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Activin receptor-like kinase 7 mediates high glucose-induced H9c2 cardiomyoblast apoptosis through activation of Smad2/3

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ABSTRACT

Cardiomyocyte apoptosis is an important pathological change of diabetic cardiomyopathy. How the elevated glucose levels cause cell apoptosis remains unknown. The aim of our study was to investigate whether activin receptor-like kinase 7 (ALK7)-Smad2/3 signaling pathway plays an important role in high glucose-induced cardiomyocyte apoptosis. H9c2 cardiomyoblasts and neonatal rat cardiomyocytes were treated with 33 mmol/l glucose. The expression of ALK7, Smad2 and Smad3 were inhibited by small interfering RNA respectively. The level of ALK7, total Smad2/3, phosphorylated Smad2/3, B-cell lymphoma-2 (Bcl-2) and cleaved Caspase3 were evaluated using western blot. The apoptosis rate was detected by flow cytometer. High glucose treatment caused the apoptosis of H9c2 cardiomyocyte and the inhibition of Smad2 or Smad3 attenuated this apoptosis. ALK7 existed in both H9c2 cardiomyoblasts and neonatal rat cardiomyocytes and high ambient glucose upregulated its expression. The increased expression level of cleaved Caspase3 and apoptosis rate and decreased expression of Bcl-2 were reversed after ALK7 was inhibited. The expression of phosphorylated Smad2/3 also decreased after the knockdown of ALK7. Our findings suggest that ALK7 mediates high ambient glucose-induced H9c2 cardiomyoblasts apoptosis through the activation of Smad2/3.

1. Introduction

Diabetic cardiomyopathy (DCM) which is characterized by systolic and diastolic dysfunction is a primary cause of death in patients with type 1 and 2 diabetes (Shaw et al., 2010; Liu et al., 2001). The apoptosis of cardiomyocyte, an important pathological change of DCM (Kuethe et al., 2007; Schroder et al., 2006), causes progressive loss of effective myocardial contractile unit (Foo et al., 2005), initiates cardiac remodeling and finally results in both systolic and diastolic dysfunction of the heart (Devereux et al., 2000). Hyperglycemia is a typical feature of diabetic metabolism disorder and plays an important role in the genesis of cardiomyocytes apoptosis (Pfister and Erdmann, 2008; Falcao-Pires and Leite-Moreira, 2012). However, the mechanism underlying high glucose-induced cardiomyocytes apoptosis remains not well understood.

Smad2 and Smad3, necessary mediators for the signal transduction of TGF-β superfamily, form a heteromeric complex with the co-Smad (Smad4) after phosphorylated and then translocate into the nucleus to regulate target gene transcription (Narayan et al., 2005). Studies have proved that hyperglycemia activity could induce the phosphorylation of Smad2 and Smad3 (Castoldi et al., 2009). Smad2 and Smad3 are important apoptosis regulatory proteins and could stimulate apoptosis-related genes such as caspase-3, caspase-6 and caspase-9 (Castoldi et al., 2009; Heger et al., 2009). However, it is not clear whether Smad2/3 participate in high glucose-induced cardiomyocyte apoptosis or not.

ALK7, a new member of type I TGF-β receptor, is firstly isolated from rat brain as an orphan receptor (Rydén et al., 1996;

Abbreviations: DCM, diabetic cardiomyopathy; TGF-β, transforming growth factor-β; ALK, activin receptor-like kinase; ActRII, activin type II receptors; Bcl-2, B-cell lymphoma-2; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; siRNA, small interfering RNA; GDF, growth and differentiation factors; BMPs, bone morphogenetic proteins; XIAP, X-linked inhibitor of apoptosis protein.

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Tsuchida et al., 1996) and consists of a transmembrane domain, a serine/threonine kinases domain and a GS domain between them (Tsuchida et al., 2008). Nodal, activin AB, activin B and GDF are ligands specific for ALK7 (Tsuchida et al., 2008; Reissmann et al., 2001) while ActRII are their primary ligands binding receptors during signal transduction processes. Once bound, ALK7 is recruited to form a complex and then its GS domain is phosphorylated by ActRII and brings about a series of transformation of downstream substrates, such as Smads proteins (Greenwald et al., 2003). Studies have shown the existence of ALK7 in human liver, intestine, adipose tissue, pancreas and brain tissue (Roberts et al., 2003; Bondestam et al., 2001; Lorentzon et al., 1996). It also participates in the proliferation and apoptosis of human ovarian epithelial cells, hepatoma cell line and pancreatic beta-cell (Bondestam et al., 2001; Xu et al., 2006; Zhang et al., 2006). ALK7 activates the same receptor-regulated Smad proteins (Smad2 and Smad3) as ALK4 and ALK5 in the signal transduction of TGF-β family (Reissmann et al., 2001). Thus, ALK7-Smad2/3 signaling pathway might play a crucial role in high ambient glucose-induced cardiomyocyte apoptosis.

To explore whether ALK7-Smad2/3 signaling pathway is involved in high glucose-induced cardiomyocyte apoptosis, H9c2 cardiomyoblasts and neonatal rat cardiomyocytes were cultured in high glucose condition. First, we analyzed the impact of high glucose on H9c2 cardiomyoblasts apoptosis; then we studied the role of Smad2 and Smad3 in high ambient glucose-induced H9c2 cardiomyoblasts apoptosis. We further analyzed the effect of high glucose on the expression level of ALK7 and the effect of ALK7 in high glucose-induced apoptosis. Finally, the phosphorylation level of Smad2 and Smad3 after inhibition of ALK7 in H9c2 cardiomyoblasts were measured.

2. Materials and methods

2.1. Reagents and antibodies

DMEM, fetal bovine serum, and trypsin were purchased from Gibco (Grand Island, NY, USA). Trizol reagent and Lipofectamine^{TM}2000 were purchased from Invitrogen (Carlsbad, CA, USA). Real-time RT-PCR assay kits were purchased from Takara (Dalian, China). PCR primers were synthesized by Biosun (Jinan, China). The siRNA specific for ALK7, Smad2 and Smad3 and scramble control siRNA sequence were designed and synthesized by Integrated Biotech (Shanghai, China). Mouse anti-rat ALK7 monoclonal antibody was purchased from R&D Systems (Minneapolis, MN, USA). Rabbit anti-rat Smad2/3 antibody, phosphorylated Smad2 antibody, phosphorylated Smad3 antibody, Bcl-2 antibody and cleaved Caspase3 antibody were purchased from Cell Signaling Technology (Beverly, MA). GAPDH antibody was obtained from Zhongshan Biotech (Guangzhou, China). Horseradish peroxidase-conjugated rabbit anti-mouse IgG secondnary antibody was obtained from Abcam (Cambridge, MA, USA). The FITC Annexin V apoptosis detection kit was purchased from Becton-Dickinson Biosciences (San Diego, CA).The chemiluminescence (ECL) kit was obtained from Amersham Pharmacia (Piscataway, NY). Other chemicals were of the highest purity grade.

2.2. Cell culture

Neonatal rat cardiac myocytes were prepared from the ventricles of 2–3-day-old Sprague-Dawley rats. This investigation conforms with the guide for the care and use of laboratory animals published by the U.S. National Institutes of Health. All experimental procedures were performed in accordance with animal protocols approved by the Shandong University Animal Care Committee. Briefly, ventricles were minced and cardiac myocytes were parepared by trypsin (0.125%) and collagenase type 2 (0.08%). The enzyme digestion was performed for about 5–10 min every time and repeated for about 8–10 times until all the tissue blocks were digested. Collected cells were preincubated for 2 h in DMEM with 10% FBS containing appropriate 5-bromo-2-deoxyuridine to reduce non-myocyte contamination and then the remaining cell suspension was plated (2.0×10^6 cells) in culture flasks. Rat cardiomyocytes and H9c2 rat cardiomyoblasts (ATCC) were maintained in low glucose (5.5 mM) DMEM supplemented with 10% FBS in 5% CO2 and 95% humidified air at 37 °C. The cells medium were replaced with DMEM containing 5.5 mM glucose for 12 h when the confluence of H9c2 cardiomyoblasts reached 50%, then with DMEM containing 5.5 mM glucose (normal glucose), 5.5 mM glucose plus 27.5 mM mannose (osmotic control), or 33 mM glucose (high glucose). After coincubation at 37 °C for 4, 8, 12, 24 and 48 h, cells were harvested for real-time RT-PCR, western blotting or flow cytometry. All tests were repeated three times at least. To examine the effect of ALK7 on cell apoptosis and the activation of Smad2/3, ALK7-siRNA plasmid was transfected 24 h before stimulations. To examine the effect of Smad 2/3 on apoptosis induced by high ambient glucose, Smad2 and Smad3 siRNA was transfected separately 24 h before stimulations.

2.3. Small interfering RNA (siRNA) transfection

ALK-7-siRNA plasmid (pGenesil-1) and siRNA specific for Smad2 and Smad3 were used to inhibit the expression of ALK7, Smad2 and Smad3, respectively. The 5′-GGUCGUUUGUGAUCAGAAACU-3′ and 5′-UUUCUGAUCACAAACGACCUU-3′ oligoribonucleotides were used to inhibit ALK7 synthesis while plasmid with randomly mixed sequences siRNA were used as a negative control. The 5′-CGAAUGUGCACCAUAAGAAtt-3′ and 5′-UUCUUAUGGUGCACAUUCGtt-3′ oligoribonucleotides were used to inhibit the expression of Smad2 while the 5′-GGAAUUUGCUGCCCUCCUAtt-3′ and 5′-UAGGAGGGCAGCAAAUUCCtt-3′ oligoribonucleotides were used to inhibit Smad3 synthesis. Randomly mixed sequences siRNA 5′-UUCUCCGAACGUGUCACGUTT-3′ and 5′-ACGUGACACGUUCGGAGAATT-3′ oligoribonucleotides were used as a negative control. The plasmids or oligoribonucleotides were transfected into cells grown in a six-well plate by using Lipofectamine^{TM}2000 Transfection according to the manufacturer's protocol. The non-fluorescent-labeled plasmid and siRNA were used in assessment of apoptosis by flow cytometry.

2.4. Assessment of apoptosis by flow cytometry

The apoptosis induced by high ambient glucose in H9c2 cardiomyoblasts was detected using Annexin V-FITC/PI staining on flow cytometer (BD, Mansfield, MA, USA). Cells were harvested for flow cytometry after exposing to high ambient glucose or control for 24 or 48 h. Briefly, cells were washed twice with cold PBS and resuspended in binding buffer at a concentration of 1×10^6 cells/ml. Then 100 μl of the solution was transferred to a 5 ml culture tube and subsequently labeled with 5 μl of Annexin V and 5 μl PI. The solution was gently vortexed and incubated for 15 min at 25 °c in the dark, then, 400 μl of the binding buffer was add to each tube. The analysis by flow cytometry should conduct within 1 h. Data were analyzed using the software FlowJo.

2.5. Real-time quantitative RT-PCR

Total RNA was preparing with trizol agent according to the manufacturer's procedures after the cells incubated at 37 °C for0, 0.5, 1, 4, 8, 12, 24, 48 h. The amount of extracted mRNA was measured by extinction at 260 nm and the purity was determined by the 260/280 ratio. The 20 μl reverse-transcription systems