inhibitors of AKT (LY294002), ERK1/2 (PD98059), and PKCα (rottlerin) significantly inhibited the Nar-induced phosphorylation of ERK1/2, PKCδ, and AKT (Figure 10C), and interestingly, suppressed Nrf2, GCL, and HO-1 activation induced by Nar, respectively (Figure 10D). These results suggest that the Nar-induced activation of Nrf2 signaling may be mediated by the phosphorylation of ERK1/2, PKCδ, and AKT. Finally, the cell viability and caspase-3 activity were evaluated to assess whether these kinases were required for the protective effects of Nar. As shown in Figure 11, H9c2 cells exposed to A/R had a decreased cell viability and increased caspase-3 activity. Nar pretreatment could significantly prevent these changes. However, LY294002, PD98059, and rottlerin partially reversed the protection of Nar on these pathological processes in H9c2 cells. These results indicated that Nar protects H9c2 cells against A/R-induced injury by activating Nrf2 signaling, which is mediated by the activation of ERK1/2, PKCδ, and AKT.

4. Discussion

Nar is a flavanone that can be derived from almost all fruits. Previous studies have shown that Nar has a strong cardioprotective effect because of its powerful antioxidant properties [26,29]. Nar has been recently reported to reduce I/R-induced myocardial injury in vivo [30]. However, the mechanism of Nar protects against I/R-induced myocardial injury is still not well defined. In accordance with previous studies [31-33], we found that A/R induced a variety of pathological changes in H9c2 cells. These changes include death, apoptosis, oxidative stress, and mitochondrial insult, as evidenced by the decreased cell viability, increased LDH leakage and MDA levels in the culture medium, increased number of apoptotic cells and ROS production, and the up regulated activity and protein expression of caspase-3/-9 (Figures 2—5). However, Nar pretreatment of H9c2 cells mitigated all these changes induced by A/R. Our study was the first to demonstrate that the Nar-induced Nrf2 translocation participated in this protective process, which is mediated by the phosphorylation of ERK1/2, PKCδ, and AKT.

Excessive ROS damages various biomolecules via lipid peroxidation, protein oxidation, and DNA damage, thereby causing mitochondrial dysfunction, caspase-3 activation and cell apoptosis, which have important functions in I/R injury [34]. In accordance with a previous study [35], our present study demonstrated that A/R exposure significantly increased ROS production in H9c2 cells, thereby increasing the levels of MDA, which is an oxidative stress marker (Figure 5). However, Nar pretreatment significantly decreased the level of intracellular ROS and MDA production. Compared with the effects of A/R exposure, Nar significantly increased the levels of intracellular antioxidant enzymes, such as SOD, GSH-Px, and CAT (Figure 6). Our data indicated that Nar might protect A/R-induced H9c2 cells against injury by increasing the endogenous antioxidants of these cells.

HO-1 is a rate-limiting enzyme that degrades heme (a potent oxidant) to bilirubin (which has antioxidant properties) [36]. This important enzyme has been shown to reduce I/R-induced myocardial damage. GSH is a thiol-reduced form of glutathione, which is present in millimolar concentrations in most cells. The major function of GSH is the detoxification of xenobiotics and/or their metabolites. GCLC is a catalytic subunit of γ-GCS, which serves a key function in GSH biosynthesis [37]. As an adaptive survival response, HO-1 and GCLC are up-regulated in cells under oxidative stress. Previous studies showed that the expression of these enzymes is regulated by the Nrf2 signaling pathway [38]. Nrf2 signaling serves an important function in protecting H9c2 cells from oxidative stress injury [21,39]. Under normal conditions, Nrf2 is located in the cytoplasm and binds with Keap1. Nrf2 can be activated by a range of oxidative and electrophilic stimuli, including ROS, heavy metals, and certain disease processes [40]. The activated Nrf2 is released from Keap1 and translocated to the nucleus, where it activates the transcription of target genes, including HO-1 and GCLC [15]. In the present study, A/R exposure induced moderate activity of the Nrf2 signaling pathway and caused serious injury to H9c2 cells. However, pretreatment with Nar significantly increased Nrf2 translocation, as well as the protein expression of Nrf2 target genes, HO-1 and GCLC, and their enzymatic activity (Figures 7A and 7C). These results suggested that Nar alone may trigger the activation of Nrf2 signaling. Thus, we treated H9c2 cells with different concentrations of Nar and found that Nar activated the Nrf2 signaling pathway in a dose-dependent manner (Figure 7B). Both Nar and A/R treatment can

activate Nrf2 signaling but these stimuli have opposite effects on H9c2 cells, possibly because Nar and A/R induce Nrf2 signaling through different molecular mechanisms.

Nrf2-siRNA transfection was conducted to further investigate the function of Nrf2 in the protective effect of Nar against A/R-induced H9c2 injury. We demonstrated that the transient transfection of Nrf2-siRNA can significantly inhibit Nrf2 translocation (Figure 8A). Our study also showed that Nrf2 inhibition could decrease the protein expression of HO-1 and GCLC in A/R-exposed H9c2 with or without Nar (Figure 8B). These results suggest that the activation of Nrf2 signaling after Nar treatment was responsible for the induction of phase II antioxidant enzymes. Moreover, we discovered that the suppression of Nrf2 signaling abrogated the protective effect of Nar against A/R-induced cell injury. As shown in Figures 8C and 9, Nar induced an increase in cell viability, as well as a decrease in the caspase-3 activity and ROS levels of H9c2 cells exposed to A/R. However, Nrf2-siRNA transfection neutralized all these effects. These results demonstrated that the protection of Nar against A/R-induced oxidative stress and apoptosis in H9c2 cells is mainly mediated by the activation of Nrf2 signaling.

Previous studies have reported that Nrf2 signaling can be regulated by several protein kinases, such as PI3K/AKT, PKCδ, and ERK1/2 [20,41,42]. In our study, we investigated the effects of Nar on the Nrf2 translocation and phosphorylation of AKT, PKCδ, and ERK1/2 at different time points. The results showed that Nar increases Nrf2 translocation, which is accompanied by ERK1/2, PKCδ, and AKT phosphorylation (Figures 10A and B). The activators of AKT and PKCδ, namely, insulin-like growth factor I (IGF-I) and phorbol-12-myristate-13-acetate (PMA), were used to further confirm that Nar could activate the PKCδ and AKT signaling pathways. As shown in Figure S1, IGF-I (1 μg/ml) and PMA (10 ng/ml) increased the phosphorylation of AKT and PKCδ, respectively, which have the same role as Nar. These results demonstrated that Nar-induced activation of Nrf2 signaling might be mediated by the phosphorylation of AKT, PKCδ, and ERK1/2. To further verify our hypothesis, the chemical inhibitors of ERK1/2 (PD98059), PKCδ (rottlerin), or AKT (LY294002) were used in the experiments. As shown in Figure 10C, these inhibitors significantly inhibited the Nar-induced phosphorylation of AKT, PKCδ, and ERK1/2, respectively. Furthermore, these inhibitors partially inhibited the Nar-induced nuclear

accumulation of Nrf2 as well as the protein expression of HO-1 and GCLC (Figure 10D). A previous study showed that Nar could alleviate oxidative stress injury in H9c2 cells by activating the Nrf2 signaling pathway [43]. However, the relationship of Nrf2 activation and the phosphorylation of AKT, PKCδ, and ERK1/2 remained unclear. Our results proved that Nar-induced Nrf2 activation and its target gene expression via the phosphorylation of ERK1/2, PKCδ, and AKT. Moreover, MTT assays and caspase-3 activity measurements showed that PD98059, rottlerin, and LY294002 can reverse the Nar-mediated suppression of A/R-induced injury in H9c2 (Figure 11). These results further proved that the phosphorylation of AKT, PKCδ, and ERK1/2 participates in the protection exerted by Nar on A/R-exposed H9c2 cells.

However, our study has several limitations. ERK1/2 is activated by MAP/ERK kinase 1 (MEK1) and MEK2 (MEK2), which are members of the MAPK kinase (MAPKK) family. MEK1 and MEK2 are activated by MAPK kinase kinase (MAPKKK)-mediated phosphorylation, which subsequently activates ERK1/2. PD98059 is a non-ATP competitive MEK inhibitor and is usually used as an inhibitor of ERK1/2 [44]. However, PD98059 also inhibits the MEK5-ERK5 pathway. ERK5, similar to ERK1/2, has a role in the regulation of cell proliferation [45]. Our study did not evaluate the effect of Nar on the activity of ERK5. Rottlerin was previously identified as a selective PKCδ inhibitor by Gschwendt in 1994 [46]. However, other studies have demonstrated that rottlerin may not directly act on PKCδ but may produce similar effects through other signal pathways [47]. Besides, PKCα also plays an important function in regulating the activity of Nrf2 [48], which was not reflected in our study. Therefore, the mechanism of the Nar-induced activation of Nrf2 signaling needs further study.

In summary, this study clearly showed that Nar can protect A/R-induced H9c2 cells from death or apoptosis by alleviating oxidative stress injury, which may be mediated by the phosphorylation of ERK1/2, PKCδ, and AKT (Figure 12). Collectively, our results suggest that Nar could be considered as a candidate drug for the prevention and treatment of AMI.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content

and writing of the paper.

Acknowledgments

This work was supported by grants (Nos. 81374010) from the National Natural Sciences Foundation of China, the Major Scientific and Technological Special Project for "Significant New Drugs Formulation" (No. 2012ZX09501001-004), Program for innovation research team in IMPLAD (IT 1301), and the Graduate Innovation Foundation of Peking Union Medical College (No. 2013-1007-03). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Figure legends

Figure. 1. Molecular structure of Nar.

Figure. 2. Protective effects of Nar against A/R-induced cell death in H9c2 cells. Cell viability was measured by MTT or LDH assays. (A) Nar had no toxic effect on cell viability. (B) Nar preconditioning protected against the A/R-induced cell death in H9c2 cells. (C) Nar preconditioning reduced the A/R-induced LDH release in H9c2 cells. (D) Nar preconditioning protected against the A/R-induced morphological changes of H9C2 cells. The results were expressed as the mean ± SD of three independent experiments. * indicates significant differences from the control (P <0.05). # indicates significant differences from treatment with A/R alone (P <0.05).

Figure 3. Protective effects of Nar against A/R-induced apoptosis in H9c2 cells. (A) Apoptotic H9c2 cells were detected by Hoechst 33342/PI double staining. (B) Quantitative analysis of Hoechst 33342/PI double staining. The results were expressed as the mean ± SD of three independent experiments. * indicates significant differences from the control (P <0.05). # indicates significant differences from treatment with A/R alone (P <0.05).

Figure 4. Effects of Nar on caspase-3 and caspase-9 activity. (A and B) Caspase-3 and caspase-9 activity was measured by fluorometric assays. (C and D) Western blot analysis of cleaved caspase-3 and caspase-9. The results were expressed as the mean ± SD of three independent experiments. * indicates significant differences from the control (P <0.05). # indicates significant differences from treatment with A/R alone (P <0.05).

Figure 5. Effects of Nar on anti-oxidant enzyme activity. (A) Nar pretreatment decreased the activity of MDA in H9c2 cells exposed to A/R. (B, C, and D) Nar preconditioning prevented the A/R-induced decrease of SOD, CAT, and GSH-PX activity in H9c2 cells. The results were expressed as the mean ± SD of three independent experiments. * indicates significant differences from the control (P <0.05). # indicates significant differences from treatment with A/R alone (P <0.05).

Figure 6. Effects of Nar on the mitochondrial transmembrane potential and ROS production. (A) H9c2 cells stained by JC-1 were observed with fluorescence microscopy and analyzed by flow cytometry. (B) H9c2 cells stained by the ROS fluorescent dye were observed with fluorescence microscopy and analyzed by flow cytometry. (C) Quantitative analysis of JC-1 staining. (D) Quantitative analysis of ROS staining. The results were expressed as the mean ± SD of three independent experiments. * indicates significant differences from the control (P <0.05). # indicates significant differences from treatment with A/R alone (P <0.05).

Figure 7. Effects of Nar on Nrf2 signaling. (A) H9c2 cells were pretreated with Nar (10, 20, and 40 μg/ml) for 4 h,

and then treated with A/R. Cytosolic and nuclear proteins were prepared for western blot analysis. (B) The effect of different concentrations of Nar on the Nrf2 translocation and the protein expression of HO-1 and GCLC. (C) Immunofluorescence assays were used to detect the effect of Nar and A/R on Nrf2 translocation. The results were expressed as the mean ± SD of three independent experiments. * indicates significant differences from the control (P <0.05). # indicates significant differences from treatment with A/R alone (P <0.05).

Figure 8. Effects of Nrf2-siRNA transfection on the Nar-mediated activation of Nrf2 signaling and cardioprotection. After 48 h of Nrf2-siRNA transfection, H9c2 cells were treated with Nar (40 μg/ml) for 4 h and then exposed in A/R. (A) Nuclear Nrf2 translocation was determined by Western blot analysis. (B) Protein expression of HO-1 and GCLC was determined by Western blot analysis. (C) A/R-induced apoptosis was determined using an MTT assay. (D) Caspase-3 activity was measured using a fluorometric assay. The results were expressed as the mean ± SD of three independent experiments. * indicates significant differences from the control (P <0.05). # indicates significant differences from treatment with A/R alone (P <0.05).

Figure 9. Effects of Nrf2-siRNA transfection on ROS production. After 48 h of Nrf2-siRNA transfection, H9c2 cells were treated with Nar (40 μg/ml) for 6 h and then exposed in A/R. ROS production was detected by flow cytometry. The results were expressed as the mean ± SD of three independent experiments. * indicates significant differences from the control (P <0.05). # indicates significant differences from treatment with A/R alone (P <0.05).

Fig. 10. Effects of Nar on the activity of ERK1/2, PKCδ, and AKT. (A) Immunoblot analysis for Nrf2 translocation in H9c2 cells that had been incubated with 40 μg/ml Nar for 15 min to 6 h. (B) Immunoblot analysis for the phosphorylation of ERK1/2, PKCδ and AKT in H9c2 cells that had been incubated in 40 μg/ml Nar for 15 min to 6 h. (C) Immunoblot analysis for the activation of phosphorylation of ERK1/2, PKCδ, and AKT in cells treated with 40 μg/ml Nar for 6 h after incubation in PD98059, LY294002, and rottlerin, respectively, for 30 min. (D) Immunoblot analysis for the activation of Nrf2, HO-1, and GCLC in cells treated with 40 μg/ml Nar for 6 h after incubation with PD98059, LY294002 and rottlerin, respectively, for 30 min. The results were expressed as the mean ± SD of three independent experiments. * indicates significant differences from the control (P <0.05). # indicates significant differences from treatment with A/R alone (P <0.05).

Figure 11. Effects of ERK1/2, PKCδ and AKT inhibition on the protective properties of Nar in H9c2 cells. After incubation with PD98059, LY294002, and rottlerin for 30 min, H9c2 cells were treated with Nar (40 μg/ml) for 6 h and then exposed to A/R. (A) Cell viability was determined by the MTT assay. (D) Caspase-3 activity was measured by a fluorometric assay. The results were expressed as the mean ± SD of three independent experiments. * indicates significant differences from the control (P <0.05). # indicates significant differences from treatment with A/R alone (P <0.05). $ indicates significant differences from the Nar and A/R co-treatment group (P <0.05).

Figure 12. Schematic of the molecular mechanism responsible for the cardioprotective effects of Nar. Nar induces the activation of ERK1/2, PKCα, and AKT, which subsequently activates and facilitates the nuclear translocation of Nrf2. In the nucleus, activated Nrf2 upregulates its target genes, including HO-1 and GCLC, which subsequently inhibits A/R-induced intracellular ROS production, lipid peroxidation, protein oxidation, DNA fragmentation, mitochondrial membrane depolarization, and caspase-3 activation.

Figure 1

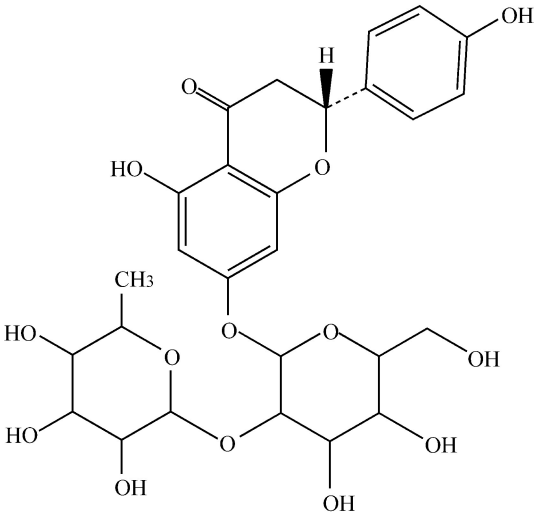

Figure 2

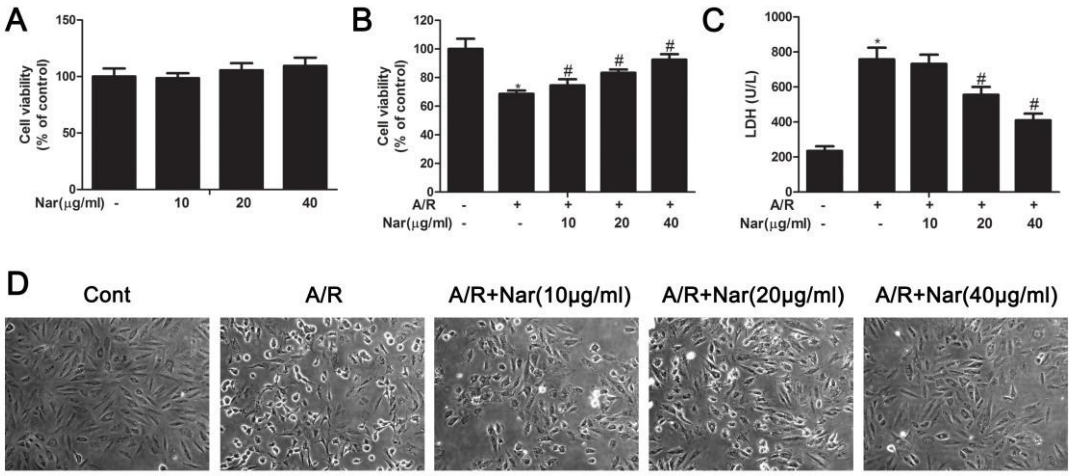

Figure 3

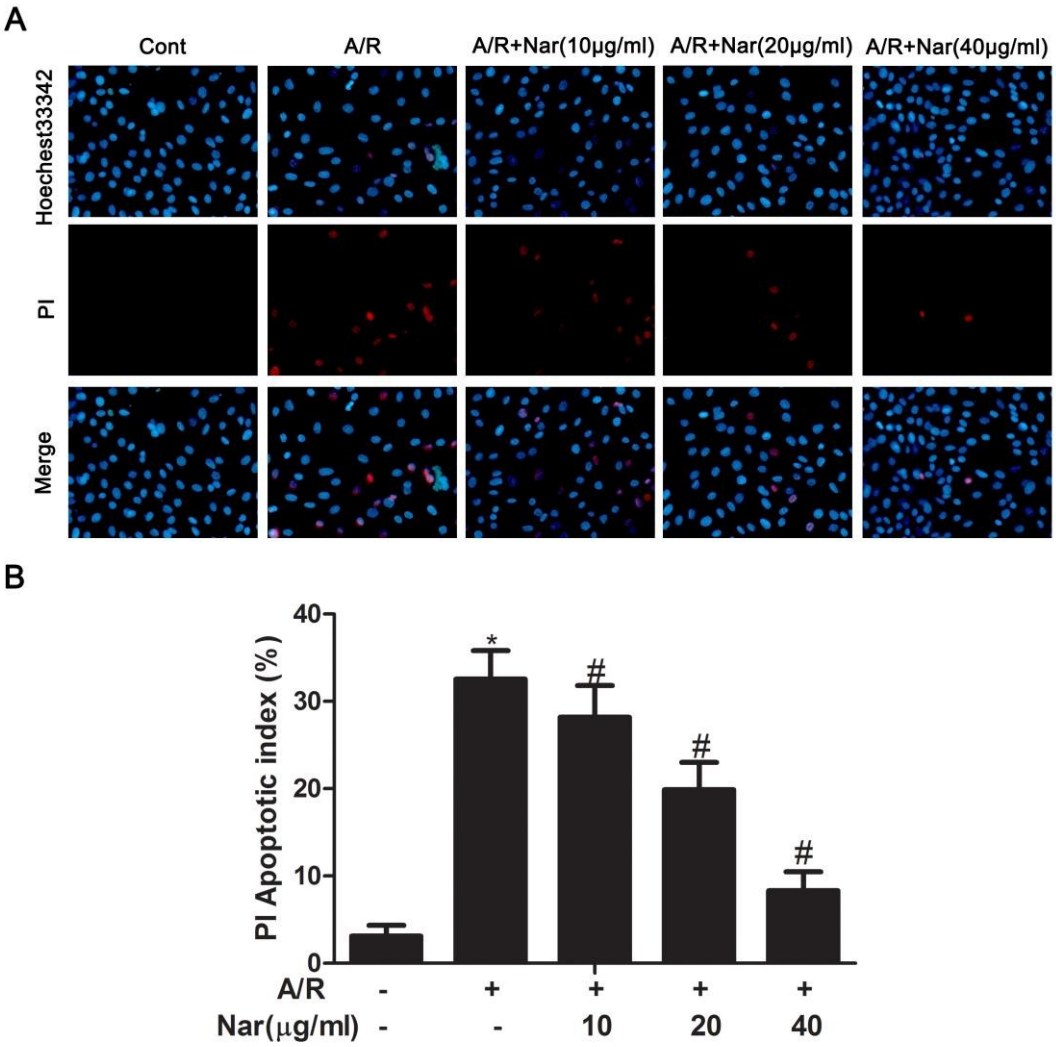

Figure 4

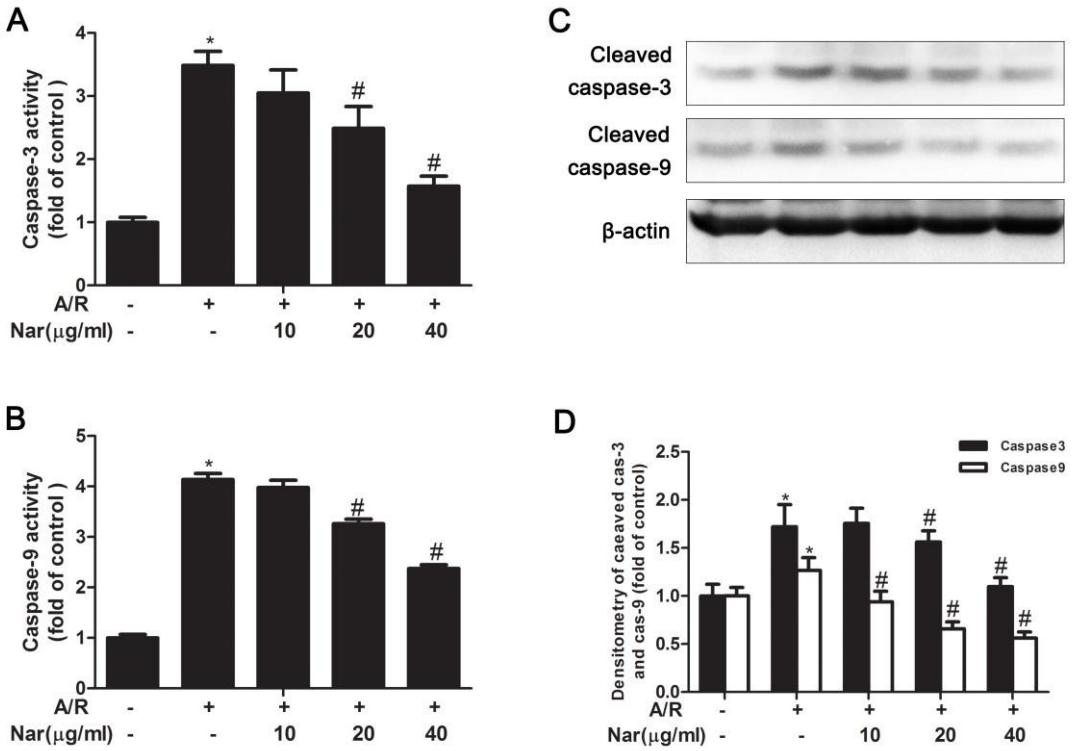

Figure 5

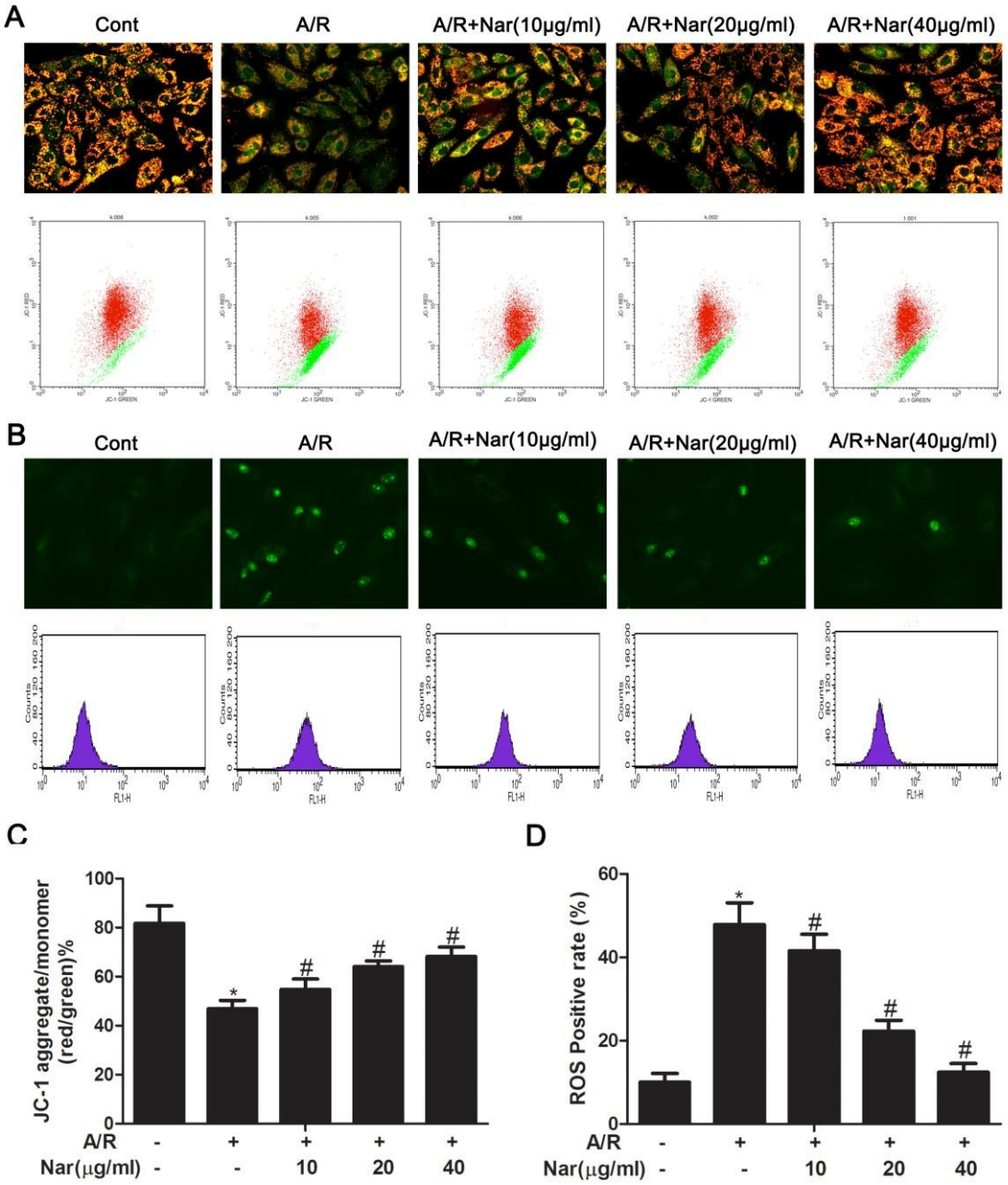

Figure 6

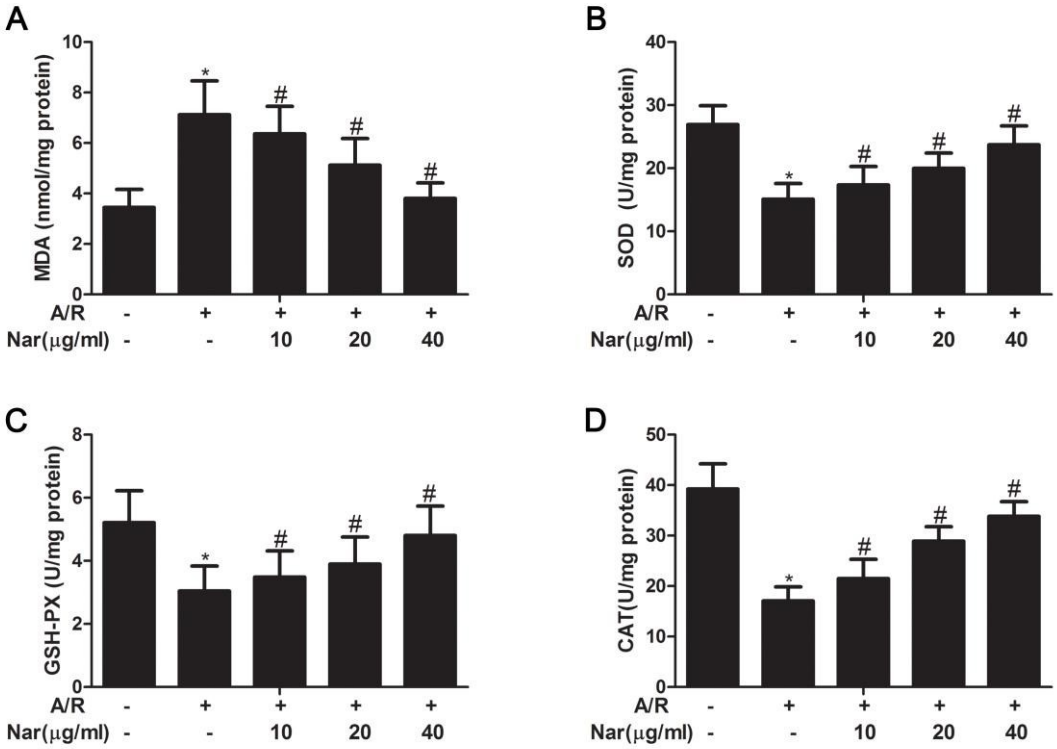

Figure 7

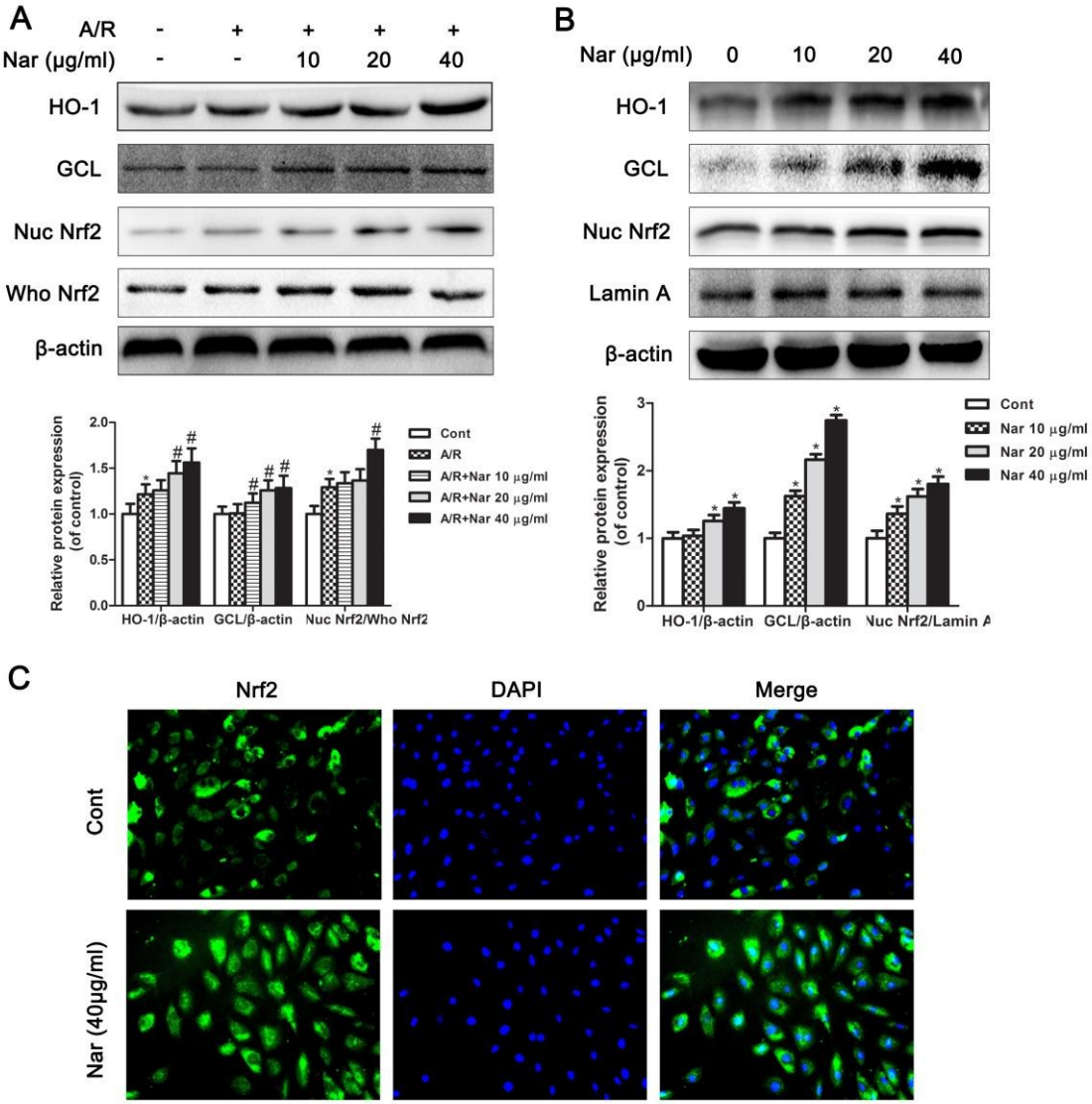

Figure 8

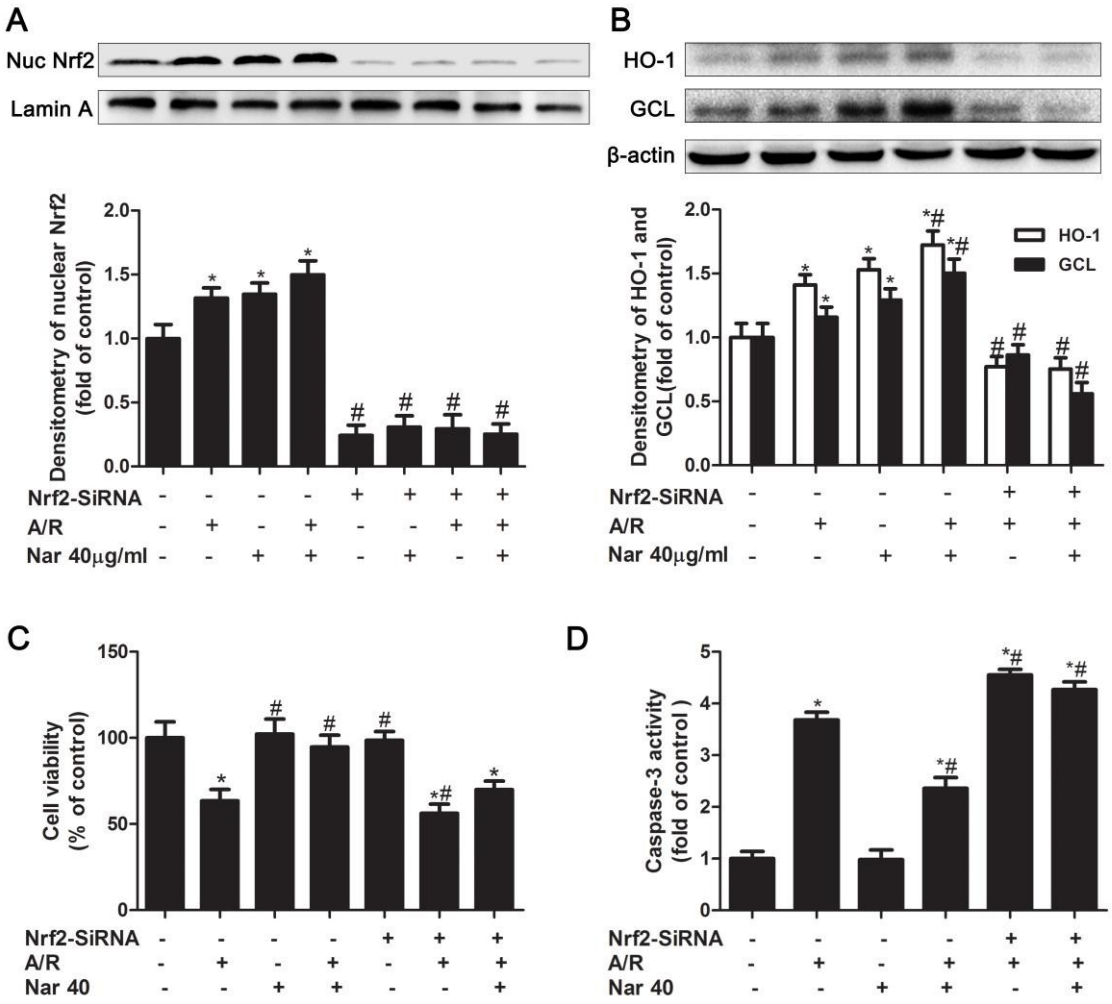

Figure 9

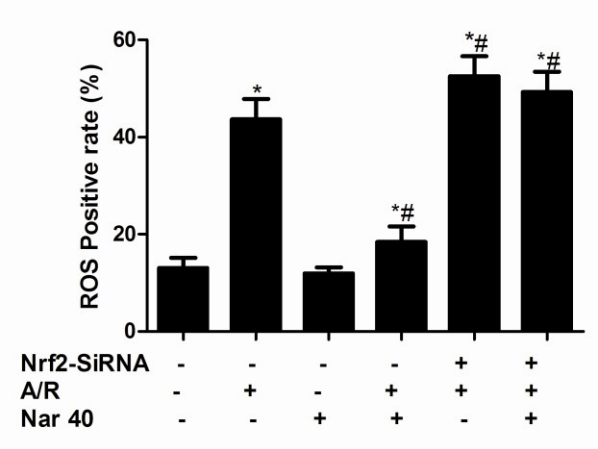

Figure 10

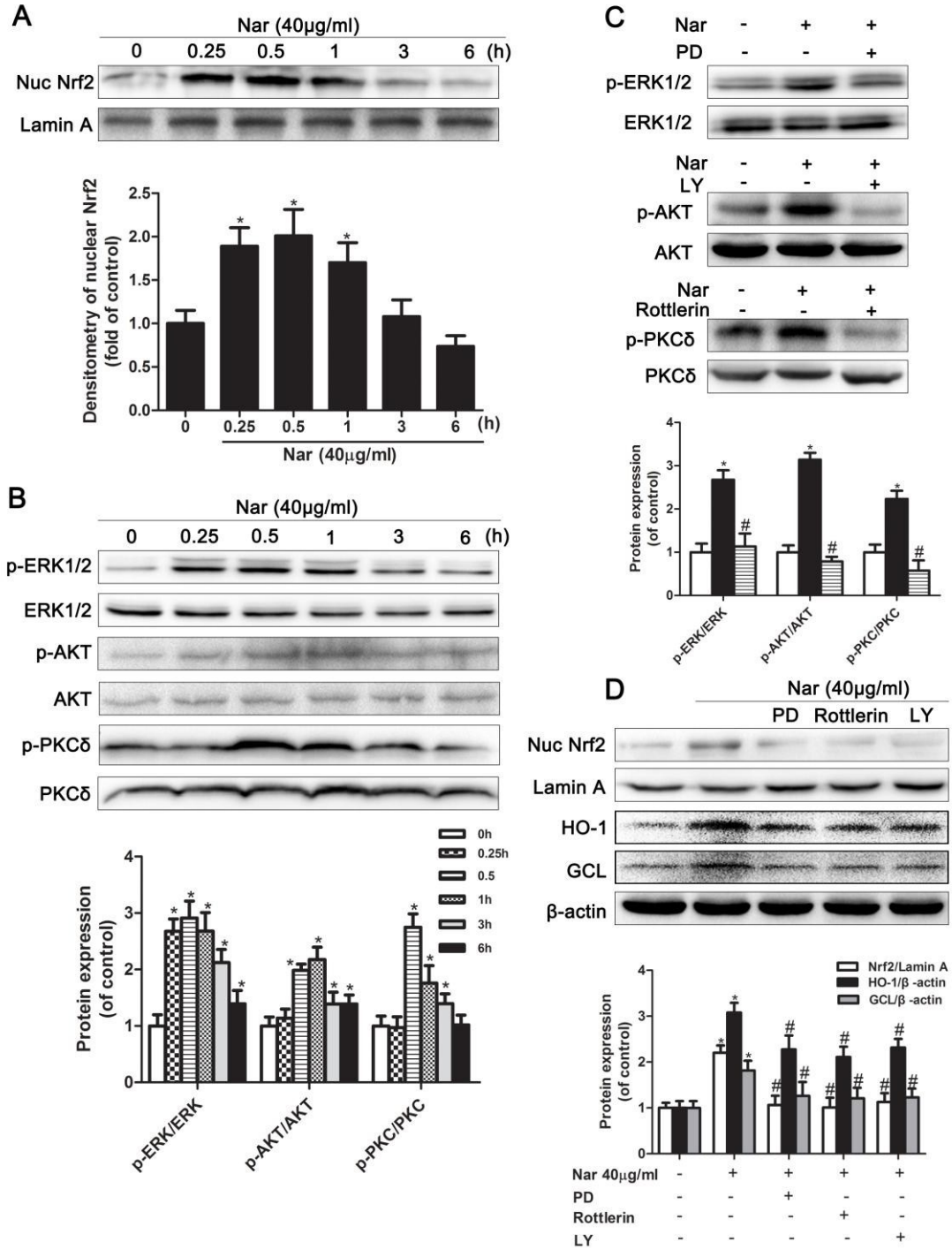

Figure 11

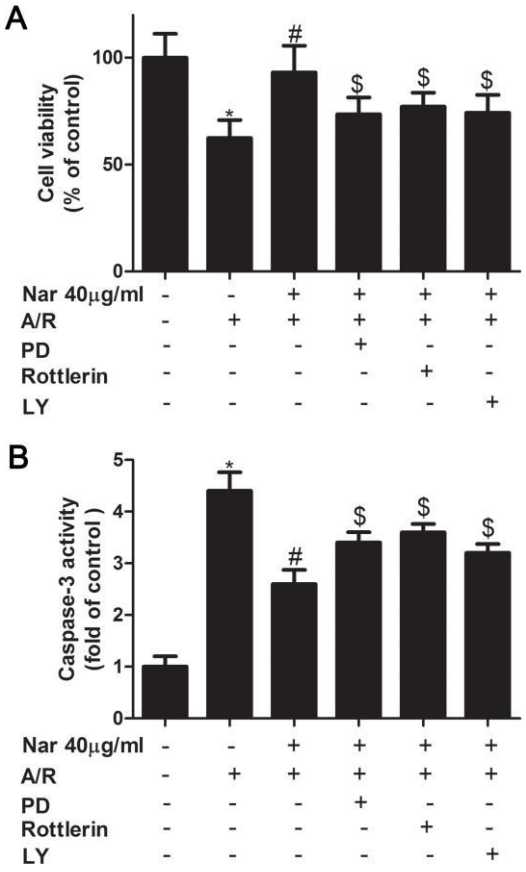

Figure 12