Naringenin Protects Rats against Ang-II Induced Cardiac Hypertrophy and Fibrosis by Downregulating TGF- β 1/Smads Signaling Pathways

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Research

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Abstract

Background: Naringenin (Nrg), a flavone found in several plant foods with various biological properties, has been shown prevention of cardiac remodeling. However, the mechanisms underlying this suppression of cardiac remodeling has not been known clearly.

Methods: Male Sprague Dawley (SD) rats were AngII infused via osmotic minipumps for 4 weeks and were given Nrg by gavage (100mg/kg/day) at the same time. In vitro experiments used cardiomyocyte and cardiac fibroblasts (CF) treated with AngII or AngII plus Nrg. Cardiac remodeling was assessed using the echocardiography and histological analysis. And, the effect of Nrg on TGF- β 1/Smad signaling pathway was investigated.

Results: Treatment with Nrg (100mg/kg/day) decreased the ratio of heart weight to tibia length and hypertrophy markers in rats given AngII infusion. In vitro experiments demonstrated that AngII-induced cardiomyocyte hypertrophy and proliferation of CFs were significantly inhibited by Nrg administration. Nrg inhibited activation of the TGF- β 1/Smad2/3 signaling pathway stimulated by AngII.

Conclusions: Nrg supplementation prevented cardiac remodeling via down-regulating the TGF- β 1/Smad2/3 signaling pathway both in cardiomyocyte and CFs, and attenuating cardiac remodeling in AngII-induced rats model.

Introduction

Cardiac remodeling is characterized by cardiac hypertrophy and fibrosis, which has been recognized as a key determinant of clinical outcome in heart disease [1]. Angiotensin II (AngII) is a crucial regulator of cardiac remodeling through inducing cardiomyocyte hypertrophy and proliferation and migration of cardiac fibroblasts (CFs). Transforming growth factor- β 1 (TGF- β 1) has been identified as a key regulator of extracellular matrix synthesis and degradation, which is believed to partially mediate AngII-induced cardiac remodeling [2].

Naringenin (Nrg) is a flavonoid compound found in several plant foods including citrus fruit, tomatoes and figs. Nrg has been identified as a potential therapeutic agent as it demonstrates anticancer [3], anti-inflammation [4], anti-atherogenic [5] and antimicrobial [6] effect. Previous studies have reported that Nrg ameliorates cardiac hypertrophy induced by high glucose [7] [8] and pressure overload [9]. Although Nrg inhibits TGF- β 1 signaling and the subsequent Smad3 phosphorylation for the downstream signal transduction [10], whether Nrg modifies AngII-induced cardiac remodeling through TGF- β 1/Smad signaling pathway remains elusive.

This study therefore explored the possible prevention by Nrg of cardiac remodeling in vivo, using the AngII-induced rat model, and in vitro on cardiomyocytes and CFs stimulated by AngII plus Nrg.

Methods

Materials

Naringenin, AngII, 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Antibiotic-antimycotic solution (10,000 units/ml of penicillin, 10,000µg/ml of streptomycin), and Tris were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trizol Reagent was purchased from Invitrogen (USA). All-In-One RT Mastermix and EvaGreen qPCR MasterMix were purchased from ABM (Canada). Antibodies against ANP, β -MHC, TGF- β 1, Smad2/3, phospho-Smad2/3 (p-Smad3), and GAPDH were purchased from Abcam CO (Cambridge, UK).

Animal

Male 8-week-old male Sprague Dawley (SD) rats (150–180 g body weight) were purchased from Beijing Vital River Laboratory Animal Technology Company (Beijing, China). All experiments involving rats were approved by the Institutional Animal Care Research Advisory Committee of the National Institute of Biological Science (NIBS) and Animal Care Committee of Zhengzhou University. All rats were kept under a 12-hr light/dark cycle with free access to water and food.

Experimental Design And Treatment Protocol

A rat model of AngII infusion induced cardiac remodeling was established as described previously [11]. In brief, SD rats were quickly anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg), then the prefilled osmotic minipumps (Alzet, Model 2002) were implanted into the subcutaneous tissue to deliver AngII (Sigma-Aldrich, A9525) at 400 ng/kg/min for 4 weeks. Rats were randomly assigned to one of the following groups: the sham group (n = 10) received subcutaneous injections of phosphate buffer (PBS) for 4 weeks; the AngII group (n = 10) received subcutaneous injections of AngII (400 ng/kg/min) via osmotic minipumps; the AngII + Nrg group (n = 10) received naringenin by gavage (100mg/kg/day) plus the daily injections of Ang as above.

Echocardiographic Study

Transthoracic echocardiography was used to measure left ventricular (LV) function variables one day before killing. Briefly, rats were placed in a supine position after the induction of general anesthesia. Rats were underwent transthoracic two dimensional guided M-mode echocardiography with a 12L MHz transducer (Sibiscope Co. Ltd.). From the cardiac short axis, the LV anterior wall end-diastolic thickness (LVAWd), the systolic LV anterior wall thickness (LVAWs), the LV internal dimension at end-diastole (LVIDd), the LV internal dimension at end-systole (LVIDs), the LV posterior wall end-diastolic thickness (LVPWd), the LV posterior wall end-systolic thickness (LVPWs) were measured. Echocardiographic measurements were averaged from at least three separate cardiac cycles.

Heart Histological Analysis

The left ventricle were fixed in 10% formalin and embedded in paraffin, and subsequently were sectioned at 4µm and stained with Masson to evaluate the cardiac collagen deposition. To evaluate the size of cardiomyocytes, tissue sections were stained with 1.0 mg/ml Alexa Fluor 488® conjugate of wheat germ agglutinin (WGA) solution (MolecularProbes, Eugene, OR, USA). Ten fields in each region of the heart were selected randomly from four nonconsecutive serial sections, and collagen content was quantified by measuring the total blue area per square millimeter using the ImageJ.

Neonatal Rat Ventricular Cardiomyocytes Isolation, Culture And Treatment

Neonatal rat ventricular cardiomyocytes and CFs were obtained from the hearts of 1–2 days old SD rats as described previously [12]. In brief, the ventricles of neonatal rats were harvested after killed by decapitation, and then were cut into ~ 1mm³ pieces in a dish with cold PBS. 0.125% and 0.05% collagenase type I were used to dissociate cardiomyocytes and fibroblasts. Cells were cultured in DMEM with 15% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified atmosphere of 5% CO₂ and 95% air at about 37°C. Naringenin were dissolved in dimethyl sulfoxide (DMSO) and diluted with DMEM. Cells were incubated with Nrg (0.1, 1, 10µM) with or without AngII 10uM for 24 h in a 6 well plate. Cell surface area analysis was performed using confocal microscopy as described previously [13].

Methylthiazolyl Tetrazolium Assay For Cell Viability

Cells were cultured at a density of 4–5 x 10⁴ cells per well in 96-well plates for 24 h. The cells were treated with different concentrations of Nrg for 24 h. And then cell viability was determined by the MTT reduction assay. Cells were incubated with MTT solution (5 mg/mL) for 4 h at 37°C. The dark blue formazan crystals that formed in intact cells were solubilized with 150 µL of DMSO, and the absorbance at 490 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA).

Western Blotting

The heart tissues or cells were lysed by RIPA lysis buffer and the protein concentration was detected by using a BCA protein assay kit. Protein (30µg) were separated using 10% SDS-PAGE and then were transferred onto a polyvinylidene difluoride membrane (PVDF). Next, PVDF membranes were blocked with 5% fat-free milk and incubated with primary antibodies overnight at 4°C. Subsequently, the membranes were washed and incubated with secondary antibodies at room temperature. The optical density of the bands was visualized by an ECL system (Pierce). GAPDH was used as an endogenous control. Data was normalized to GAPDH levels.

Rna Isolation And Quantitative Real-time Pcr

Total RNA was extracted from the frozen tissues or cultured cells using Trizol reagent (Invitrogen, USA). First strand cDNA was synthesized using an RT kit (Invitrogen, USA). qPCR analysis were performed in a MiniOpticon Real-Time PCR Detection System (BioRad Laboratories, USA). Results were expressed as fold differences relative to GAPDH using the $2^{-\Delta\Delta CT}$ method. All the primers were synthesized by Sangon Biotech (Shanghai, China) and the sequence are listed in Table 1.

Table 1
Primers used for reverse transcription and real-time PCR

Primer Names		Sequences
ANP	Sense	CCTTCTCCATCACCAA
	Anti-Sense	TGTTATCTTCGGTACCG
β -MHC	Sense	GCCGAGTCCCAGGTCAACAA
	Anti-Sense	GTAATTCGAGGGCAGGAACCC
α -SMA	Sense	GCAAACAGGAATACGACGAAGC
	Anti-Sense	GCTTTGGGCAGGAATGATTTG
Co1 I	Sense	ACTCAGCCCTCTGTGCCT
	Anti-Sense	CCTTCGCTTCCATACTCG
GAPDH	Sense	GACATCAAGAAGGTGGTGAAGC
	Anti-Sense	TGTCATTGAGAGCAATGCCAGC

Statistical analysis

All data are presented as means $\pm$ SEM. SPSS 21.0 was used to perform statistical analysis of the data. Statistical differences were calculated with the 2-tailed Student t test when comparing 2 conditions, and ANOVA was used when comparing ≥ 2 conditions. A value of $P < 0.05$ was considered statistically significant.

Results

Nrg alleviated AngII-induced proliferation and collagen expression of CFs

Firstly, we detected the cytotoxicity of Nrg on CFs. The results of methylthiazolyl tetrazolium assay showed that Nrg had no cytotoxic effects on CFs at concentrations less than 200 μ M (Fig. 1A). Thus, in the following experiments, Nrg concentrations of 200 μ M were chosen. Then, we examined whether Nrg

could inhibit AngII induced proliferation of CFs. CFs were pretreated with Nrg (200 μ M) following with AngII (0.1 μ M) for 72h. Our results demonstrated that Nrg could inhibit AngII-induced proliferation of CFs in a concentration-dependent manner (Fig. 1B). Then we examined the effect of Nrg on collagen expression in cardiac fibroblasts (CFs). Following AngII administration, the fibrotic markers α -SMA and Col1a1 gene expression were increased in CFs, and Nrg treatment prevented AngII induced CF collagen expression (Fig. 1C and D).

Nrg Attenuated Angii-induced Cardiomyocyte Growth In Cultured Cardiomyocytes

To assess the protective role of Nrg on the development of cardiac hypertrophy, cardiomyocytes were treated with AngII 0.1 μ M for 24 h, and cell surface area and hypertrophic markers were measured. AngII treatment induced significant increase of hypertrophic markers (ANP and β -MHC) and cell surface area compared to the control group (Fig. 1B, C and D). However, compared with AngII group, combined treatment with Nrg significantly reversed AngII-induced increases of hypertrophic markers (ANP and β -MHC) and cell surface area (Fig. 1B, C and D).

Nrg Ameliorated Angii-induced Cardiac Hypertrophy

Here we analyzed cardiac effects of Nrg treatment in an animal model of AngII-induced cardiac hypertrophy in rats. AngII infusion rats showed a significant increase in the ratio of weight/tibia length (HW/TL), and the cell size of cardiomyocytes (Fig. 3A and D). Examination by echocardiography revealed that the thickness of the left ventricular post wall at the end-diastole (LVPWd) and the end-systole (LVPWs) was higher in AngII infusion rats (Fig. 3B and C). Compared with AngII group, Nrg treatment markedly ameliorated AngII-induced cardiac hypertrophy, as demonstrated by a significantly decrease in HW/TL, cardiomyocyte size, the thickness of the left ventricular post wall at the end-diastole (LVPWd) and the end-systole (LVPWs) (Fig. 3A, B, C and D). Meanwhile, AngII infusion induced increased protein levels of ANP and β -MHC, while their expression was inhibited in Nrg-treated rat (Fig. 3E).

Nrg Attenuated Angii-induced Cardiac Fibrosis

To determine the effect of Nrg on cardiac fibrosis, heart sections were stained with Masson's staining. Quantitative data revealed increased collagen deposition in AngII-induced rats, while was significantly attenuated in Nrg-treated rats (Fig. 4A). As showed in Fig. 4B, AngII infusion induced a significant increase in protein levels of α -SMA and Col I, and Nrg treatment reversed cardiac fibrosis as evidenced by a decreased in collagen deposition and α -SMA and Col I protein level (Fig. 4A and B). Collectively, Nrg treatment can attenuated AngII-induced cardiac hypertrophy and fibrosis.

Suppression of TGF- β 1/Smad2/3 signaling contributes to the anti-hypertrophy effect of Nrg

Furthermore, we explored the mechanism underlying the anti-hypertrophy effect of Nrg. As shown in Fig. 5A, the expression of TGF- β 1 and phosphorylated Smad2/3 were increased in AngII-induced rat model, which could be attenuated by treatment with Nrg. In cultured cardiomyocytes and cardiac fibroblasts, AngII induced upregulation of TGF- β 1 and phosphorylated Smad2/3 (Fig. 5B and C), and Nrg treatment inhibited TGF- β 1/Smad2/3 signaling as evidenced by attenuating protein expression of TGF- β 1 and phosphorylated Smad2/3 (Fig. 5B and C).

Discussion

Cardiac remodeling is a major driving force in the development and progression of cardiovascular diseases including cardiac hypertrophy, heart failure and myocardial infarction. However, no therapeutic intervention directly targets the fibrotic response. A better understanding of the mechanisms underlying cardiac remodeling is important for developing more effective diagnostic and therapeutic strategies. In the present study, one of the important findings is that Nrg treatment markedly improved AngII-induced cardiac hypertrophy and fibrosis through downregulating TGF- β 1 signaling pathway.

AngII, the main effector peptide of renin-angiotensin system (RAS), has been shown to induce TGF- β 1 expression and its subsequent signaling and mediates cardiac remodeling [14–16]. Pharmacological inhibition of angiotensin converting enzyme and AngII receptor have shown their therapeutic effects for cardiac remodeling [17]. Our previous study also demonstrated that suppressing TGF- β 1/Smads signaling pathway inhibits cardiac fibrosis and improves cardiac function [18]. Nrg, a natural flavanone with many pharmacological effects, has been proved to reduce Smad3 phosphorylation and expression in the presence of TGF- β 1 [19], and exerted anti-fibrosis effect [20]. In this work, we therefore attempted to characterize the potential role of TGF- β 1 signaling pathway in Nrg inhibition of Ang II-induced cardiac remodeling *in vitro* and *in vivo*. Our results showed that Nrg markedly improved AngII-induced cardiac hypertrophy and fibrosis through inhibiting the TGF- β 1 signaling pathway. Thus, our result suggest that Nrg might play a protective role in AngII-mediated cardiac remodeling by targeting the TGF- β 1 signaling pathway.

However, it remains obscure what's the inhibition mechanism of Nrg on the AngII-TGF- β 1 signaling pathway. One possible explanation is that Nrg can reduced the binding probability of TGF- β 1 to its specific TGF- β 1 type II receptor (T β RII). TGF- β 1 binding to T β RII is the initial step of TGF- β signaling. Thus the effect of Nrg on TGF- β ligand-receptor interaction induced inhibition of the receptor dimerization and activation for the signaling complex formation and the subsequent Smad3 phosphorylation for the downstream signal transduction [10].

Conclusions

In present study, we arrived at a conclusion that Nrg targeting TGF- β 1/Smad signaling pathway, and promoted cardiac hypertrophy and fibrosis in AngII-induced rats. Our study supports the notion Nrg has the potential to be developed as a novel inhibitor target for TGF- β signaling, and might be considered as potential prevention strategy for cardiac hypertrophy and fibrosis.

Our results might help to deepen the understanding of the role and function of TGF- β signaling in cardiac hypertrophy. These findings offer important insights into fundamental mechanisms underlying functions of Nrg, meanwhile, would provide a potential therapeutic targets for cardiac hypertrophy.

Abbreviations

Nrg: Narinngenin; SD: Sprague Dawley; Transforming growth factor- β 1 (TGF- β 1); LV: left ventricular; LVAWd: the LV anterior wall end-diastolic thickness; LVAWs: the systolic LV anterior wall thickness; LVIDs: the LV internal dimension at end-systole; LVPWd: the LV posterior wall end-diastolic thickness; LVPWs: the LV posterior wall end-systolic thickness; WGA: wheat germ agglutinin; PVDF: polyvinylidene difluoride membrane; CFs: cardiac fibroblasts.

Declarations

Acknowledgment

Not applicable

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Availability of data and materials

All data generated of analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Author's contributions

HDL designed the study; XWC and XZ conducted the experiments; HW did sample analysis and data analysis, HDL wrote the manuscript; HDL revised the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the ethics committee of Zhengzhou University.

Consent for publication

Not applicable

Competing interest

The authors have no conflicts of interest to disclose.

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Figures

Figure 1

Naringenin (Nrg) treatment inhibited AngII-induced cardiac fibroblasts proliferation and collagen expression in vitro. Cardiac fibroblasts (CFs) were exposed to AngII(0.1 μ M) with or without Nrg 200 μ M for 24 h, and then the cell viability was measured by MTT assay (A). CFs were pretreated with Nrg (50, 100 and 200 μ M), and then stimulated with AngII (0.1 μ M) for 72h. The proliferation of CFs was measured by MTT assay (B). mRNA level of α -SMA (C) and Col1a1 (D) were quantified using real-time PCR. Data are presented as mean $\pm$ SEM, ^{##}P<0.01 vs Vehicle; ^{**}P<0.01 vs Ang II; ^{*}P<0.05 vs Ang II.

Figure 2

Naringenin (Nrg) treatment inhibited AngII-induced hypertrophy in cultured cardiomyocytes in vitro. Cardiomyocytes were exposed to AngII 0.1 μ M with or without Nrg 200 μ M for 24 h. (A) Surface area was determined. mRNA level of ANP (B) and β -MHC (C) were quantified using real-time PCR. Data are presented as mean $\pm$ SEM, ##P<0.01 vs Vehicle; **P<0.01 vs Ang II; *P<0.05 vs Ang II.

Figure 3

Naringenin (Nrg) ameliorated AngII-induced cardiac hypertrophy in vivo. (A) Heart weight to tibia length ratio of different groups, n=10. (B) Left ventricular post wall thickness in diastole (LVPWd). (C) Left ventricular post wall thickness in systole (LVPWs). (D) Representative wheat germ agglutinin-stained of the left ventricles to cardiomyocyte size and quantification of the cardiomyocyte size in the indicated groups (n=8 per group). (E) Western blot images and densitometric quantitation for ANP and β -MHC

protein in different groups (n=6 per group). Data are presented as mean ±SEM,##P<0.01 vs Sham; **P<0.01 vs Ang II.

Figure 4

Naringenin (Nrg) ameliorated AngII-induced cardiac fibrosis in vivo. (A) Representative Masson-staining of the left ventricles to assess cardiac fibrosis and quantification of the fibrosis area in different groups (n=9 per group). (B) Western blot images and densitometric quantitation for Co1 I and α-SMA protein in different groups (n=6 per group). Data are presented as mean ±SEM,## P<0.01 vs. Sham, **P<0.01vs. AngII.

Figure 5

Naringenin (Nrg) downregulated TGF- β 1 signaling in vivo and in vitro. (A) Western blot images and densitometric quantitation for TGF- β 1, phosphorylated (P-) Smad2 and P-Smad3 protein levels in heart tissues (n=6 per group). (B) Western blot images and densitometric quantitation for TGF- β 1, phosphorylated (P-) Smad2 and P-Smad3 protein levels in cultured cardiomyocytes (n=6 per group). (C) Western blot images and densitometric quantitation for TGF- β 1, phosphorylated (P-) Smad2 and P-

Smad3 protein levels in cultured fibroblasts (n=6 per group). Data are presented as the mean $\pm$ SEM, ## P $\leq$ 0.01 vs. Sham, **P $\leq$ 0.01 vs. AngII.