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TGF-β1-elevated TRPM7 channel regulates collagen expression in hepatic stellate cells *via* TGF-β1/Smad pathway

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ABSTRACT

Transdifferentiation of hepatic stellate cells (HSCs) into myofibroblasts plays a critical role in the development of liver fibrosis, since myofibroblasts are the key cells responsible for excessive deposition of ECM proteins. Transient receptor potential melastatin 7 (TRPM7), a non-selective cation channel with protein serine/threonine kinase activity, has been demonstrated to function in the proliferation of activated HSCs. Here, we investigated the functional role of TRPM7 in collagen deposition in activated HSC-T6 cells (a rat hepatic stellate cell line). TRPM7 mRNA and protein were measured by Real-time PCR and Western blot in TGF-β1-activated HSC-T6 cells *in vitro*. Results demonstrated that TRPM7 protein was dramatically increased in fibrotic human livers. Stimulation of HSC-T6 cells with TGF-β1 increased TRPM7 mRNA and protein level in a time-dependent manner. Nevertheless, TGF-β1-elicited upregulation of TRPM7 in HSC-T6 cells was abrogated by SB431542 (TGF-β1 receptor blocker) or SIS3 (inhibitor of Smad3 phosphorylation). Additionally, blockade of TRPM7 channels with non-specific TRPM7 blocker 2-APB or synthetic siRNA targeting TRPM7 attenuated TGF-β1-induced expression of myofibroblast markers, as measured by the induction of α-SMA and Col1α1. Silencing TRPM7 also increased the ratio of MMPs/TIMPs by increasing MMP-13 expression and decreasing TIMP-1 and TIMP-2 levels. Strikingly, phosphorylation of p-Smad2 and p-Smad3, associated with collagen production, was decreased in TRPM7 deficient HSC-T6 cells. These observations suggested that TGF-β1 elevates TRPM7 expression in HSCs *via* Smad3-dependant mechanisms, which in turn contributes Smad protein phosphorylation, and subsequently increases fibrous collagen expression. Therefore, TRPM7 may constitute a useful target for the treatment of liver fibrosis.

Introduction

Hepatic fibrosis refers to an excessive wound healing response to chronic liver injury such as hepatitis viral infection, ethanol and is characterized by excessive accumulation of extracellular matrix (ECM) (Bataller and Brenner, 2005; Gao and Bataller, 2011). Hepatic stellate cells (HSCs) are the primary ECM-producing cells, resulting in the deposition of fibrous tissue and scar formation (Hernandez-Gea and Friedman, 2011). In response to liver injury of any etiology, the resting HSCs undergo a complex activation process and ultimately differentiate into fibrogenic myofibroblast-like cells (Moreira, 2007). This process is driven by a variety of inflammatory and growth factors, where transforming growth factor-β1 (TGF-β1) is thought to be the main profibrogenic mediator and platelet-derived growth factor (PDGF) is recognized as the major inducer of HSC proliferation (Friedman, 1999). TGF-β1 classically transmits intracellular signaling *via* Smad proteins, then triggers the differentiation of HSCs into myofibroblasts, identified by upregulated expression of alpha-smooth muscle actin (α-SMA) and collagen type 1 alpha 1 (Col1α1) (Dooley et al., 2000; Friedman, 1999; Schnabl et al., 2001). Accordingly, studies from others and our own lab have demonstrated that blockade of TGF-β1/Smad signaling pathway suppresses collagen production and eventually alleviates hepatic fibrosis (Bian et al., 2014; He et al., 2012; Liu et al., 2013; Szuster-Ciesielska et al., 2013).

Transient receptor potential melastatin 7 (TRPM7) channel, a member of the transient receptor potential melastatin (TRPM) subfamily, possesses both an ion channel and a functional serine/threonine α-kinase domain, whose expression has been detected in HSCs and livers

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; α-SMA, α-smooth muscle actin; AKT, protein kinase B; Col1a1, collagen type 1 alpha 1; ECM, extracellular matrix; eEF2-k, eukaryotic elongation factor-2 kinase EGF, epidermal growth factor; HSC, hepatic stellate cell; MMPs, matrix metalloproteinases; PDGF, platelet-derived growth factor; PLCγ2, phospholipase Cγ2; siRNA, small interference RNA; TGF-β, transforming growth factor beta; TIMPs, tissue inhibitor of metalloproteinases; TRPM7, transient receptor potential melastatin 7.

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from rat hepatic fibrosis models. A good deal of studies shows that TRPM7 channel participates in many physiological and pathophysiological processes including cell proliferation (Du et al., 2010; Fang et al., 2013; Inoue and Xiong, 2009; Sun et al., 2013), survival (Yee et al., 2012; Zierler et al., 2011), differentiation (Du et al., 2010; Zhang et al., 2012), adhesion (Du et al., 2010; Visser et al., 2013), migration (Middelbeek et al., 2012; Rybarczyk et al., 2012), and neurotransmitter release (Montell, 2006; Oancea et al., 2006). Moreover, TRPM7 is essential for embryonic development, as evidenced by the findings that genetic ablation of TRPM7 results to embryonic lethality (Jin et al., 2008, 2012). Recent studies indicated that TRPM7 plays a contributory role in cardiac and lung fibrosis (Du et al., 2010; Yu et al., 2013; Zhang et al., 2012). Accordingly, silencing TRPM7 with small interference RNA (siRNA) impedes angiotensin II (Ang II)-triggered phenotypic switching of vascular smooth muscle cells (VSMCs) of ascending aortic (Zhang et al., 2012). Moreover, blockade of TRPM7 channel with pharmacological agents or siRNA leads to the inhibition of lung fibroblast proliferation and differentiation elicited by TGF-β1 (Yu et al., 2013).

Abnormal overexpression of TRPM7 channel had been observed in rat fibrotic livers and the channel was required for HSC-T6 (a rat hepatic stellate cell line) cell proliferation induced by PDGF-BB (Fang et al., 2013). In addition, a potential correlation between TRPM7 channel and HSC-T6 cell apoptosis induced by tumor necrosis-related apoptosis-inducing ligand (TRAIL) was proposed (Liu et al., 2012). Nevertheless, whether upregulation of TRPM7 channels by TGF-β1 contributes to the collagen production in HSCs is still unknown. Moreover, the mechanism by which TGF-β1 increasing TRPM7 expression remains obscure. In this study, we found that TGF-β1 enhanced TRPM7 expression *via* TGF-β receptor-mediated Smad signaling in HSC-T6 cells and upregulation of TRPM7 was involved in the overproduction of α-SMA and Col1α1 in activated HSC-T6 cells. Intriguingly, elimination of TRPM7 also impaired phosphorylation of Smad2 and Smad3. Finally, upregulation of TRPM7 protein was observed in the fibrotic livers from human hepatic fibrosis patients.

Materials and methods

Rat model of liver fibrosis. Male Sprague–Dawley (200–250 g) rats were provided by the Experimental Animal Center of Anhui Medical University (Hefei, China). All animals received humane care. The animal experimental protocol was approved by the University Animal Care and Use Committee. Rats ($n = 10$) were treated with carbon tetrachloride (CCl_4, Shantou Xilong Chemistry Plant, China) diluted (1:1) in olive oil (1 ml of CCl_4/kg body weight) by intraperitoneal injection twice-weekly for 12 weeks (Lafyatis, 2006). Vehicle control animals were treated intraperitoneally with 1 ml of olive oil per kg bodyweight at same time intervals ($n = 10$). Twenty four hours after the final CCl_4 administration, the rats were euthanized and the livers were harvested for further analysis.

Immunohistochemistry. Liver tissues were fixed in 10% neutral buffered formalin solution, embedded in paraffin, and stained for routine histology. The sections were dewaxed in xylene and dehydrated in alcohol. Antigen retrieval was achieved by microwaving in citric saline for 5 min. Thin sections were treated with 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. The sections were further blocked by 5% bovine serum albumin and were then incubated overnight at 4 °C with primary rabbit polyclonal antibody against TRPM7 (Abcam, UK, 1:50). After rinsing (Phosphate Buffered Saline), the sections were incubated with biotinylated secondary antibody (Cowin Bioscience, China) for 30 min at room temperature. TRPM7 expressions were visualized by 3,3′-diaminobenzidine tetrahydrochloride (DAB, Cowin Bioscience, China) staining. The sections were counterstained with Mayers Hematoxylin for 5 min and dehydrated, and TRPM7 positive areas within the fibrotic region were then observed.

Cell culture and cell treatment with TGF-β1. The rat HSC-T6 cell line was obtained from Shanghai Fumeng Gene Biological Corporation (Shanghai, China). HSC-T6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, and 10% fetal calf serum (FCS, Sijiqing, China). The cells were maintained at 37 °C at an atmosphere of 5% CO_2. HSC-T6 cells were cultured for 48 h and serum-starved with 0.5% FCS for 24 h before adding 10 ng/ml recombinant murine TGF-β1 (Peprotech, UK).

2-Aminoethoxydiphenyl borate (2-APB) treatment. HSC-T6 cells were seeded overnight in 6-well plates in the presence of 100 μM 2-APB (Sigma, USA) or PBS (control) which were refreshed every 24 h for a total of 48 h treatment.

Quantitative Real-time PCR. Total RNA was extracted from rat liver tissues and HSC-T6 cells using TRIzol reagent (Invitrogen, USA). The first-strand cDNA was synthesized from total RNA using Thermoscript RT-PCR synthesis kit (Fermentas, Canada) according to the manufacturer's instructions. Real-time PCR was performed under standard protocol using the following primers: β-actin (forward: 5′-CACC CGCGATACAACCTTC-3′; reverse: 5′-CCCATACCCACCATCACACC-3′), TRPM7 (forward: 5′-GGAAAGTATGGGGCAGAAGTC-3′; reverse: 5′-TATC AAAGCCACCACAGGAAC-3′), α-SMA: (forward: 5′-CGAAGCGCAGAGCA AGAGA-3′; reverse: 5′-CATGTCGTCCCAGTTGGTGAT-3′), Col1a1: (forward: 5′-TGTTCAGCTTTGTGGACCT; reverse: 5′-CCGTTCTGTACGCAGGTGAT-3′), MMP-2 (forward: 5′-GGAAGCATCAAATCGGACTG-3′; reverse: 5′-GGGC GGGAGAAAGTAGCA-3′), MMP-13 (forward: 5′-GGGACGCCCATTTTGATG-3′; reverse: 5′-AGCTCATGGGCAGCAACAAT-3′), TIMP-1 (forward: 5′-AGCCCTGCTCAGCAAAAGG-3′; reverse: 5′-CTGTCCACAAGCAATGACTG TCA-3′), TIMP-2 (forward: 5′-TGCACCCGCAACAGGCGTTTT-3′; reverse: 5′-TTCCTCCAACGTCCAGCGAGA-3′). Real-time PCR conditions were as follows; 42 °C for 30 min; 95 °C for 10 min, 40 cycles of amplification at 95 °C for 20 s, 62 °C for 30 s, 72 °C for 30 s. Melt curve analysis was performed at 95 °C for 30 s, 60 °C for 30 min, and 95 °C for 30 s. The experiment was repeated at least three times from three independent RNA samples, while each of them was extracted from at least three lobes or wells of HSC-T6 cells. The fold-change for mRNA relative to β-actin was determined by the formula: $2^{-\triangle\triangle Ct}$.

RNA interference analysis. SiRNA oligonucleotides against TRPM7 genes or scrambled sequences were designed and synthesized by the Shanghai Gena Pharma Corporation (Shanghai, China) and contained the following sequences: TRPM7 siRNA (sense: 5′-GGCACCUUUAUAUCAUUA ATT-3′ and antisense: 5′-UUAAUGAUAUAAAGGUGCCTT-3′); si-control with scrambled sequence: (sense: 5′-UUCUCCGAACGUGUCACGUTT3-3′ and antisense: 5′-CGUGACACGUUCGGAGAATT-3′). HSC-T6 cells (1×10^5 cells) were cultured in 6 well plates with antibiotics-free DMEM for 24 h and then subjected to transfection with siRNA using Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer's protocol. Knockdown efficiency was determined by Real-time PCR and Western blot analysis. Three independent transfection experiments were performed.

Western blot analysis. Rat liver tissues, human liver tissues (derived from the liver bank of the Department of Laboratory Medicine of the First Affiliated Hospital, Anhui Medical University) and HSC-T6 cells were lysed with RIPA lysis buffer (Beyotime, China). Whole extracts were prepared, and protein concentrations were measured using a BCA protein assay kit (Boster, China). Total proteins (30 or 50 mg) were separated by SDS-PAGE and blotted onto PVDF membranes (Millipore, USA). After blockade of nonspecific protein binding with 5% milk, nitrocellulose blots were incubated for 6 h with primary antibodies diluted in a dilution buffer (Beyotime, China). Rabbit polyclonal antibody recognizing TRPM7 (Abcam, UK) was used at 1:1500, rabbit polyclonal anti-α-SMA (Boster, China) was diluted at 1:600, mouse monoclonal