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Activin A stimulates the proliferation and differentiation of cardiac fibroblasts via the ERK1/2 and p38-MAPK pathways

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ABSTRACT

Activin A is a key regulator of cardiac fibrosis. However, little is known about the mechanisms by which it contributes to cardiac fibrosis. Our study explored the effects of activin A on proliferation and differentiation of adult rat cardiac fibroblasts (CFs) via the activin A receptor, activin receptor-like kinase 4 (ALK4). CF proliferation was measured by CCK8 and EdU assays, while differentiation, fibrosis and signaling were measured by western blot analysis of α-smooth muscle actin, collagen type I, phosphorylated extracellular signal-regulated kinase (ERK)1/2 and p38 mitogen-activated protein kinase (p38-MAPK) expression. Activin A levels were measured by ELISA and western blot analysis. We demonstrated that CFs express activin A and its expression was significantly enhanced by angiotensin II (Ang II), but follistatin (activin A inhibitor) significantly reversed Ang II-induced activin A upregulation, CF proliferation, differentiation, collagen type I expression as well as ERK1/2 and p38-MAPK pathways activation. Conversely, recombinant activin A largely increased these parameters in both the presence and absence of Ang II. Interestingly, p38-MAPK (SB203580) and ALK4 (SB431542) inhibitors significantly reduced all activin A-mediated responses; however, an ERK1/2 inhibitor (PD98059) could only significantly reduce CF proliferation and collagen type I expression but not differentiation. Importantly, the most significant effects were observed in the presence vs. absence of Ang II. Thus, activin A promotes basal and Ang II-induced CF proliferation and differentiation via ALK4, and the effects are partly mediated through the ERK1/2 and p38-MAPK pathways. These data suggest that activin A is a potential therapeutic target for cardiac fibrosis.

1. Introduction

Cardiac fibroblasts (CFs) account for approximately two-third of all cells in the myocardium (MacKenna et al., 2000). Cardiac fibrosis plays an important role in arrhythmias, myocardial infarction, hypertension and heart failure (Berk et al., 2007; Vasquez et al., 2011; Pan et al., 2012; Song and Wang, 2015). This response includes CF proliferation and differentiation into myofibroblasts, which then synthesize and release extracellular matrix proteins such as collagen I and collagen III, which are regarded as the main elements of cardiac fibrosis (Porter and Turner, 2009). Therefore, inhibition of CF proliferation and differentiation may prove to be an effective strategy to delay the pathological process of cardiac fibrosis.

Activin A, a transforming growth factor-β superfamily member, plays an important role in cell proliferation, differentiation, fibrosis, inflammation and immune regulation (Hedger and de Kretser, 2013). The effects of activin A are exerted through the type II (ACVR2A or ACVR2B) and type I (activin receptor-like kinase 4, ALK4, ACVR1B) activin receptors (Hedger and de Kretser, 2013). Studies have also suggested an important role for activin A in cardiac fibrosis in various animal and cell models (Bergestuen et al., 2010; Wei et al., 2013; Venteclef et al., 2015). Furthermore, activin A is mitogenic and can trigger proliferation and differentiation of many cells types. For example, thrombin and Ang II induce activin A production leading to an increase in DNA synthesis in rat aortic smooth muscle cells (Pawlowski et al., 1997). Activin A can also induce DNA synthesis in porcine thyroid cells (Kotajima et al., 1995), as well as enhance proliferation of

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fibroblast-like synoviocytes in rheumatoid arthritis (Mor et al., 2005). Additionally, activin A induces the proliferation and differentiation of human lung fibroblasts, primary renal interstitial fibroblasts and NRK-49F cells (Ohga et al., 1996; Yamashita et al., 2004). Thus, it is of significant interest to determine whether activin A can regulate CF proliferation and differentiation.

Extracellular signal-regulated kinase (ERK)1/2 and p38 mitogen activated protein kinase (p38-MAPK) pathways play an important signaling role in CF proliferation (Zhang and Liu, 2002). The p38-MAPK pathway has been specifically shown to play a critical role in CF differentiation (Davis and Molkentin, 2014). Angiotensin II (Ang II) is also an important upstream mediator of CF proliferation and differentiation (Stockand and Meszaros, 2003; Davis et al., 2012). Growing studies have linked activin A to the ERK1/2 and p38-MAPK signaling pathways. Activin A promotes the expression of a proliferation-inducing ligand via the ERK1/2 pathway in mouse macrophages (Lee et al., 2011) as well as induces erythroid differentiation via the p38-MAPK pathway in K562 cells (Huang et al., 2004). However, little is known of the effects of activin A on CF function as well as the involvement of ERK1/2 and p38-MAPK pathways in this context. Therefore, the goal of our study was to explore whether activin A stimulated CF proliferation and differentiation in an ALK4-dependent manner under basal and Ang II conditions at least in part via the ERK1/2 and p38-MAPK pathways.

2. Materials and method

2.1. Reagents and antibodies

Recombinant rat activin A, human follistatin-300 (follistatin) and an ALK4 inhibitor (SB431542) were purchased from R&D Systems (Minneapolis, MN, USA). The ERK1/2 inhibitor (PD98059) and p38 inhibitor (SB203580) were purchased from Promega (Madison, WI, USA). Ang II was purchased from Sigma (St. Louis, MO, USA). Antibody sources were as follows: activin A (AF338) was from R&D Systems; ERK1/2 (#4695), phospho-ERK1/2 (p-ERK1/2, #4370), p38-MAPK (#8690) and phospho-p38-MAPK (p-p38, #4511) were from Cell Signaling Technology (Danvers, MA, USA); α-smooth muscle actin (α-SMA, #ab5694), collagen type I (ab6308) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, ab37168) were from Abcam (Cambridge, UK); horseradish peroxidase-conjugated anti-rabbit/mouse/goat IgG was from Amersham Biosciences (Buckinghamshire, UK).

2.2. Preparation of adult rat left ventricular CFs

All animal procedures were performed in accordance with the Guidelines of the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication #85–23, revised 1996) and the rules for the Care and Use of Laboratory Animals from the Institutional Animal Care and Use Committee of Wuhan University, China. Left ventricular CFs were isolated from adult male Sprague–Dawley rats (8–14 weeks old) and cultured under sterile conditions as described previously (Wang et al., 2013). Rats were anesthetized with sodium pentobarbital (40 mg/kg i.p.; Sigma), and the hearts were quickly excised and placed in cold phosphate-buffered saline (PBS). The atria and valves were removed. The left ventricles were dissected, minced into small pieces, washed twice with Dulbecco's Modified Eagle's Medium (DMEM), and then digested with 0.04% collagenase and 0.08% trypsin for 15 min at 37 °C. The digestion was repeated until the tissues were completely digested. The combined cell suspension was centrifuged at 1000 × *g* for 10 min, and the cell pellet was suspended in DMEM (with 5 mM glucose; Gibco) supplemented with 10% fetal bovine serum (FBS, Invitrogen), streptomycin (100 μg/ml), and penicillin (100 U/ml). The cell suspension was incubated in culture flasks for 1.5 h to allow selective adhesion of CF, and the unattached myocytes were removed. CF were maintained in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. The identity of the CF was confirmed by positive staining for the fibroblast marker vimentin and lack of staining for von Willebrand factor (an endothelial-cell-specific marker) and α-actinin (myocardial-cell-specific marker). CF from the first and second passages were serum starved for 24 h before use in experiments. After the experiments, the culture supernatants were harvested for ELISA analysis and the cells were collected for protein extraction, cell proliferation assays, and immunofluorescence staining.

2.3. Cell proliferation assays

CF proliferation was determined using a Cell Counting kit (CCK-8; Dojindo Kumamoto, Japan) and an EdU assay kit (Ribobio). For the CCK-8 assay, cells were seeded in 96-well plates, serum starved, and then treated with the experimental reagents as described in the text. At the end of the incubation, CCK-8 solution was added for 4 h and the absorbance at 450 nm ($A_{450\ nm}$) was measured with a microplate reader (Sunrise). DNA synthesis was measured with the EdU incorporation assay according to the manufacturer's instructions. Cells were cultured in 12-well plates, serum starved, and treated with reagents. At the end of the incubation, EdU was added for 2h, and the cells were fixed and treated with the kit Apollo reaction reagent. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and the cells were visualized with a fluorescence microscope (Olympus BX51, Tokyo, Japan). Proliferating cells were quantified by counting the number of EdU-positive (green) cells relative to the total number of cells (blue nuclei) using Image Pro-Plus 6.0 software (Wang et al., 2013).

2.4. Western blot analysis

Total protein was extracted from cultured CF and protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA). Samples containing equal concentrations of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The blots were blocked with 3% bovine serum albumin (BSA) (for detection with phosphoprotein-specific antibodies) or 5% non-fat milk (for all other antibodies) in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature, and then incubated with primary antibodies activin A (0.1 μg/ml), ERK1/2 (1:1000), p-ERK1/2 (1:2000), p38-MAPK (1:1000), p-p38 (1:1000), α-SMA (1 μg/ml), collagen type I (1:1000), or GAPDH (1:10,000) at 4 °C overnight. The membranes were washed three times and incubated with horseradish peroxidase (HRP)-coupled secondary antibodies to detect proteins of interest. Finally, the blots were developed with a enhanced chemiluminescence kit (ECL; St. Louis, MO, USA) and the bands were quantified densitometrically using a Bio-Rad imaging system (Hercules, CA). The relative band intensity of each sample was normalized to the GAPDH signal in the same lane. The relative intensity of the protein of interest was normalized to that of the control group, which was set as 1 (100%).

2.5. Immunofluorescence staining

CF were cultured on coverslips, washed with PBS, fixed with 4% paraformaldehyde for 20 min, and permeabilized with 0.1% Triton X-100 in PBS added to the coverslips with a syringe. The coverslips were then blocked in 3% BSA for 30 min and incubated with a