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Research Paper

Piperine Attenuates Pathological Cardiac Fibrosis Via PPAR-γ/AKT Pathways

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ABSTRACT

Mitogen-activated protein kinases (MAPKs) and AMP-activated protein kinase α (AMPKα) play critical roles in the process of cardiac hypertrophy. Previous studies have demonstrated that piperine activates AMPKα and reduces the phosphorylation of extracellular signal-regulated kinase (ERK). However, the effect of piperine on cardiac hypertrophy remains completely unknown. Here, we show that piperine-treated mice had similar hypertrophic responses as mice treated with vehicle but exhibited significantly attenuated cardiac fibrosis after pressure overload or isoprenaline (ISO) injection. Piperine inhibited the transformation of cardiac fibroblasts to myofibroblasts induced by transforming growth factor-β (TGF-β) or angiotensin II (Ang II) in vitro. This anti-fibrotic effect was independent of the AMPKα and MAPK pathway. Piperine blocked activation of protein kinase B (AKT) and, downstream, glycogen synthase kinase 3β (GSK3β). The overexpression of constitutively active AKT or the knockdown of *GSK3β* completely abolished the piperine-mediated protection of cardiac fibroblasts. The cardioprotective effects of piperine were blocked in mice with constitutively active AKT. Pretreatment with GW9662, a specific inhibitor of peroxisome proliferator activated receptor-γ (PPAR-γ), reversed the effect elicited by piperine in vitro. In conclusion, piperine attenuated cardiac fibrosis via the activation of PPAR-γ and the resultant inhibition of AKT/GSK3β.

1. Introduction

Cardiac hypertrophy is an adaptive response to increased biomechanical loads and is characterized by myocyte hypertrophy, fibroblast activation, and extracellular matrix accumulation, ultimately leading to congestive heart failure and sudden death (Frey and Olson, 2003; Heineke and Molkentin, 2006). Increasing evidence suggests that mitogen-activated protein kinases (MAPKs) and AMP-activated protein kinase α (AMPKα) play critical roles in the process of pathological cardiac hypertrophy (Chan and Dyck, 2005; Lorenz et al., 2009; Ravingerova et al., 2003; Zhang et al., 2008). Therefore, pharmacological interventions in these signaling pathways could be of great therapeutic interest for treating cardiac hypertrophy.

In addition to cardiomyocytes, cardiac fibroblasts (CFs) are involved in orchestrating a pathological hypertrophic response (Kamo et al., 2015). The expression of transforming growth factor-β (TGF-β) was increased in the hypertrophied hearts induced by pressure overload (Dobaczewski et al., 2011). TGF-β directly induced the transformation of fibroblasts to myofibroblasts, which secrete hypertrophic and profibrotic factors and result in extracellular matrix protein deposition (Butt et al., 1995; Eghbali et al., 1991). Furthermore, fibrosis impaired the electrical coupling of cardiomyocytes and reduced cardiac capillary density (Sabbah et al., 1995; Swynghedauw, 1999). Thus, cardiac fibrosis plays key roles in the process of cardiac remodeling.

Piperine is a phenolic component of black pepper and long pepper (Srinivasan, 2007). Previous studies have shown that piperine possesses a number of pharmacological activities. Pharmaceutically, piperine has been reported to protect against hepatotoxicity (Piyachaturawat et al., 1995), attenuate depressive disorders (Bhutani et al., 2009), and mitigate obesity and diabetes (Nogara et al., 2016). It is noteworthy that the administration of piperine and its derivatives resulted in the activation of AMPKα signaling in mice (Choi et al., 2013; Kim et al., 2011). Piperine also decreased the phosphorylation of extracellular signal-

Abbreviations: AB, (aortic banding); AKT, (protein kinase B),; AMPKα, (AMP-activated protein kinase α),; ANP, (atrial natriuretic peptide),; BW, (body weight),; BNP, (brain natriuretic peptide),; CF, (cardiac fibroblast),; ERK, (extracellular signal-regulated kinase),; FS, (fractional shortening),; GAPDH, (glyceraldehyde 3-phosphate dehydrogenase),; GSK3β, (glycogen synthase kinase 3β),; HW, (heart weight),; ISO, (isoprenaline),; LVIDd, (left ventricle end-diastolic internal diameter),; MAPK, (mitogen-activated protein kinases),; β-MHC, (β-myosin heavy chain),; PE, (phenylephrine),; PI3K, (phosphatidylinositol-3-kinase),; PPAR-γ, (peroxisome proliferator activated receptor-γ),; α-SMA, (α-smooth muscle actin),; TGF-β, (transforming growth factor-β),; TL, (tibial length).

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regulated kinase (ERK) in vitro (Hwang et al., 2011). These findings raised the possibility that piperine could protect against cardiac hypertrophy.

In the current study, we found that piperine-treated mice had similar hypertrophic responses as those treated with the vehicle but developed limited cardiac fibrosis after long-term pressure overload or repeated isoprenaline (ISO) injection independent of the AMPK and MAPK pathway. We also demonstrated that piperine acted as an agonist of peroxisome proliferator that activated receptor-γ (PPAR-γ) and blocked the activation of protein kinase B (AKT).

2. Materials and Methods

2.1. Reagents

Piperine (≥97% purity, as determined by high-performance liquid chromatography) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Isoprenaline (ISO, I5627), TGF-β (T7039), angiotensin II (Ang II, A9525) and phenylephrine (PE, P6126) were purchased from Sigma-Aldrich. The primary antibodies against the following proteins were purchased from Cell Signaling Technology: T-AKT (4691, 1:1000), phospho-AKT (P-AKT, 4060, 1:1000), T-glycogen synthase kinase 3β (GSK3β, 9315, 1:1000), P-GSK3β (9323P, 1:1000), T-P38 (9212P, 1:1000), P-P38 (4511P, 1:1000), T-AMPKα (2603P, 1:1000), P-AMPKα (2535, 1:1000), T-ERK (4695, 1:1000), P-ERK (4370P, 1:1000), T-SMAD3 (3103S, 1:500), P-SMAD3 (3101, 1:500) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 2118, 1:1000). α-Smooth muscle actin (α-SMA, ab7817, 1:500) was obtained from Abcam (Cambridge, UK). Antibodies against α-actinin (ab90776) were obtained from Merck Millipore (Massachusetts, United States), and anti-vimentin (sc-5565) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The anti-rabbit/mouse EnVisionTM+/HRP reagent used for immunohistochemistry was purchased from Gene Technology (Shanghai, China), and Alexa Fluor 488-goat anti-mouse secondary antibody was purchased from LI-COR Biosciences (Lincoln, USA). The BCA protein assay kit was from Pierce (Rockford, IL, USA).

2.2. Animals and Treatments

All the animal experimental procedures were carried out under the guidance of the Animal Care and Use Committee of Renmin Hospital of Wuhan University, which is also in agreement with the Guidelines for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication, revised 2011). All the animal experimental procedures, including surgery and subsequent analyses, were performed without knowledge of the treatments. C57/B6 mice (male, age: 8–10 weeks; body weight: 25.5 ± 2 g), purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China), were anaesthetized with 3% pentobarbital sodium (50 mg/kg, Sigma) by an intraperitoneal injection. Then, the mice were subjected to aortic banding (AB) or sham surgery as described previously (Jiang et al., 2014). Briefly, the left hemithorax of mouse was shaved, the left chest was opened by performing a ministernotomy, and the thoracic aorta was identified at the second intercostal space. Subsequently, the thoracic aorta was ligated with a 27-G needle using a 7-0 silk suture. Subsequently, the needle was removed, and the thoracic cavity was closed. During the surgery, a heating pad was used to keep the mouse warm. Temgesic (qd, 0.1 mg/kg) was used to relieve postoperative pain. The adequacy of constriction was confirmed via Doppler analysis without knowledge of the treatment. Beginning one week after surgery, the mice were orally treated for 3 weeks with piperine (50 mg/kg diluted in 0.1% DMSO) or vehicle. The dose of piperine was determined according to a previous article (Choi et al., 2013). The chemical structure of piperine has been reported previously (Taqvi et al., 2008). Four weeks after surgery, the mice were sacrificed with an overdose of sodium pentobarbital (200 mg/kg; i.p.) to harvest the hearts. To determine the effect of piperine on agonist-induced cardiac remodeling, we exposed the mice to continuous injection of ISO (50 mg/kg dissolved in sterile saline) for 14 days according to our previous study (Ma et al., 2016a,b).

2.3. Echocardiography and Hemodynamics

Transthoracic echocardiography was performed according to our previous studies (Ma et al., 2016a,b; Wei et al., 2016). Briefly, the mice were anaesthetized by 1.5% isoflurane, and then the left hemithorax was shaved and covered with the pre-warmed ultrasound gel. Transthoracic echocardiography was performed by a MyLab 30CV ultrasound (Esaote SpA, Genoa, Italy) with a 10-MHz linear array ultrasound transducer to obtain M-mode images at the papillary muscle level for measurement of wall thickness, chamber dimensions and cardiac function.

Invasive hemodynamic monitoring was performed to evaluate hemodynamics by cardiac catheterization, which was connected to a Millar Pressure-Volume System (MPVS-400; Millar Instruments). In brief, the mice were anaesthetized with 1.5% isoflurane and ventilated. Then, a 1.4-French Millar catheter transducer (SPR-839; Millar Instruments, Houston, TX) was placed into the left ventricle through the isolated carotid artery for the measurement of left intraventricular pressure. The obtained data were analyzed using PVAN data analysis software.

2.4. Morphometric Analyses and Immunohistochemistry

Hearts obtained from euthanized mice were arrested in diastole and then fixed with 4% formaldehyde overnight. The hearts were embedded in paraffin, sectioned into 5-μm slices, and stained with hematoxylin and eosin (H&E) to count the cardiomyocyte area, and further stained with picrosirius red (PSR) to measure cardiac fibrosis. The cross-sectional area and average collagen volume were counted using a digital analysis system (Image-Pro Plus 6.0, Media Cybernetics, Bethesda, MD, USA). For the detection of the cardiomyocyte area, 50 cells per slide were analyzed. For the determination of fibrosis, >60 fields per group were assessed.

To further evaluate cardiac fibrosis, we performed immunohistochemical staining for α-SMA. Briefly, the paraffin-embedded sections were incubated with anti-α-SMA (ab7817, Abcam, 1:100) overnight at 4 °C and EnVisionTM+/HRP reagent at 37 °C for 30 min. Then, the sections were visualized with diaminobenzidine (DAB) for 2 min at room temperature and mounted with neutral gums. Sections were assessed by light microscopy (Nikon H550L, Tokyo, Japan) and were examined in a blinded fashion by two authors.

2.5. Adenoviral Vectors and Injection

The constitutively active AKT1 and GFP adenoviral (Ad) vectors used in our study were generated by Hanbio Biotechnology Co. (Shanghai, China). To overexpress activated AKT1, mice were given an intramyocardial injection of 1×10^9 viral genome particles (Ad-*Akt* or Ad-*Gfp*, diluted in 15 μL PBS) in 3 locations of the left ventricle (Ma et al., 2016a,b). One week after adenoviral injection, these mice were subjected to injection of ISO for 14 days.

2.6. Western Blot and Quantitative Real-Time PCR

Protein extraction, SDS-PAGE, and immunodetection were performed according to previous articles (Ma et al., 2016a,b; Wei et al., 2016). Protein expression levels were normalized to the matched total proteins or GAPDH. Total mRNA was extracted from tissues or cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription and Real-time PCR were performed as described previously (Ma et al., 2016a,b; Wei et al., 2016). GAPDH was used as an internal control. The primers used are described in Table S1.

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