Biochemical and Biophysical Research Communications 468 (2015) 758–765

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Effect of acid-sensing ion channel 1a on the process of liver fibrosis under hyperglycemia

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ARTICLE INFO

Article history:
Received 3 November 2015
Accepted 4 November 2015
Available online 10 November 2015

Keywords:
Acid-sensing ion channel
Diabetes
Hepatic fibrosis
Hyperglycemia

ABSTRACT

Metabolic syndrome characterized by hyperglycemia contributes to nonalcoholic steatohepatitis-associated liver fibrosis. This study was to investigate the effects of Acid-sensing ion Channel 1a (ASIC1a) on the process of liver fibrosis under hyperglycemia. Results showed that high glucose significantly worsen the pathology of liver fibrosis in vivo. In vitro, high glucose stimulated proliferation, activation and extracellular matrix (ECM) production in HSCs, and enhanced the effect of PDGF-BB on the activation and proliferation of HSCs. These effects could be attenuated by ASIC1a specific inhibitor Psalmotoxin-1(PcTx1) or specific ShRNA for ASIC1a through Notch1/Hes-1 pathways. These data indicate that ASIC1a plays an important role in diabetes complication liver fibrosis.

1. Introduction

Hepatic fibrosis is the wound healing process that occurs in response to many causes of chronic injury. It is characterized by the accumulation of extracellular matrix (ECM) following liver injury. Hepatic Stellate Cells (HSCs) are the main liver cells that produce matrix such as a-SMA and Collagen I. For fibrogenesis to occur, quiescent HSCs must be activated, and transform to proliferating myofibroblast-like cells. This event was regarded as the pivotal point of the progression of liver fibrosis [1–4].

Diabetes mellitus (DM) is a complex, multisystem disease characterized by hyperglycemia [5]. It causes damage/dysfunction of multiple organs such as kidney [6,7], heart [8,9], and retina [10,11]. Hepatic damage is also a common complication but has not been well addressed. The standardized mortality rate from end-stage liver disease (i.e., fibrosis, cirrhosis) is higher in the patients with diabetes than those without diabetes [12]. The development of diabetes is accompanied by an increase in the levels of many harmful factors, some of which stimulate the activation and proliferation of HSCs, such as hyperglycemia [13,14]. However, little attention has been paid to the effects of high glucose on HSCs activation, and on T2DM -associated hepatic fibrogenesis.

Acid-sensing ion Channels (ASICs) are a subfamily of degenerin/epithelial Na^+ channel family of non-voltage gated cation channels [15]. Until now, six subtypes of the ASIC family have been identified that arise from four genes, ASIC1, ASIC2, ASIC3, and ASIC4. ASIC1a, 1b and 2a, 2b are splice variants [16]. Among all ASICs, ASIC1a is also unique in that homomultimeric ASIC1a channels conduct Ca^{2+} and Na^+, and may play an important role in pathology and physiology [17]. Early studies on ASIC1a mainly focused on CNS, however, recent studies reported the presence of ASIC1a in rat liver and HSCs, the expression of ASIC1a was increased in activated HSCs and liver tissues of CCl4-treated rats [18,19]. However, the role of ASIC1a in the proliferation and activation of HSC cells involved in high glucose has not been explored. In this study, we demonstrated for the first time the presence of ASIC1a in rat liver and HSCs exposed to high glucose concentration.

2. Materials and methods

2.1. Animals treatment

Male Sprague–Dawley (100–140 g) rats were provided by the Experimental Animal Center of Anhui Medical University (Hefei,

Abbreviations: HSC, Hepatic stellate cells; ASIC1a, acid-sensing ion channel 1a; STZ, streptozocin; ECM, extracellular matrix; PcTx1, Psalmotoxin-1; ShRNA, short hairpin RNA; DM, Diabetes mellitus; a-SMA, alpha-smooth muscle actin.

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http://dx.doi.org/10.1016/j.bbrc.2015.11.029
0006-291X/

China) and housed under controlled temperature (23 °C) and a 12 h light/dark cycle. All animals received humane care. The animal experimental protocol was approved by the University Animal Care and Use Committee. After 7 days adaption, rats were randomly divided into control group (n = 6), diabetes group (n = 45) and hepatic fibrosis group (n = 15). The Non-diabetes group was fed with standard diet while the diabetes group was fed with high-fat diet (10% lard, 20% sucrose, 10% yolk powder, and 1% cholesterol combined with 59% standard diet). After 6 weeks feeding with high-fat diet, diabetes was induced by intraperitoneal injection of streptozotocin (STZ; 45 mg/kg; Sigma, USA) dissolved in citrate buffer with a concentration of 0.1 mM and continue with high-fat feed. 72 h after STZ or vehicle injection, glycemia was verified and rats with fasting blood-glucose greater than 7 mmol/L, in addition to polyuria and other diabetic features, were defined as diabetic. Four weeks later, randomly selected diabetic rats (n = 25) and the hepatic fibrosis group rats were treated with carbon tetrachloride (CCl4, Shantou Xilong Chemistry Plant, China) diluted (3: 2) in olive oil (4 ml of CCl4/kg bodyweight for the first dose, and 2 ml/kg for the remaining doses) by intraperitoneal injection twice-weekly for 8 weeks. Vehicle control animals were treated intraperitoneally with 2 ml of olive oil per kg bodyweight at the same time intervals. 72 h after the final CCl4 administration, the rats were sacrificed and livers and blood samples were harvested for further analysis.

2.2. Histopathologic assessment and immunohistochemical staining

The liver tissues were fixed in 10% buffered formalin and embedded in paraffin, then cut at 5 μm using a microtome, and stained with Hematoxylin and Eosin (HE staining) and the indicated antibodies, including anti-ASIC1 (Millipore, USA, 1: 100), anti-α-SMA (Bioss, China, 1: 100) and anti-Collagen I (Bioss, China, 1: 100). For each tissue section, the slides were examined under a light microscope with a photodocumentation facility (Olympus, Tokyo, Japan). The images were photographed with same magnification for H&E and immunohistochemical staining (× 200).

2.3. Cell culture and treatment with PDGF-BB

Rat HSC-T6 cells, a cell line of human hepatic fibroblasts (KeyGEN Bio TECH, Nanjing, China), were incubated in Dulbecco's modified Eagle's medium (DMEM, D-Glucose 1000 mg/l, KeyGEN, China), supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 10% fetal bovine serum (FBS). HSC-T6 cells were also cultured in DMEM with 10% FBS and incubated in 5% CO2 at 37 °C to 60–80% confluence, and growth arrested for 24 h in DMEM with 0.5% FBS before adding high glucose and recombinant human PDGF-BB (Peprotech, USA).

2.4. MTT [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide] cell viability assay

HSC-T6 cells (5×10^3) were seeded into each well of 96-well culture plates and then cultured in DMEM for 48 h. After culture, 5 mg/ml MTT reagent was added to each well, and the plates were then cultured at 37 °C for 4 h before adding DMSO to dissolve formazan crystals and examining in triplicate at 490 nm wavelength using a Thermomax microplate reader (bio-tekEL, USA).

2.5. RNA interference analysis

Short hairpin RNA (ShRNA) oligo nucleotides against ASIC1a genes or scrambled sequences were designed and synthesized by the Shanghai Gena Pharma Corporation (Shanghai, China). The sequence for ASIC1a RNAi was 5′-CACCGCCA AGAAGTTCAA-CAAATCGTTCAAGAGACGATTTGTTGAACTTCTTGGCTTTTTG-3′ (forward) and 5′-GATCCAAAAAAGCCAAGAAGTTCAACAAATCGTCT CTTGAACGATTTGTTGAACTTCTTGGC -3′ (reverse) and the sequence for the control RNAi was 5′- CACCGTTCTCCGAACGTGTCACGTTT-CAAGAGAACGT GACACGTTCGGAGAATTTTTTG-3′ (forward) and 5′-GATCCAAAAAATTCTC CGAACGTGTCACGTTCTCTTGAAACGTGA-CACGTTCGGAGAAC-3′ (reverse). The synthesized shRNA fragment containing the open reading frame of interfering-ASIC1a expression cDNA was inserted into the HindIII site of the pGCsi–U6–Neo–GFP cloning vector. HSC-T6 cells (2×10^5 cells) were cultured in 6 well plates with antibiotics-free DMEM for 24 h and then subjected to transfection with ShRNA using Lipofectamine TM2000 (Invitrogen, USA) according to the manufacturer's protocol.

2.6. Western blotting analysis

Rat liver tissues and HSC-T6 cells were lysed with RIPA lysis buffer (Beyotime, China). Extracts were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA), which were incubated with primary antibodies against ASIC1a (1: 500, Alomone, USA), a-SMA (1: 200, Bioss, China), Collagen I (1: 500, Bioss, China), CyclinD1 (1: 500, Bioss, China), Notch1 (1: 500, Cell signaling, USA), Hes-1 (1: 500, Cell signaling, USA) and β-actin (1: 500, Bioss, China). The membranes were then washed in TBS/Teween 20, and incubated with secondary antibodies correspondingly. After extensive washing in TBS/Tween 20, protein bands were visualized with ECL chemiluminescent kit (ECL-plus, Thermo Scientific).

2.7. Cell cycle analysis

To analyze the intracellular DNA content, HSCs were fixed in 70% ethanol at 4 °C overnight after 48 h treatment with high glucose, PcTx1 or ASIC1a-ShRNA. HSCs were centrifuged at 1000 g for 5 min and re-suspended in PBS. After then, cells were stained with 0.5 ml of propidium iodide (PI) staining buffer (Beyotime, China), which contains 200 mg/ml RNase A, 50 μg/ml PI, at room temperature for 30 min in the dark. Flow cytometric analysis (FACS) was performed on Beckman Coulter.

2.8. Data analysis

Data are represented as Mean ± SE. Statistical significance was determined by either the Student's t-test for comparison between means or one-way analysis of variance with a post hoc Dunnett's test. In all cases, $p < 0.05$ was regarded statistically significant.

3. Result

3.1. Upregulation of ASIC1a expression in the fibrotic livers from CCl4 and STZ treated rats

To determine hyperglycemia in the development of hepatic fibrosis, the expression of several related key proteins in liver tissues from experimental rats were measured by immunohistochemistry and Western blot assays. As shown in the result of H&E staining, in the control group, the liver tissue organization was normal and the hepatic lobules were well-arranged without liquid droplets. In contrast, in the diabetes group, the structure of liver tissues was light damaged, and inflammatory cells and fatty degeneration were also observed. In the liver fibrosis group, extensive fatty change, necrosis, and inflammatory cell infiltration