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Angiotensin IV protects against angiotensin II-induced cardiac injury via AT4 receptor

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ABSTRACT

Angiotensin II (Ang II) is an important regulator of cardiac function and injury in hypertension. The novel Ang IV peptide/AT4 receptor system has been implicated in several physiological functions and has some effects opposite to those of Ang II. However, little is known about the role of this system in Ang II-induced cardiac injury. Here we studied the effect of Ang IV on Ang II-induced cardiac dysfunction and injury using isolated rat hearts, neonatal cardiomyocytes and cardiac fibroblasts. We found that Ang IV significantly improved Ang II-induced cardiac dysfunction and injury in the isolated heart in response to ischemia/reperfusion (I/R). Moreover, Ang IV inhibited Ang II-induced cardiac cell apoptosis, cardiomyocyte hypertrophy, and proliferation and collagen synthesis of cardiac fibroblasts; these effects were mediated through the AT4 receptor as confirmed by siRNA knockdown. These findings suggest that Ang IV may have a protective effect on Ang II-induced cardiac injury and dysfunction and may be a novel therapeutic target for hypertensive heart disease.

1. Introduction

The renin–angiotensin system (RAS) has a pivotal role in the pathogenesis of cardiovascular diseases. The main effector peptide of the RAS, angiotensin II (Ang II), mediates the immediate physiological effects of vasoconstriction and blood pressure regulation and is implicated in inflammation, endothelial dysfunction, hypertension and heart failure [42]. Emerging evidence indicates that Ang II exerts these actions by binding to its receptors, including Ang type 1 and type 2 receptors (AT1R and AT2R, respectively) [42]. The functions of Ang II in the cardiovascular system and brain are predominantly mediated through AT1R activation [42]. Inhibition of AT1R and angiotensin-converting enzyme 1, as targets of treatment, have been effective in reducing the risk of cardiovascular morbidity and mortality, including myocardial infarction, heart failure, and stroke [23].

In addition to Ang II and its receptors, a novel Ang receptor, AT4 receptor (AT4R), was found widely expressed in many tissues, including kidney, heart, blood vessels, and brain and upregulated in arteries in response to injury [8,21]. Ligands for this receptor include the Ang II metabolite (3–8) Ang IV, a hexapeptide derived by the cleavage of 2 N-terminal amino acids from Ang II by aminopeptidases [8]. Interestingly, rabbit myocardium showed 10-fold more AT4R than AT1R. AT4R has a high affinity for Ang IV but very low affinity for Ang II [1,5]. Increasing evidence suggests that Ang IV has an important physiological function by binding to AT4R [8]. The Ang IV–AT4R system may counteract AT1R-mediated cardiovascular events such as blood pressure control, cell growth, and cardiac function [22,26]. For example, chronic Ang IV treatment reversed endothelial dysfunction in ApoE-deficient mice [30]. Pharmacological doses of Ang IV were protective against acute cerebral ischemia by triggering AT4R-mediated, nitric oxide-dependent intracerebral hemodynamic mechanisms [13]. In contrast, deletion of AT4R reduced fibrinolysis by downregulating plasminogen activator inhibitor type 1 and preventing the subsequent formation of arterial thrombosis [22]. Ang IV also enhanced cognition in animal models [14,26]. Interestingly, there are some studies that have reported that Ang IV has similar effects to those of Ang II via AT1R [10,20]. For example, Ang IV significantly decreases the baseline mean arterial pressure (MAP), renal cortical blood flow (CBF) and cortical vascular resistance (CVR) in AT1a knockout mice

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than wild-type mice [39]. However, the effect of this system on cardiac dysfunction and injury induced by Ang II has not been studied.

In the present study, we sought to determine whether Ang IV could attenuate Ang II-induced cardiac dysfunction and injury via AT4R. We provide important insights into the novel association of Ang IV and Ang II-mediated cardiac injury and demonstrate a pivotal role of Ang IV/AT4R pathway in the pathogenesis of hypertensive heart diseases.

2. Materials and methods

2.1. Materials

Sprague-Dawley (SD) male rats (200–250 g) and neonatal SD rats less than 3 days old were from the Animal Department, Capital Medical University. The experimental protocol was approved by the Animal Care and Use Committee of Capital Medical University. Ang II and Ang IV were from Sigma (St. Louis, MO, USA). Collagenase, Trizol, Lipofectamine 2000 and double-strand small interfering RNA (siRNA) targeting AT1R (NM_030985) and AT4R (U76997.1) were from Invitrogen (San Diego, CA). The cytotoxicity detection kit was from Roche (Switzerland). Kits to detect lactate dehydrogenase (LDH) and hydroxyproline content were from Jian-Cheng Biochemical Engineering (Nanjing, China). ^{3}H-Leucine (^{3}H-Leu) and ^{3}H-thymidine (^{3}H-TdR) were from NEN Life Science Products (Boston, MA). Fetal calf serum (FCS), Dulbecco modified Eagle medium (DMEM), mitomycin C and streptomycin/penicillin were from Gibco BRL (Life Technologies, Paisley, UK). Other chemicals and reagents were of analytical grade.

2.2. Langendorff model of myocardial ischemia/reperfusion (I/R) injury

SD rats were anaesthetized by intraperitoneal injection of 20% urethane (6 ml/kg). Hearts were excised and perfused retrogradely by the aorta at a constant flow with 8 ml Krebs–Henseleit (K–H) buffer with constant oxygenation (95% O_2 and 5% CO_2, v/v), pH 7.4 at 37 °C as described [40]. Isolated rat hearts were randomly assigned to groups for treatment (n = 6 per group) and stabilized for 20 min, then underwent 40 min global ischemia (achieved by total perfusion arrest) and 30 min reperfusion (I/R). Except for the control group (no perfusion arrest) and I/R group, Ang II and Ang IV were diluted in the reperfusion buffer alone or together to obtain a final concentration of 100 nM. Myocardial temperature (thermal probe) and heart function (ventricular balloon) were monitored throughout the perfusion periods. The LDH activity in the perfusion solution was measured by LDH release kit (Cat No: A001-1, Jian-Cheng Biochemical Engineering, Nanjing, China).

2.3. Cell culture and Ang II or IV treatment

Neonatal rat cardiomyocytes and fibroblasts were isolated from SD rat hearts as described [40]. Briefly, minced hearts were digested with 0.0625% trypsin, 0.05% collagenase I and 0.025% collagenase II in a 37 °C water bath. Digested cells were mixed with DMEM containing 20% FCS and seeded in plates for 30 min to allow selective adhesion of cardiac fibroblasts. The cardiomyocytes were cultured in DMEM containing 20% FCS, 1% streptomycin/penicillin and 2 μg/ml mitomycin C at 37 °C [30]. Cardiac fibroblasts from passages 2 to 4 and primary cardiomyocytes were identified with α-actinin and vimentin antibody. Cells were treated with Ang II and/or Ang IV for the indicated doses.

2.4. Measurement of cell apoptosis, injury and hydroxyproline concentration

Neonatal cardiomyocytes and fibroblasts were treated with ischemia buffer that contained 118 mM NaCl, 24 mM $NaHCO_3$, 1.0 mM NaH_2PO_4, 2.5 mM $CaCl_2$–$2H_2O$, 1.2 mM $MgCl_2$, 20 mM sodium lactate, 16 mM KCl, 10 mM 2-deoxyglucose (pH 6.2) for 2 h and reperfused with normal medium [35] containing Ang II (100 nM) and Ang IV (100 nM) for 24 h. Apoptosis was analyzed with In situ Cell Death Detection Kit, according to manufacturer's instructions (Roche, Cat No: 11684817910). The number of TUNEL-positive cells was analyzed using NIH Image software. The LDH activity in the medium was measured by use of a cytotoxicity detection kit (Roche, Cat No: 11644793001). Fibroblasts were treated with Ang II (100 nM) and concentrations of Ang IV (0–100 nM) for 24 h, and hydroxyproline content was measured by use of hydroxyproline kit (Cat No: A030-1, Jian-Cheng Biochemical Engineering, Nanjing, China).

2.5. siRNA transfection

Cardiomyocytes and fibroblasts were transfected with siRNA-AT1, -AT4 or -control with use of Lipofectamine 2000 according to the manufacturer's instructions [31]. The double strand small interfering RNA (siRNA) targeting AT1R (NM_030985) and AT4R (U76997.1) was purchase from Invitrogen (San Diego, CA). The siRNA sequences were for siRNA-AT1R-1: 3′-UUACAUAGGUGAUUGCCGAAGGGCC-5′, 3′-GGCCCUUCGGCAAUCACCUAUGUAA-5′; siRNA-AT1R-2: 3′-UUCUUUAGAGCUUUCCAAAUAAGGG-5′, 3′-CCCUUAUUUGGAAAGCUCUAAAGAA-5; siRNA-AT4R-1: 5′-UAGCGUUGAGGAAUAAUGGCAGUGG-3′, 5′-CCACUGCCAUUAUUCCUCAACGCUA-3′; siRNA-AT4R-2: 5′-UGAUAAUGCAGUAUGGUGCUCAUCC-3′, 5′-GGAUGAGCACCAUACUGCAUUAUCA -3′. The AT4 receptor siRNA is targeting the insulin-regulated aminopeptidase.

2.6. Real-time PCR

At 48 h after transfection, the expression of AT1R and AT4R mRNA was detected by RT-PCR analysis. Total RNA was extracted from cells with use of Trizol reagent according to the manufacturer's instruction. In total, 1 μg RNA was reverse-transcribed into single-strand cDNA. Primer sequences were for AT1: 5′-TCTGGGCTTCTTGTTCCCTTTCCT-3′, 5′-AACAAAGGTTCCTTGCCCTTTGGG-3′; AT4: 5′-GGCTAAACGAAGGCTTTGCCACTT-3′, 5′-ATCTGTCCATGTCTGCTGCTGGAT-3′; and GAPDH: 5′-TGACTCTACCCACGGCAAGTTCAA-3′, 5′-TTGTCATTGAGAGCAATGCCAGCC-3′. The amplification products were electrophoresed on 1% agarose gel and visualized by ethidium bromide (0.5 g/ml) staining. Gel images were photographed and analyzed by use of the ABI 7700 Prism Sequence Detection System (PE-ABI). GAPDH mRNA was an internal control. The RT-PCR conditions for AT1R, AT4R and GAPDH was as follows: force-denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 61.5 °C for 60 s, and elongation at 72 °C for 45 s, and a final extension at 72 °C for 5 min.

2.7. ^{3}H-Leu incorporation assay

Protein synthesis was evaluated by ^{3}H-Leu incorporation into cardiomyocytes as described [33]. Briefly, cardiomyocytes were seeded at 2×10^5 cells/well in 24 pores plates, transfected with siRNA for 24 h and then treated with Ang II (100 nM) and concentrations of Ang IV (1–100 nM). At 10 h before the end of incubation, 1 μCi/well of ^{3}H-Leu was added in the culture medium. Cultures were incubated with 10% trichloroacetic acid on ice for 20 min and