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Puerarin attenuates angiotensin II-induced cardiac fibroblast proliferation *via* the promotion of catalase activity and the inhibition of hydrogen peroxide-dependent Rac-1 activation

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[ABSTRACT] The aims of the present study were to evaluate the effects of puerarin on angiotensin II-induced cardiac fibroblast proliferation and to explore the molecular mechanisms of action. Considering the role of H_2O_2 in nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation, we hypothesized that modulating catalase activity would be a potential target in regulating the redox-sensitive pathways. Our results showed that the activation of Rac1 was dependent on the levels of intracellular H_2O_2. Puerarin blocked the phosphorylation of extracellular regulated protein kinases (ERK)1/2, abolished activator protein (AP)-1 binding activity, and eventually attenuated cardiac fibroblast proliferation through the inhibition of H_2O_2-dependent Rac1 activation. Further studies revealed that angiotensin II treatment resulted in decreased catalase protein expression and enzyme activity, which was disrupted by puerarin *via* the upregulation of catalase protein expression at the transcriptional level and the prolonged protein degradation. These findings indicated that the anti-proliferation mechanism of puerarin was mainly through blocking angiotensin II-triggered downregulation of catalase expression and H_2O_2-dependent Rac1 activation.

[KEY WORDS] Angiotensin II; Puerarin; Catalase; Hydrogen peroxide

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Introduction

Cardiac fibroblasts mediate heart remodeling *via* proliferating, differentiating, and secreting extracellular matrix proteins, and excessive proliferation leads to interstitial fibrosis and impairment of diastolic function. Angiotensin II, an effective stimulator of NADPH oxidase, activates many of the redox-sensitive signal pathways, leading to cells proliferation [1]. Thus, attenuation of NADPH oxidase activation may demonstrate an effective way to suppress cardiac fibroblast proliferation. Puerarin (7, 4-dihydroxy-8-*β*-D-glucosylisoflavone, $C_{21}H_{20}C_9$), an isoflavone polyphenolic antioxidant purified from the root of the plant *Puerara lobata*, has comprehensive pharmacological actions for the treatment of cardiovascular disease [2]. Our previous study has indicated that puerarin inhibits angiotensin II-induced cardiac hypertrophy *via* the redox-sensitive ERK1/2, p38, and NF-*κ*B pathways [3]. However, little information is available concerning the mechanisms of the effect of puerarin on catalase and Rac1 activation after angiontensin II induction.

It is well known that the stable H_2O_2 acts as a pivotal role in physiological signaling pathways in a variety of cells and tissues. There is document providing evidence for a signaling pathway in vascular endothelium in which elevation of intracellular H_2O_2 levels leads to the activation of the small GTPase Rac1 after stimulation of the angiotensin type 1 (AT1) receptor [4]. However, the signaling pathways connecting H_2O_2 accumulation and NADPH oxidase activation in mouse cardiac fibroblasts are less well understood. The predominantly enzymatic mechanisms that regulate intracellular H_2O_2 levels are mediated by catalases. A growing body of evidence has confirmed that puerarin protects different tissues from oxidative stress-induced damage by promoting the activities of catalase [5-6], but the underlying molecular mechanism is not clarified. The present study elucidated the mechanisms of anti-proliferative effect of puerarin, providing evidence for a pathway linking angiontensin II-dependent H_2O_2 generation

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to neonatal mouse cardiac fibroblast proliferation via a signaling pathway involving the key Rac1 activation and manipulation of catalase. Our findings would provide more convincing basis to support that puerarin may be a candidate to treat heart remodeling.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium/F12 medium (DMEM/F12) and fetal bovine serum (FBS) were purchased from Hyclone (Waltham, MA, USA). Anti-vimentin antibody, puerarin, apocynin, PD98059, propdium iodide (PI), actinomycin D, cycloheximide, 3-aminotriazole, polyethylene glycol (PEG)-catalase, losartan, and angiontensin II were bought from Sigma-Aldrich (St. Louis, MO, USA). NSC23766 was purchased from Millipore (Billerica, MA, USA). Anti-cyclinD1, anti-proliferating cell nuclear antigen (PCNA), anti-phospho-specific ERK1/2, and anti-ERK1/2 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-GAPDH, anti-Na^+/K^+-ATPase, anti-p47phox, and anti-p67phox antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). All the other reagents used in the present study were of an analytical grade and commercially available.

Neonatal mouse primary cardiac fibroblast culture

All the experiments with animals were approved by the Animal Ethical and Welfare Committee of Capital Medical University (Permit Number: AEEI-2015-116), and all animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85−23, revised 1996). Primary cultures of neonatal mouse cardiac fibroblasts were prepared as previously described [3, 7]. Cardiac fibroblasts grown in DMEM/F12 supplemented with 10% FBS from passage 2 to 3 were used in the experiments and were greater than 99% positive for vimentin antibodies. When cellular confluence reached 60%−70%, the cells were maintained in DMEM/F12 containing 0.1% FBS. After 24 h of serum starvation, the cardiac fibroblasts were pretreated for 1 h with 100 μmol·L^{-1} of puerarin, 10 μmol·L^{-1} of losartan, 1 000 IU of PEG-catalase, 10 μmol·L^{-1} of apocynin, 10 μmol·L^{-1} of PD98059 or 20 μmol·L^{-1} of NSC23766 and then stimulated with 1 μmol·L^{-1} of angiontensin II or 100 μmol·L^{-1} of H_2O_2 for the indicated times. The cells were then harvested and extracted for the analysis. Puerarin was dissolved in dimethyl sulfoxide (DMSO). This stock solution was diluted to the final concentrations with the extracellular solution Dulbecco's modified Eagle's medium/F12 medium (DMEM/F12) before application. The final concentration of DMSO did not exceed 0.1%, and no effects of the vehicle were found in our experiments.

Cardiac fibroblast proliferation assay

The rate of cellular proliferation was determined by cell counting and quantifying 5-bromo-2′-deoxyuridine (BrdU) incorporation as described previously [8]. The cells (1 × 10^5/well) were seeded into 6-well plates and cellular confluence reached 60%−70%. After 24 h serum starvation, the cells were treated with or without different reagents for 48 h. The cells were removed from the culture dish by addition of 0.125% trypsin and then centrifuged (1 000 *g* for 5 min). The pellet was resuspended in 1 mL of DMEM/F12, and the cells were counted in an automatic cell counter (TC20 ™, Bio-Rad, Hercules, CA, USA). The cells (8 × 10^3/well) were incubated in 96-well plastic plates. When cellular confluence reached 60%−70%, the cells were treated with or without different reagents for 24 h after 24 h serum starvation. Then, the incorporation of BrdU was measured with a BrdU Cell Proliferation Assay Kit (BioVision Inc, Milpitas, CA, USA), according to the manufacturer's instructions.

Cell cycle analysis by flow cytometry

After the starved cells were treated with or without different reagents for 24 h, the cells were trypsinized, washed once with phosphate buffer saline (PBS), and then fixed with cold 70% ethanol at 4 °C overnight. The fixed cells were washed twice with PBS and incubated with 100 μg·mL^{-1} of RNaseA at 37 °C for 30 min and then stained in PBS containing 50 μg·mL^{-1} of PI for 1 h. The fluorescence intensity was detected using a BD FACSCalibur cytometer and the cell cycle distribution was determined using the ModFit LT software (BD Biosciences, San Diego, CA, USA).

Detection of intracellular H_2O_2 production and catalase activity

The cells (1 × 10^5/well) were seeded into 6-well plates, grown for 48 h in DMEM/F12 containing 10% FBS, and quiesced for an additional 24 h in DMEM/F12 containing 0.1% FBS. The cells were then stimulated with different reagents for indicated times. Intracellular H_2O_2 levels and catalase activity were measured by Hydrogen Peroxide Colorimetric / Fluorometric Assay Kit (BioVision Inc, Milpitas, CA, USA) and Catalase Activity Colorimetric / Fluorometric Assay Kit (BioVision Inc, Milpitas, CA, USA), respectively, according to the manufacturer's instructions.

Measurements of NADPH oxidase activity

NADPH oxidase activity was determined using an assay based on the lucigenin-enhanced chemiluminescence assay, as described previously [9]. Photoemissions were measured over 10 min in quadruplicate with a Lumat LB9507 luminometer (Berthold Technologies GmbH & Co. KG, Germany) in 1-min intervals. The results were expressed as relative light units (RLUs) per microgram of cell extract.

Measurements of Rac1 activation

Activated Rac1 was determined by p21-binding domain of p21-activated protein kinase 1 pull-down assay, according to the manufacturer's protocol for the Rac1 Activation Assay Kit (Cell Biolabs, San Diego, CA, USA).

Preparation of cell fraction extracts and Western blotting analysis

After various treatments, plasma membrane and cytoplasmic protein enriched fractions of cardiac fibroblast were prepared using a Plasma Membrane Protein Extraction Kit