Original Article

Astragalus polysaccharide suppresses palmitate-induced apoptosis in human cardiac myocytes: the role of Nrf1 and antioxidant response

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Abstract: Objective: Previous studies have shown that Astragalus polysaccharides (APS) can be used to ameliorate cardiotoxicity due to chemotherapy and improve the cardiac function. However, the mechanism by which APS mediate this effect is unclear. In the present study, the effects of APS, which suppressed ROS-mediated apoptosis through Nrf1 accumulation in human cardiac myocytes (HCMs), was investigated. Methods: The cell viability was detected by the CCK8 assay. The cell apoptosis was assessed by annexin V-PI double-labeling staining. Expression of genes and proteins were analyzed by real-time PCR and western blotting respectively. Nrf1 gene was overexpressed using a lentiviral expression vector in HCMs in vitro, in order to explore the mechanism by which the Nrf1 promoted cell growth. Results: CCK8 and Annexin V-PI double-labeling showed that PAL induced cell death in a concentration-dependent manner, and suppressed HCMs proliferation. The combination PAL with APS was significantly decreased the percentage of the early phase of apoptosis cells. ROS levels were increased in HCMs by exposure to PAL. APS treatment significantly inhibited generation of ROS in response to palmitate. Moreover, PAL administration significantly decreased the mRNA and proteins expression of Bcl-2 as well as increased the mRNA expression of BAX and the protein expression of caspase-3 and caspase-8 as compare to those of control group, but APS treatment could reverse PA-induced HCMs apoptosis. The levels of reactive oxygen species (ROS), which was an oxidative stress marker, was significantly increased in cardiomyocytes by exposure to PAL, but overexpressing Nrf1 could ameliorate ROS-induced cardiomyocyte toxicity and increase the expression of SOD1 and SOD2 in HCMs by overexpressing Nrf1. Conclusions: This study demonstrated that the PAL could induce HCMs apoptosis. However, APS could reverse PAL-induced cardiomyocyte toxicity, at least partially, through suppression ROS and Nrf1 accumulation in HCMs.

Keywords: Palmitate, HCMs, ROS, APS, nuclear respiratory factor 1

Introduction

Progressive cardiomyopathy attends diverse stresses ranging from aberrant calcium signaling to inflammation to direct cardiomyocyte toxicity [1]. Reactive oxygen species (ROS), a product of normal cellular metabolism, are usually handled effectively by the cellular defense systems, thereby having little bearing on cellular health. Cellular redox balance is maintained by antioxidant enzymes, such as superoxide dismutase and catalase, and by signaling mechanisms to conserve a state of oxidative homeostasis [2]. However, under situations of exaggerated stress or hypoxia, the cellular defenses may be insufficient to overcome ROS overload. Oxidative stress has been clinically shown to be relevant in the progression of cardiac diseases and heart failure [3, 4]. Excess ROS can cause a variety of cellular damage including mitochondrial dysfunction, DNA damage, and ultimately lead to apoptosis with apoptosis of cardiomyocytes being critical in tissue damage and eventually heart failure. Hence, protection of cardiomyocytes and their increased survival is a putative target for cardioprotection [5].

Palmitate (PAL) is the main saturated free fatty acid in the bloodstream. The exposure of endothelial cells to PAL leads to cell necrosis [6] and the release of inflammatory cytokines IL-6 [7]. Moreover, PAL-induced increase in the genera-
tion of reactive oxygen species, the activation of NADPH oxidase, the up-regulation of inducible nitric oxide synthase (iNOS) and down-regulation endothelial nitric oxide synthase (eNOS) [8]. In vivo studies, the exacerbation of the progression of endothelial dysfunction was reported in C57BL/6 mice after long-term exposure to high-calorie and high-cholesterol diets [9]. Similarly, type 2 diabetic mice induced by a high-fat diet combined with a single injection of low-dose streptozotocin exposure to exacerbate coronary endothelial dysfunction and increase mitochondrial reactive oxygen species (ROS) concentration [10]. Increasing evidence suggests that PAL treatment can induce apoptosis in cardiomyocytes [11, 12]. Recent research has shown that PAL-induced loss of caveolin-3 results in cardiac contractile dysfunction via a defect in calcium-induced calcium release [13].

The dry roots of Astragalus membranaceus, which is also known as Huang Qi in China and belongs to the Fabaceae family, have long been used as an important component of many herbal prescriptions in traditional Chinese medicine [14, 15]. Astragalus polysaccharide (APS), the extract from Astragalus membranaceus, exerts strong anti-tumor [16] and effectively alleviates inflammation-induced artery endothelium cell injury [17] and atherosclerosis [18] and insulin resistance [19]. Moreover, APS is used to ameliorate doxorubicin-induced cardiotoxicity in mice [20] and inhibit isoprenaline-induced cardiac hypertrophy [21] and improve the cardiac function in Sjögren’s syndrome model rats [22]. However, whether APS could reverse PAL-induced cardiotoxicity is not understood. In the current study, we investigated the effects of APS on ROS and Nrf1 in HCMs in vitro.

Materials and methods

Cell culture

The Human Cardiac Myocytes (HCMs) were obtained from the Chinese Academy of Sciences (Institute of Shanghai Cell Biology and Chinese Type Culture Collection, China), and maintained in DMEM (Dulbecco’s modified Eagle’s medium; Invitrogen), supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 100 units/ml penicillin, and 100 mg/ml streptomycin (Invitrogen) at 37°C in a humidified, 5% CO₂, 95% air atmosphere. The medium was replenished every day. Confluent cells were treated with Astragalus polysaccharide (APS) at 200 μg/ml.

Cell viability detection by CCK8

HCMs (1.0 × 10⁵/well) were plated and treated in 96-well plates (three wells per group) with vehicle, PAL, APS (200 μg/ml) or the combination PAL with APS for 24, 48 or 72 hour respectively. 10 μL of CCK8 (Dojindo, Kumamoto, Japan) was added to the cells, and the viability of the cells was measured at 490 nm using an ELISA reader (BioTek, Winooski, VT, USA) according to the manufacturer’s instructions.

Measurement of ROS production

HCMs (1.0 × 10⁵ cells/well) in a 96-well plate were treated with or without APS for 1 h, followed by incubation with palmitate for 24 h. ROS generation was measured by incubation of the cells with 10mM DCFH-DA for 45 min. The fluorescence, corresponding to intracellular ROS, was measured on a Spectra Max M5 microplate reader (Molecular Devices, USA) at 485 nm excitation and 530 nm emission wavelengths.

Quantification of apoptosis by flow cytometry

Apoptosis was assessed using annexin V, a protein that binds to phosphatidylserine (PS) residues which are exposed on the cell surface of apoptotic cells. HCMs were treated with vehicle, PAL, APS (200 μg/ml) or the combination PAL with APS for indicated time intervals. After treatment, HCMs were washed twice with PBS (PH = 7.4), and re-suspended in staining buffer containing 1 μg/ml PI and 0.025 μg/ml annexin V-FITC. Double-labeling was performed at room temperature for 10 min in the dark before the flow cytometric analysis. Cells were immediately analyzed using FACScan and the Cellquest program (Becton Dickinson). Quantitative assessment of apoptotic cells was also assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick endlabeling (TUNEL) method, which examines DNA-strand breaks during apoptosis by using BD ApoAlert™ DNA Fragmentation Assay Kit. Briefly, cells were incubated with honokiol for the indicated times. The cells were trypsinized, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton-X-100 in 0.1% sodiumcitrate. After being washed, the...
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Figure 1. APS suppressed PAL-induced cell apoptosis in HCMs. HCMs were incubated with vehicle, PAL (50 mM), APS (200 μg/ml) or the combination PAL with APS for 24 h, 48 h or 72 h, and the cell viability was examined by CCK8 assay (A). Cells were treated with vehicle, PAL (50 mM), APS (200 μg/ml) or the combination PAL with APS for 48 h, the percentage of apoptotic cells was also analyzed by flow cytometric analysis of annexin V/PI double staining (B). The percentage of apoptotic cells is analyzed by flow cytometric analysis of annexin V/PI double staining (C). The intracellular levels of ROS were determined by fluorescence using a fluorescence microplate reader with excitation/emission set to 485/530 nm. Cells were plated at 1 × 10^5 cells/mL and pretreated with or without APS for 0.5 h, followed by incubation with or without PAL for 24 h (D). Values are expressed as mean ± SEM, n = 3 in each group. *P < 0.05, versus control group; #P < 0.05, versus PAL group.
cells were incubated with the reaction mixture for 60 min at 37°C. The stained cells were then analyzed with flow cytometer.

**Real time-polymerase chain reaction**

The Human hepatoma cells RNA extraction was performed according to the TRIzol manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). RNA integrity was verified by agarose gel electrophoresis. Synthesis of cDNAs was performed by reverse transcription reactions with 2 μg of total RNA using moloney murine leukemia virus reverse transcriptase (Invitrogen) with oligo dT (15) primers (Fermentas) as described by the manufacturer. The first strand cDNAs served as the template for the regular polymerase chain reaction (PCR) performed using a DNA Engine (ABI 7300). PCR with the following primers: Bcl-2, forward 5'-GTAGATGGGCCGTTCACTTTGTTGTC-3', reverse 5'-CGGGTTTCTCTGAAAACTCCGGAT-3'; BAX, forward 5'-AGCTATTAGGTTCGATTTTCCGAT-3'; reverse 5'-ATTGAGGAGGATCGTTAGTCTAG-3'; SOD1, forward 5'-GTAGATGGGCCGTTCACTTTGTTGTC-3'; reverse 5'-CGGGTTTCTCTGAAAACTCCGGAT-3'; SOD2, forward 5'-AGCTATTAGGTTCGATTTTCCGAT-3'; reverse 5'-ATTGGAGGAGGATCGTTAGTCTAG-3'; Nrf1, forward 5'-GCGGGCCGTTCACTTTGTTGTC-3', reverse 5'-GAGGAAGGATCGTTAGTCTAG-3'; GAPDH, forward 5'-GCGGGCCGTTCACTTTGTTGTC-3', reverse 5'-GAGGAAGGATCGTTAGTCTAG-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control was used to normalize the data to determine the relative expression of the target genes. The reaction conditions were set according to the kit instructions. After completion of the reaction, the amplification curve and melting curve were analyzed. Gene expression values are represented using the 2^(-ΔΔCt) method.

**Western blotting**

The HCMs were homogenized and extracted in NP-40 buffer, followed by 5-10 min boiling and centrifugation to obtain the supernatant. Samples containing 50 μg of protein were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After saturation with 5% (w/v) non-fat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with the following antibodies, cas-
pase3, caspase8, SOD1, SOD2, Bcl-2, BAX, Nrf-1 and 4-HNE (Santa Cruz Biotechnology, CA, USA), at dilutions ranging from 1:500 to 1:2,000 at 4°C over-night. After three washes with TBST, membranes were incubated with secondary immunoglobulins (Igs) conjugated to IRDye 800CW Infrared Dye (LI-COR), including donkey anti-goat IgG and donkey anti-mouse IgG at a dilution of 1:10,000-1:20,000. After 1 hour incubation at 37°C, membranes were washed three times with TBST. Blots were visualized by the Odyssey Infrared Imaging System (LI-COR Biotechnology). Signals were densitometrically assessed (Odyssey Application Software version 3.0) and normalized to the β-actin signals to correct for unequal loading using the mouse monoclonal anti-β-actin antibody (Bioworld Technology, USA).

**Transfection and selection of stable human hepatoma cells lines**

For the transfection of the HCMs, lentiviral vectors harboring Nrf-1 were constructed and the HCMs were infected. Briefly, the HCMs were cultured in McCoy’s 5α medium containing 10% FBS and when they reached the exponential growth phase, 1.0 × 10⁵ cells per well were plated in 96 plates. Next, 300 μl complete culture medium, containing recombinant lentiviruses, control lentiviruses or McCoy’s 5α medium (all containing 6 μg/ml polybrene; Sigma) was added into the plates when the cells reached 50-60% confluence. Two days later, the virus-containing medium was replaced with fresh complete medium.

**Statistical analysis**

The data from these experiments were reported as mean ± standard errors of mean (SEM) for each group. All statistical analyses were performed by using PRISM version 4.0 (GraphPad). Inter-group differences were analyzed by one-way ANOVA, and followed by Tukey’s multiple comparison test as a post test to compare the group means if overall P < 0.05. Differences with P value of < 0.05 were considered statistically significant.

**Results**

**APS suppressed PAL-induced cell apoptosis in HCMs**

To evaluate the potential cell apoptosis of PAL in HCMs, we analyzed the effect of PAL on cell survival in HCMs. The CCK8 assay was used to measure cell viability. The viabilities of HCMs treated with PAL were significantly lower than with combination therapy or those of untreated group (P < 0.05). Treatment of HCMs with PAL induced cell death in a time and dose-dependent manner by using CCK8 assay, but...
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Figure 4. PAL-induced HCMs apoptosis was suppressed by overexpressing Nrf1 in HCMs. HCMs were incubated with vehicle, PAL (50 mM), or overexpressing Nrf1 for 24 h, 48 h or 72 h, and the cell viability was examined by CCK8 assay (A). The percentage of apoptotic cells was also analyzed by flow cytometric analysis of annexin V/PI double staining (B). The percentage of apoptotic cells is analyzed by flow cytometric analysis of annexin V/PI double staining (C). The proteins expression of Nrf1 is measured by western blotting (D). Intracellular levels of ROS were determined by fluorescence using a fluorescence microplate reader with excitation/emission set to 485/530 nm (E). Values are expressed as mean ± SEM, n = 3 in each group. *P < 0.05, versus control group; #P < 0.05, versus PAL group.
APS could reverse PAL-induced cell apoptosis in HCMs (Figure 1A). We next investigated whether PAL induces cell death through an apoptotic mechanism. Annexin V-PI double-labeling was used for the detection of PS externalization, a hallmark of early phase of apoptosis. Consistent with the CCK8 assay, the results showed that the proportion of the early phase of apoptosis cells had gained as compared to untreated group (P < 0.05) (Figure 1B and 1C). Moreover, the percentage of the early phase of apoptosis cells with combination treatment was significantly lower than with PAL single treatment (P < 0.05). Oxidative stress has been implicated in the pathogenesis of cardiomyocyte. It is suggested that increased ROS levels are an important trigger for cardiomyocyte toxicity. We next investigated whether ROS levels were altered by palmitate and APS treatment. As shown in Figure 1D, ROS levels were increased in HCMs by exposure to PAL. APS treatment significantly inhibited generation of ROS in response to palmitate.

APS regulated apoptosis-related mRNA and proteins expression

The apoptotic response was further investigated by measuring apoptosis-related mRNA and proteins expression. PAL administration significantly decreased the mRNA and proteins expression of Bcl-2 as well as increased the mRNA expression of BAX and the protein expression of caspase-3 and caspase-8 as compared to those of control group (Figure 2A-D). However, the combination PAL with APS induced strong specific suppression of protein expression of caspase-3 and caspase-8 and mRNA expression of BAX as compared to those of PAL group (Figure 2B-D). Additionally, the expression of Bcl-2 was statistically up-regulated in the PAL combination with APS-treated group as compared to PAL single treatment group. Therefore, our data suggest that APS regulation apoptosis-related mRNA and proteins expression could reverse PA-induced HCMs apoptosis.

APS regulated Nrf1 and anti-oxidation genes expression

A genome-wide analysis has revealed that Nrf1 binding elements are present in genes involved in DNA replication, mitosis, and cytokinesis, suggesting that Nrf1 plays an important role in cell cycle regulation and apoptosis. We determined the mRNA and proteins expression of Nrf1 in HCMs by exposure to PAL (Figure 3A and 3B). The mRNA and protein level for Nrf1 were decreased as compared to those of control group. However, the combination PAL with APS increased the mRNA and protein expression of Nrf1 as compared to those of PAL group. 4-hydroxynonenal (4-HNE) was an oxidative stress marker, which increased in HCMs by exposure to PAL. APS treatment significantly inhibited the protein expression of 4-HNE in response to PAL (Figure 3B and 3C). At the protein level, we observed change in SOD1 and SOD2 in HCMs by exposure to PAL, however, APS treatment could reverse the protein expression of SOD1 and SOD2 (Figure 3B and 3C). The results in Figure 3 suggested that the antioxidant response was compromised in HCMs by exposure to PAL, but APS could ameliorate cardiomyocyte toxicity.

PAL-induced HCMs apoptosis was suppressed by overexpressing Nrf1

In this study we proposed that Nrf1 involved in PAL-induced HCMs apoptosis. The CCK8 assay shows that PAL-induced HCMs apoptosis was suppressed by overexpressing Nrf1 (Figure 4A). To investigate the anti-apoptosis effect of overexpressing Nrf1 in HCMs, the Annexin V-PI double-labeling results showed that the proportion of the early phase of apoptosis cells had gained with PAL treatment, but overexpressing Nrf1 could suppress early phase of apoptosis cells (Figure 4B and 4C). Moreover, in an attempt to explore the influence of PAL on Nrf1 in HCMs when PAL induce HCMs apoptosis. The protein expression of Nrf1 was significantly decreased in HCMs with PAL treatment group (Figure 4D). We considered that enhanced oxidative stress might be a possible cause of HCMs apoptosis. The levels of reactive oxygen species (ROS), which was an oxidative stress marker, was significantly increased in cardiomyocytes by exposure to PAL, but overexpressing Nrf1 could ameliorate ROS-induced cardiomyocyte toxicity (Figure 4E). The apoptotic response was further investigated by measuring apoptosis-related mRNA expression in recombinational HCMs. Overexpressing Nrf1 significantly increased the mRNA and protein expression of Bcl-2 as well as decreased the
mRNA and protein expression of BAX as compared to those of PAL group (Figure 5A, 5C and 5D). At the gene and protein level, we observed markedly increasing the expression of SOD1 and SOD2 in HCMs by overexpressing Nrf1 (Figure 5B-D). The results in Figure 5 suggested that the antioxidant response was accumulated in HCMs by overexpressing Nrf1.

Discussion

In this study, we undertook a comprehensive and integrative approach to explore the role of Nrf1 and antioxidant response in PAL-induced human cardiac myocytes apoptosis. We demonstrated that Nrf1 was associated with proliferation of human cardiac myocytes. According to the CCK8 assay and Annexin V-PI double-labeling staining, PAL induced cell death through an apoptotic mechanism, the proportion of the early phase of apoptosis cells had significantly gained, but the combination PAL with APS was markedly suppressed cell apoptosis and promoted cell proliferation. Moreover, the mRNA and protein expression of Nrf1 was suppressed with PAL treatment, but the combination PAL with APS was reversed the expression of Nrf1. Nrf1 was overexpressed in the human cardiac myocytes using pLenti-Nrf1 and then the stable and overexpressed KRT6B mRNA and protein cell were screened. The results indicated that the mRNA and protein levels of Nrf1 in the human cardiac myocytes were higher than the levels in the vehicle group, while the overexpression of Nrf1 clearly suppressed PAL-induced human cardiac myocytes apoptosis. The finding suggested the concept that Nrf1 might play a role in the development of heart failure [23]. Our observations are consistent with some studies suggesting that Nrf1 activation prevents diabetic cardiomyopathy [24]. Nrf-1 silencing functionally blocks succinate oxidation in aerobic heart cells prior to NADH-linked substrate, and that loss of NRF-1 expression increases HIF-1α stability and glucose transporter and glycolytic gene expression characteristic of pseudo-hypoxia [25].

Nrf1, in addition to regulating the expression of genes involved in mitochondrial transcription and replication, is known to modulate antioxidant gene expression [23]. We observed mark-
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edly increasing the expression of SOD1 and SOD2 in HCMs by overexpressing Nrf1 as well as decreasing ROS in HCMs. Oxidative stress, which may be precipitated by hyperglycemia and hyperlipidemia, plays a pivotal role in the development of heart failure [26]. ROS overproduction can lead to impairment of intracellular signaling pathways and development of cardiomyopathy [27]. Oxidative stress has been proposed as a link between Nrf1 and cardiotoxicity [23]. Consequently, reducing oxidative stress by lowering ROS production is crucial in the management of cardiac function. We showed that PAL stimulated ROS formation and APS presented effective suppression on PAL-induced ROS overproduction and prevented cell apoptosis in HCMs.

In C57BL/6J mice, doxorubicin reduced cardiomyocyte viability and induced heart failure with concurrent elevated ROS generation and apoptosis, which, however, was attenuated by APS treatment [20]. APS inhibits the cohesion between human cardiac microvascular endothelial cells (HCMECs) and polymorphonuclear leukocyte during ischemia-reperfusion injury through the downregulation of p38 MAPK signaling and the reduction of cohesive molecule expression in HCMECs [28]. In animal model, APS can improve the cardiac function in Sjögren’s syndrome model rats [22] and lead to the inhibition of PPARα-mediated lipotoxicity in the pathogenesis of diabetic cardiomyopathy in MHC-PPARα mice [29]. In this study, we showed that APS presented effective suppression on PAL-induced ROS overproduction and advanced cell proliferation HCMs, well demonstrating its antioxidant potency against PAL insult. Therefore, we proposed that there might be cross-talk between Nrf and antioxidant responses in cell apoptosis progression.

In conclusion, our studies provide a basis for investigating the novel mechanisms of APS suppressing PAL-induced HCMs apoptosis and targeting restoration of NRF1 function in maintaining antioxidant status to prevent the toxicity of cardiomyocytes.

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Disclosure of conflict of interest

None.

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