Naringenin Protects Against Septic Cardiomyocyte Injury Via PI3K- AKT Signaling Pathway

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Research Article

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Abstract

**Background:** Sepsis is one of the most common critical illnesses, with approximately 40% of patients experiencing complications of myocardial injury. Naringenin (Nar) is a natural flavonoid with benign biological effects, including anti-inflammatory, anti-bacterial, anti-oxidant, and anti-cancer effects. However, the specific effect of Nar on myocardial injury in sepsis has not yet been determined.

**Methods:** In vivo, the severity of cardiac histopathology and serum inflammatory factors were detected in the mouse model of lipopolysaccharide (LPS)-induced sepsis with/without naringenin treatment. Similarly, in vitro, a myocardial cell injury model was constructed by treating H9C2 cells with lipopolysaccharide (LPS), and the cell activity and apoptosis was detected by LDH and flow cytometry. Carry out transcriptome sequencing, bioinformatics analysis, Western blot and the molecular docking analysis to determine its molecular mechanism.

**Results:** Our study found that Nar treatment improved cardiac histopathological manifestations and reduced serum levels of inflammatory markers in a model of lipopolysaccharide (LPS)-induced sepsis. In addition, Nar attenuated LPS-induced cardiomyocyte apoptosis *in vivo* and *in vitro*. Mechanistically, Nar inhibited nuclear factor-kappa B translocation by activation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway. Molecular docking results showed that Nar binds directly to the PI3K protein.

**Conclusion:** Nar has novel therapeutic potential for LPS-induced myocardial dysfunction, which provides a new target for the clinical prevention and treatment of septic cardiomyopathy.

Introduction

Sepsis is one of the most common critical diseases among the global population. The incidence of sepsis is increasing annually by 0.3–0.5%, and the mortality rate is approximately 25–30%; therefore, sepsis is regarded as a global public health problem [1]. The main reason for the poor prognosis of sepsis is organ failure due to dysregulation of the body's response to infection. In particular, myocardial damage plays a key role during sepsis-induced organ failure and is known as one of the primary contributors to the high morbidity and mortality rates in patients with sepsis [2]. Although progress has been made in understanding the mechanisms and clinical outcomes of myocardial injury in sepsis, there are no effective and specific pharmaceuticals for the clinical treatment of myocardial injury during sepsis.

The mechanisms of myocardial injury caused by sepsis are complex and related to multiple factors, including inflammation, oxidative damage, and cardiomyocyte apoptosis [3]. During sepsis, cellular apoptotic signaling pathways can be activated by numerous inducers, such as inflammation, oxidative stress, mitochondrial damage, energy metabolism dysfunction, and disturbed intracellular calcium homeostasis. Therefore, controlling inflammatory responses, inhibiting oxidative stress, and diminishing cardiomyocyte apoptosis are potential strategies for the treatment of sepsis-induced secondary myocardial damages [4, 5].
The phosphoinositide 3-kinase kinase/protein kinase B (PI3K/AKT) signaling pathway plays an important role in cell survival and apoptosis, cardiac electrophysiology, and mitochondrial energy metabolism. The PI3K/AKT signaling pathway is implicated in different stages of apoptosis by directly or indirectly regulating apoptotic regulators; thus, making it closely connected to cardiomyocyte apoptosis during the development of cardiovascular disease. It has been demonstrated that the PI3K/AKT signaling cascade acts as one of the most important pro-proliferative and anti-apoptotic signaling pathways during the regulation of cardiomyocyte survival and functions [6].

Naringenin (Nar), a natural flavonoid, is mainly found in citrus fruits, such as grapefruit, and has various biological effects, including anti-inflammatory, anti-bacterial, anti-oxidation, and anti-cancer effects [7]. Nar has been used to prevent atherosclerosis, hypertension, arrhythmias, and other cardiovascular diseases [8]. Interestingly, studies that have examined sepsis-induced secondary organ damage have demonstrated that naringenin ameliorates lipopolysaccharide (LPS)-induced inflammation in the lungs of ALI mice [9, 10]. However, there are few studies that have investigated the protective effects and potential mechanisms of action of Nar on myocardial damage caused by sepsis. Accordingly, this study aimed to investigate the protective effects of Nar on myocardial injury during sepsis and the underlying mechanistic pathways involved.

**Materials & Methods**

**Reagents**

LPS was purchased from Sigma-Aldrich (St. Louis, MO, USA), Nar was purchased from Aladdin (Aladdin Bio-Chem Technology Company, Shanghai, China), Dulbecco’s modified eagle medium (DMEM), fetal bovine serum (FBS) and 0.25% trypsin were purchased from gibco, and penicillin-streptomycin solution was purchased from Beyotime (Beyotime, Shanghai, China). Anti-AKT antibody, anti-p-AKT antibody, anti-PI3K antibody, anti-p-PI3K antibody, anti-p-NF-κB p65 and anti-NF-κB p65, the secondary antibodies of anti-rabbit and anti-mouse IgG which link Horseradish peroxidase (HRP) – were purchased from Cell Signaling Technologies (Beverly, MA, USA); DAPI was purchased from Solarbio (Beijing, China); lactate dehydrogenase (LDH) cytotoxicity assay kits and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) apoptosis assay kits were purchased from Beyotime (Shanghai, China).

**Animals**

In this study, experimental procedures involving animals were performed according to the Laboratory Animal Care Specification (NIH Publication No. 85Y23, revised 1996) and approved by the Ethics Committee for Experimental Animals of Yangzhou University (No. 202202004). The ICR male mice (mass: 20–25 g) were housed in a specific pathogen-free environment, fed standard rodent food and water, maintained at appropriate humidity and temperature (25 ± 2 °C), and kept under cyclic lighting (12 h light/12 h dark).
RNA sequencing

H9C2 cells were incubated by LPS or co-treated with Nar which were subjected to RNA sequencing (n=5 per group) and the sequencing depth was 6G. The power analysis calculation of this experimental design, calculated on the website (https://rodrigo-arcoverde.shinyapps.io/maseq_power_calc/) was 0.85. Total RNA was extracted using the TRIzol reagent according to the manufacturer’s protocol. The purity and quantification of were assessed by the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and RNA integrity was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA) was performed to construct the libraries according to the manufacturer’s instructions. The read counts of each gene were obtained by HTSeq-count, Fragments per Kilobase Million[11] of each gene was calculated using Cufflinks. The analysis of genes that differently expressed was performed using the DESeq (2012) R package[12]. P value < 0.05 and foldchange > 2 or foldchange < 0.5 was set as the threshold for significantly differential expression. Pathway enrichment analysis of the Kyoto Gene and Genome Encyclopedia (KEGG) [13] for differentially expressed genes was performed using R language.

2.4 Animal model of sepsis and treatment.

Three groups of male ICR mice were randomly assigned (n=7–10 per group): 1) control group, administered sterile saline; 2) LPS group, administered an intraperitoneal injection of LPS (10 mg/kg); and 3) LPS + Nar (100 mg/kg) group. Nar (100 mg/kg) was administered intraperitoneally for 1 h before administering LPS (10 mg/kg), and execution was performed 24 h after LPS treatment by giving intramuscular injection of Zoletil®50 (60 mg/kg) for anesthesia.

Histological analysis

Heart tissue specimens were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. The embedded heart tissue samples were then cut into sections 5 μm in diameter, pasted hematoxylin and eosin on slides, dewaxed, dehydrated, and stained with (HE). The slides were observed under an optical microscope. Histopathological scores were assigned to each heart tissue section according to the area of cardiac injury: 0 (<10%), 1 (10–25%), 2 (25–50%), 3 (50–75%), and 4 (>75%) [14].

Tunel staining

An Apoptosis Detection Kit (C1098; Progame) was used for TUNEL staining. Heart paraffin sections were dewaxed in xylene for 5–10 min, replaced with fresh xylene, and dewaxed again for 5–10 min. The samples were sequentially placed in anhydrous ethanol for 5 min, 90% ethanol for 2 min, 70% ethanol for 2 min, and distilled water for 2 min. Then, 20 μg/mL DNase-free Proteinase K was added dropwise, and the mixture was incubated for 15 min at 37 °C. Next, 50 μL TUNEL assay solution was added to the samples and incubated for 60 min at 37 °C in the dark, then the samples were rinsed with PBS three times for 3 min each. Finally, the nuclei were stained with DAPI for 5 min. Photographs were taken using a confocal fluorescence microscope, and the density of TUNEL fluorescence was calculated for analysis.
Enzyme-linked immunosorbent assay

The levels of pro-inflammatory inflammatory factors (MCP1, TNF-α, and IL-1β) were measured according to the instructions provided by the enzyme-linked immunosorbent assay (ELISA) kits (Thermo Fisher Scientific, MA, USA).

Culture and treatment of cardiomyocytes

Rat H9c2 cardiomyocytes which was purchased from ATCC ((Manassas, VA, USA) were cultured in high-glucose DMEM supplemented with 10% FBS and 1% antibiotics (100 U/mL penicillin and 100 U/ml streptomycin) at 37 °C in an incubator containing 5% CO₂. H9c2 cells were pre-treated with Nar (2.5, 5, 7.5, and 10 μM) 30 min before LPS (25 μg/mL) treatment, and control cells were treated with the same concentration of dimethyl sulfoxide (DMSO). H9C2 cells collected 8 h after LPS stimulation were subjected to experimental manipulation.

LDH assay

The effect of Nar on H9C2 cardiomyocyte viability was assessed using the LDH Cytotoxicity Assay Kit (Beyotime). After LPS and/or Nar treatment, the cell culture supernatants were transferred to clear 96-well plates. Then, 60 mL of mixed working solution was added according to the manufacturer's recommendations, incubated at 37 °C for 30 min, and cell activity was calculated by measuring the absorbance at 490 nm using an enzyme marker.

Annexin/PI cell flow staining

H9C2 cardiomyocytes were assayed for apoptosis using an Annexin V-FITC/PI kit (BD). After 8 h of LPS treatment, the cells were collected in 100 μL buffer, reacted with 10 μL membrane linked protein V-FITC and 5 μL propidium iodide for 15 min, then incubated in the dark at room temperature. Following supplementation with 300 μL buffer, apoptosis was detected using a flow cytometer and apoptosis rates were calculated.

Western blot analysis

Total protein was extracted from H9c2 cells using RIPA lysis buffer (Beyotime, Shanghai, China) containing a protease inhibitor (Roche, Shanghai, China) and a phosphatase inhibitor (Beyotime, Shanghai, China). The protein concentration was measured using a BCA Protein Quantication Kit (Beyotime, Shanghai, China). Equivalent amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were then blocked with 5% skim milk/BSA for 2 h at room temperature. The membranes were incubated with anti-AKT (1:1000 dilution), anti-p-AKT (1:1000 dilution), anti-PI3K (1:1000 dilution), anti-p-PI3K (1:1000 dilution), anti-NF-κB p65 (1:1000 dilution), anti-p-NF-κB p65 (1:1000 dilution), and anti-p PI3K antibodies (1:1000 dilution), p-NF-κB p65, and β-actin (1:1000 dilution) overnight at 4 °C. The next day, the membranes were washed with TBST (3 × 15 min)
and incubated at room temperature for 2 h with the corresponding horseradish peroxidase-conjugated secondary antibody (1:5000 dilution). After washing with TBST three times, ECL Plus chemiluminescence was used to detect the protein bands, and ImageJ was used to analyze the grayscale values.

**Molecular docking analysis**

The open-source Autodock Vina v1.1.2 software (Scripps Research, CA, United States) was performed the molecular docking analysis between naringenin and PI3K. Two-dimensional coordinates of naringenin were obtained from the PubChem website (https://pubchem.ncbi.nlm.nih.gov), then the three-dimensional structural ligand compounds in the format mol2 were prepared using ChemOffice software. The crystal structure of the molecular docking model of PI3K (PDB:2V4L) was obtained from the Protein Data Bank. Proteins structures were hydrogenated and dehydrated using PyMOL 1.7.2.1 software and saved as PDBQT files. The molecular docking and the calculation of the binding affinity were performed by Autodock Vina v1.1.2 software. Protein molecular docking maps were drawn using PyMOL 1.7.2.1 software.

**Statistical analysis**

GraphPad Prism software (version 8.0) was used for the statistical analysis. R 4.2.1 software was used to analyze RAN sequencing and KEGG data. Differences between two groups were analyzed using independent t-tests, and differences between more than two groups were analyzed using one-way ANOVA. Data are presented as mean ± standard deviation (SD), and $P$-values <0.05 are considered statistically significant for all two-tailed tests. For statistical comparison, at least three independent experiments were performed.

**Results**

**Naringenin ameliorates LPS-induced cardiomyocyte injury in sepsis**

To investigate the role of Nar in cardiotoxicity, an LPS-induced acute myocardial injury model was applied in our study. A flowchart of the experiments is shown in Figure 1A. As shown in Figure 1B, we observed that the ratio of heart mass-to-body mass was smaller in the mice treated with Nar than in the LPS group. HE staining of the hearts in the normal control group showed normal, healthy, and well-aligned cardiomyocytes (Figure 1C). In contrast, LPS-treated mice exhibited cardiomyocyte edema, inflammatory cell infiltration, and widened intercellular gaps. Nar treatment significantly attenuated these pathological changes. As shown in Figure 1D, LPS stimulation significantly increased the cardiac histopathology scores, but after Nar treatment, the pathology scores were significantly reduced. In addition, we measured the serum levels of pro-inflammatory cytokines (IL-1$\beta$, MCP-1, and TNF-α) to assess the systemic inflammatory response in mice with sepsis. As shown in Figure 1E–G, Nar decreased the serum levels of IL-1$\beta$, MCP-1, and TNF-α compared with the LPS group, which was consistent with the degree of myocardial cell injury. These results suggest that Nar protects against LPS-induced cardiomyocyte injury in mice with sepsis.
Naringenin attenuates LPS-induced cardiomyocyte apoptosis

Apoptosis of cardiomyocytes plays an important role in LPS-induced myocardial injury. As shown in Figures 2A and 2B, the number of apoptotic cardiomyocytes were significantly reduced in mice treated with Nar compared to that in the LPS group. Similarly, an in vitro model of myocardial injury was established using H9C2 cardiomyocytes following LPS stimulation, and the results showed a decrease in LDH release in naringenin-treated H9C2 cells compared to the LPS group (Figure 2C). Flow cytometry results showed that the apoptosis rate of neurons was significantly increased after LPS treatment (Figures 2D and 2E); whereas, the release rate of LDH and apoptosis rate of cells in the Nar pretreatment group were significantly decreased (Figure 2C–E), suggesting that Nar attenuated LPS-induced cardiomyocyte apoptosis in vivo and in vitro.

Naringenin improves myocardial injury through the PI3K/AKT pathway

To explore the myocardial protection mechanism of Nar, RNA-seq was conducted on H9c2 cardiomyocytes of the LPS and LPS + Nar groups, which suggested that the PI3K/AKT signaling pathway was significantly altered in the Nar group compared with the LPS group (Figure 3A). Accordingly, we investigated changes in the PI3K/AKT signaling pathway before and after Nar treatment. The results showed that the PI3K/AKT signaling pathway was inhibited after LPS stimulation, and administration of Nar removed the inhibition of both the PI3K/AKT pathway and the activation of P65 (Figure 3B–E).

Binding mode of naringenin and PI3K

We performed molecular docking analyses of Nar and PI3K. Wei et al. [15] concluded that the stability of the binding conformation of the target protein to the ligand (small molecule) depends on the binding energy of the docking complex, which of the binding energies lower than -6 kcal/mol represents strong interactions. As shown in Figure 4A, Nar binds directly to PI3K (affinity -8.6 kcal/mol), which showed the strong interaction exists between PI3K and Nar. In the crystal structure of the PI3K complex, Nar forms hydrogen bonds with its surrounding amino acids, Glu 880, Arg 849, and Arg 690 (Figures 4B and 4C). In conclusion, these results suggest that Nar binds directly to PI3K.

Discussion

Sepsis cardiomyopathy is a nonischemic myocardial dysfunction that occurs in patients with sepsis[16, 17]. It is one of the main causes of death in patients with sepsis. Unfortunately, the pathophysiological mechanisms and pathogenesis involved in sepsis cardiomyopathy are not clear, which leads to a lack of effective therapeutic drugs to control the progression of this disease and improve the quality of life of patients. Our results indicate that Nar could decrease the inflammatory responses, attenuate the apoptosis of septic cardiomyocytes, and alleviate the damage of septic myocardial tissues in an LPS-induced sepsis mouse model. Mechanistically, Nar exerted these protective effects on septic cardiomyocytes by activating cellular PI3K/AKT signaling pathways.
Previous studies have shown that Nar, a flavonoid extracted from citrus fruits, grapes, tomatoes, and other fruits, mitigates the progression of obesity, diabetes, pancreatic diseases, tumors, and other diseases by activating various signaling pathways or secreting cytokines [18-21]. Nar also acts as a good preventive and therapeutic agent for cardiovascular diseases. For example, Nar has shown to have a protective effect against myocardial ischemia-reperfusion injury [22-24]. Both in vivo and in vitro studies by Yu et al. [24] found that Nar could protect against myocardial injury caused by ischemia/reperfusion-induced cardiomyocyte apoptosis by activating the cyclic guanosine phosphate-protein kinase G signaling pathway. Furthermore, Nar has been applied for the protection against acute lung and kidney injury caused by sepsis [24, 25].

Here, we used Nar to investigate an LPS-induced septic mouse model and the results indicate that Nar significantly attenuates sepsis-induced cardiomyocyte apoptosis in vivo and in vitro, suggesting a protective effect of Nar on septic cardiomyocyte injury. In addition, Nar decreased serum levels of TNF-α and IL-6, indicating that Nar may reduce systemic inflammatory responses. During sepsis, the immune system produces many inflammatory cytokines, which can travel through the circulatory system to the heart; thus, inducing excessive inflammatory damage to cardiac muscle. In addition, LPS, an outer-membrane component of Gram-negative bacteria, binds to Toll-like receptor 4 (TLR 4) expressed by cardiomyocytes, which stimulates the production of numerous pro-inflammatory mediators, such as IL-1β, TNF-α, and iNOS [26].

The PI3K/AKT signaling pathway, downstream from the TLR 4 receptor, is closely implicated in cardiomyocyte injury. Studies have shown that herbal components, such as Astragalus polysaccharide and curcumin, play an important role in reducing inflammatory responses, inhibiting cardiomyocyte apoptosis, and alleviating oxidative stress by activating the PI3K/AKT signaling pathway and are therefore involved in the development of cardiovascular diseases [27, 28]. These studies confirmed that the PI3K/AKT signaling pathway plays a classical role in myocardial protection. However, unlike other organs or tissues, PI3K/AKT signaling, which has anti-inflammatory effects, is downregulated in cardiomyocytes during inflammation. RNA sequencing of Nar in LPS-induced septic cardiomyocytes suggested a significant upregulation of PI3K/AKT signaling, which has been verified by experiments that have used the relevant inhibitors[26]. Our study outcomes are similar to those reported by Shang et al. [29], in which PI3K/AKT signaling significantly mediated myocardial injury.

Conclusions

Our study demonstrates that the protective effect of Nar on LPS-induced myocardial injury is mediated by activation of the PI3K/AKT signaling pathway. In clinical applications, Nar may be used as a therapeutic drug to prevent and treat septic cardiomyopathy.

Abbreviations

Naringenin (Nar)
LPS, lipopolysaccharide
PI3K, phosphoinositide 3-kinase
AKT, protein kinase B
LPS, lipopolysaccharide
DMEM, Dulbecco's modified eagle medium
FBS, fetal bovine serum
HRP, Horseradish peroxidase
DMEM, Dulbecco's modified eagle medium
FBS, fetal bovine serum
TUNEL, transferase-mediated dUTP nick end-labeling
LDH, Lactate dehydrogenase
HE, hematoxylin and eosin
TLR 4, Toll-like receptor 4

**Declarations**

**Availability of data and materials**

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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Contributions

Jiajia Pan, Lijun Meng and Rujun Li performed the literature searches, participated all the experiments, analyzed the data, and wrote the manuscript draft. Zicheng Wang aided in the data analysis. Wenjie Yuan and Yucheng Li participated in animal experiments. Lin Chen and Qinhao Shen participated in cell experiments. Li Zhu and Weili Liu designed the study and revised manuscript. All authors have read and approved the manuscript, and ensure that this is the case.

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Ethics declarations

Ethics approval and consent to participate

All animal procedures were performed according to the Laboratory Animal Care Specification (NIH Publication No. 85Y23, revised 1996) and approved by the Ethics Committee for Experimental Animals of Yangzhou University (No. 202202004).
Consent for publication

Not applicable.

Competing Interests

The authors declare there are no competing interests.

References


Figures

**Figure 1**

*Naringenin ameliorates LPS-induced cardiomyocyte injury in sepsis.* (A) Flow chart of animal experiments. (B) Comparison of heart-to-mass ratio. (C) Representative HE staining of cardiac tissues in magnifications 100× and 400×. (D) The pathological scores of cardiac tissues. (E-G) The serum levels of...
interleukin-1β (IL-1β), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-α (TNF-α) were detected by enzyme-linked immunosorbent assay (ELISA). N=6 in each group. *P < 0.05, **P < 0.01 and ***P < 0.001.

Figure 2

Figure 3

The effect of Nar on the protein level of PI3K/AKT/NF-κB Pathway against LPS-induced cardiomyocyte injury. (A) KEGG pathway enrichment analysis of all differentially expressed genes of RNA-seq data, (lipopolysaccharide [LPS] versus LPS + naringenin [Nar]). (B) The H9C2 cardiomyocytes were pre-treated with Nar (2.5 μM) for 1 h and then stimulated with LPS (25 μg/mL) for 8 h. Protein levels of p-phosphoinositide 3-kinase (PI3K), p-protein kinase B (AKT), and p-PI3K in H9C2 cells were analyzed by Western blotting. These blots were cut prior to hybridisation with antibodies during blotting. (C–E) Relative protein expression of p-PI3K, p-AKT and p-P65. PI3K, AKT and P65 were used as control for protein loading respectively. *P < 0.05, **P < 0.01 and ***P < 0.001.
Figure 4

The molecular docking analysis of Nar and PI3K complex was performed using Autodock 4.2. (A) The superposition of the naringenin (Nar) and the phosphoinositide 3-kinase (PI3K) complex. (B) 3D conformation of Nar with PI3K complex. (C) 2D diagram of Nar with PI3K complex.

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