Effects of endothelin-1 on hepatic stellate cell proliferation, collagen synthesis and secretion, intracellular free calcium concentration

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INTRODUCTION

Hepatic fibrosis associated with the activation of hepatic stellate cells (HSCs), the major source of extracellular matrix (ECM) proteins[1,2]. It is generally believed that HSCs are the main cells producing ECM, from resting state to active myofibroblasts, which is the key point of formation and development of hepatic fibrosis[3-6]. Endothelin-1 (ET-1) is currently known as a polypeptide with a stronger activity to contract blood vessel. So, based on prophase researches[7-9], we chose ET-1 to observed its direct effect on DNA ingestion and synthesis as well as collagen synthesis and secretion of HSCs in cultivating. Meanwhile, as we know that Ca$^{2+}$ is an important intracellular messenger, relate to HSC proliferation and ECM synthesis[10-13]. The effects of ET-1 on regulation and intracellular [Ca$^{2+}$], of HSCs isolated and cultivated in vitro were studied.

MATERIALS AND METHODS

MATERIALS

Animals Wistar male rats, weighting (450±50) g, were provided by Shanghai Experimental Animals Center of Chinese Academy of Sciences.

Reagents ET-1, calcium fluorescence probes Fura-2/AM, Triton X-100, trypsin, DMEM, DAB-H$_2$O$_2$, were from Sigma; verapamil from Knoll; collagenase from Medical Industry Academy of Shanghai; RPMI 1640 from Gibco; HEPES from EMK; H-L-proline from Academy of Atomic Energy in China (66.6 GBq/mmol, radioactivity purity >90%). H-TdR was from Institute of Atomic Energy in Shanghai (814 GBq/mmol, radioactivity purity >95%).

Methods Isolation and cultivation of rat HSCs Rat HSCs were isolated referring to Knook[14-17]. Rats were anaesthetized with pentobarbitone (200 mg/kg) by abdominal injection, then heparin sodium (10 mg/kg) was injected into the caudal vein. The abdominal cavity was opened and portal vein and dorsal vein were exposed. Blood was released through vein and D-Hank’s solution was perfused (20-25 mL/min) until pale yellow appeared. Liver was taken out and undergone extracorporeal circulation when perfusion fluid was changed to GBSS containing 0.5 g/L proline E, 0.5 g/L collagenase and 10 mmol L$^{-1}$ HEPES. Circle perfusion was performed for 30 min (15 mL/min). Liver was taken out and cut to pieces, then put into GBSS containing 0.25 g/L proline E, 0.25 g/L collagenase and 10 mmol L$^{-1}$ HEPES, shocked at 37 °C for 30 min, little suspended deposit was put in culture media on the top of three-layer density gradient centrifugation fluid containing 80 g/L and 130 g/L metrizamide, 2 800 r/min centrifugation for 20 min, Cells were sucked between top layer and 80 mL/L density layer. DMEM containing 200 mL/L calf serum was used to regulate the number of cells to 1×10$^5$/mL.

DNA and collagen synthesis of HSCs HSCs in 2 to 4 th generation were digested by pancreatin and cultured with DMEM supplemented with 100 g/L calf serum and 100 mL/L horse serum. Cells were adjusted to 1×10$^5$/mL and inoculated on a 48-well plate, cultured for 24 h, then different concentration of ET-1 and the same dosage of drug was added, respectively.
and triplicated for each concentration. ³H-TdR and ³H-proline were used to assay the incorporation.

**HSC ingestion of DNA** ³H-TdR 18.5 GBq/mmol was added at 10, 20, 30 and 60 min respectively, washed 3 times with PBS of 1×10³ mmol/L, centrifuged 1 000 r/min 10 min, the top layer fluid was removed, 2 mL of 100 g/L TCA was added and centrifuged 1000 r/min 10 min again. Top layer fluid was collected and deposited, washed 3 times with 800 mL/L ethanol at 4 °C. The top layer fluid was removed and dried in vacuum. 1 mol/L NaOH was used to lyse the deposit and 1N HCl was used to adjust pH to 7.0. Radioactivity of specimens was measured on Beckman scintillation counter.

**Collagen secretion of HSC** In experiment of ³H-TdR, before transferred to F₃₀ filter paper, 1 mL culture media was taken out and put into a tube. A 5 mmol/L acetic acid was used to adjust pH to 2 to 3, then 25 μL of 2.5 g/L pepsin was added to digest. A 50 μL of proline was added at 4 °C for 3 h, 1, 1.2 mol/L trichloroacetic acid was fixed for 2 h, transferred to F₃₀ filter paper, closed with saline, 0.6 mol/L trichloroacetic acid was used again, then bleached with anhydrous alcohol, baked at 80 °C. Radioactivity of specimens was measured on YSJ-75 liquid scintillation counter.

[Ca²⁺]ᵢ in Fura-2/AM loaded HSC HSCs were cultured on a rectangle glass when HSCs grew and covered the glass. Then cells were taken out of the glass and RPMI 1640 containing Fura-2/AM (10 mmol/L) was added to incubate at 37 °C for 50 min, D-Hank’s solution was used to wash extracellular free Fura-2/AM and incubated for another 30 min, 1 g/L trypsin was used to digest the cells and the number of cells was adjusted to 10⁶/mL by buffer.

**Fluorescence spectrum** About 2 mL of Fura-2/AM loaded HSCs was suspended for the test with a fluorescence spectrophotometer. Raster (EX) 5 nm, radiate raster (EM) 10 nm were used to excite at a middle scan speed (32 mm/min), excitation light scan ranged 300-400 nm, emission light scan ranged 440-540 nm.

**Intracellular fluorescence intensity** Fluorescence intensity F was detected first (laser wave-length 340 nm, EX 5 nm, emission wave-length 510 nm, EM 10 nm), then different concentrations of ET-1 and verapamil and EGTA (last concentration 8 mmol/L) were added for the detection of minimum fluorescence intensity (Fₐᵢ). Calculation of [Ca²⁺]ᵢ, Intracellular [Ca²⁺]ᵢ (nmol/L) = kᵢ(Fᵢ-Fᵢ)/Fᵢ(Fᵢ/min)(Fᵢ/max-Fᵢ). Kᵢ is a dissociation constant to Fura-2/Ca²⁺ compound which equals to 224 mmol/L.

**Statistical analysis** Variance homogeneity tests were used to make comparisons.

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**RESULTS**

**HSC activity**

Trypan blue staining revealed an activity above 90% for HSCs. The purity of HSCs was more than 80% assessed by fluorescence microscope. The nuclei of HSC were stained blue among the desmin-positive satellite cells.

**Effect of ET-1 on HSC DNA synthesis**

As shown in Table 1, ET-1 could accelerate ³H-TdR incorporation into HSCs and HSC DNA synthesis and proliferation (P<0.05), in a concentration-dependent manner.

**Effect of ET-1 on HSC ingested ³H-TdR**

ET-1 could accelerate the rate of HSC ingested DNA, the rate increased with the time prolonged (P<0.05 or P<0.01, Figure 1).

**Effect of ET-1 on HSC collagen synthesis**

ET-1 could accelerate ³H-Proline incorporation into HSCs and collagen synthesis at the concentration of 5×10⁻⁴ mol/L (P<0.05), in a concentration-dependent manner.

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**Effect of ET-1 on HSC collagen secretion**

As shown in Tablet 1, ET-1 could remarkably accelerate HSC collagen secretion compared with the control group (P<0.05).

**Effect of ET-1 (10⁻⁸ mol/L) on intracellular [Ca²⁺]**

As shown in Figure 2, when ET-1 was added to the suspension of Fura-2/AM loaded HSCs and kept for 25 min (n = 3), [Ca²⁺]ᵢ in HSCs rose from (392±4) mol/L (resting state) to (165±51) mol/L (P<0.01) and rose to peak (422±98) mol/L (P<0.001) after another 2 min, then it began to go down slowly and remained a higher concentration even after another 18 min compared with the resting [Ca²⁺]ᵢ (P<0.01). It suggested that the effect of ET-1 on [Ca²⁺]ᵢ in HSCs could be divided into 2 phases, a fast phase (I P) and a slow phase (II P).

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**Figure 1** Effect of ET-1 on HSC ingested ³H-TdR.

**Figure 2** Effect of ET-1 on [Ca²⁺]ᵢ peak value in HSCs.
It has been reported that ET could raise \([\text{Ca}^{2+}]_i\) in smooth muscle in hepatic sinusoid secrete endothelins that can activate HSCs, suggesting that \(\text{Ca}^{2+}\)-free buffer had no remarkable effect on IP cells\(^{27-29}\). This study showed that ET-1 could raise \([\text{Ca}^{2+}]_i\) in HSCs and appeared double phase reaction, fast phase and slow phase. Both phases had a dose-dependent manner. It turns out that when the cells are at resting state, if there is extracellular \(\text{Ca}^{2+}\), the \([\text{Ca}^{2+}]_i\) in HSCs will be higher than that without extracellular \(\text{Ca}^{2+}\). ET-1 can remarkably raise \([\text{Ca}^{2+}]_i\) in HSC with or without extracellular \(\text{Ca}^{2+}\). It implies that ET-1 can accelerate HSC release of intracellular \(\text{Ca}^{2+}\).

Three different ways have been found to elevate \([\text{Ca}^{2+}]_i\)\(^{30-33}\). Plenty of calcium flows into cell through Ca\(^{2+}\) channel, Ca\(^{2+}\)-ATP enzyme or Na\(^+\)-Ca\(^{2+}\) changing system is restrained which can transfer \(\text{Ca}^{2+}\) out of cells; \(\text{Ca}^{2+}\) storing systems such as mitochondrion and endoplasm increase \(\text{Ca}^{2+}\). We used \(\text{Ca}^{2+}\)-free buffer and found it had no effect on \([\text{Ca}^{2+}]_i\) in IP in HSC excited by ET-1 but could block \([\text{Ca}^{2+}]_i\) in II P. It implies that elevated \([\text{Ca}^{2+}]_i\) in IP is caused by increased \(\text{Ca}^{2+}\) stored in cells, while elevated \([\text{Ca}^{2+}]_i\) in II P is caused by \(\text{Ca}^{2+}\) flowing out of cells. It has been currently accepted by some of scholars that the raise of \(\text{Ca}^{2+}\) in HSC is through the way of phospholipase C (PLC)-inositol triphosphate (IP\(_3\))-diacylglycerol (DAG)\(^{34-35}\). ET-1 excites PLC on cell membrane through G protein that makes 4,5-biphosphate inositol divide into IP\(_3\) and DAG-IP\(_3\). Mitochondrion, endoplasm and sarcoplasm that make \(\text{Ca}^{2+}\) in cell release to cytoplasm and increase free \([\text{Ca}^{2+}]_i\) in cells. IP\(_3\) works only a very short time , and is quickly converted to IP\(_4\) by special enzymes. So peak IP lasts for a very short time, but IP\(_4\) can accelerate the opening of \(\text{Ca}^{2+}\) channel on cell membrane, which makes an increase of calcium flowing out of cells and at last results in a fast raise of \([\text{Ca}^{2+}]_i\) in cells.

Physiological and pathological significance of elevated free \(\text{Ca}^{2+}\) in HSCs excited by ET is still not clear. Maybe it could participate the series of signals in cells and physiological effect of ET\(^{25,27}\). In conclusion, ET-1 can remarkably accelerate HSC proliferation, collagen synthesis and secretion, increase of \([\text{Ca}^{2+}]_i\) in HSC and of release of \(\text{Ca}^{2+}\) in cells, thus accelerating proliferation of fibrous tissues and repair of injury tissues.

**REFERENCES**

1. Huang ZG, Zhai WR, Zhang YE, Zhang XR. Study of heterosperm-induced rat liver fibrosis model and its mechanism. World J Gastroenterol 1998; 4: 206-209
2. Kawada N. The hepatic perisinusoidal stellate cell. Histol Histopathol 1997; 12: 1069-1080
3. Schuppun D, Dorov Y. Hepatic fibrosis: From bench to bedside. J Gastroenterol Hepatol 2002; 17(Suppl 3): S300-S305
4. Marra F, Pinzani M. Role of hepatic stellate cells in the pathogenesis of portal hypertension. Nefrologia 2002; 22(Suppl 5): 34-40
5. Mann DA, Smart DE. Transcriptional regulation of hepatic stellate cell activation. Gut 2002; 50: 891-896
6. Ramadori G, Saile B. Mesenchymal cells in the liver–one cell type or two? Liver 2002; 22: 283-294
7. Hasegawa T, Kimura T, Sasaki T, Okada A. Plasma endothelin-1 level as a marker reflecting the severity of portal hypertension in biliary atresia. J Pediatr Surg 2001; 36: 1609-1612
8. Tsuchiya Y, Suzuki S, Inaba K, Sakaguchi T, Baba S, Miwa M, Konno H, Nakamura S. Impact of endothelin-1 on microcirculatory disturbance after partial hepatectomy under ischemia/reperfusion in thioacetamide-induced cirrhotic rats. J Surg Res 2003; 111: 100-108
9. Hasselblatt M, Bunte M, Dringen R, Tabernero A, Medina JM, Giaume C, Siren AL, Ehrenreich H. Effect of endothelin-1 on astrocytic protein content. Glia 2003; 42: 390-397
10. Rauke F. Epidemiology of medullary thyroid carcinoma. Recent Results Cancer Res 1992; 125: 47-54
11. Rauke F, Zink A. Measurement of free cytosolic calcium in single cells: method and application. Methods Find Exp Clin Pharmacol 1992; 14: 327-332
12. Lyali V, Alam RI, Phan TH, Phan DQ, Heck GL, DeSimone JA. Excitation and adaptation in the detection of hydrogen ions by taste receptor cells: a role for cAMP and Ca(2+). J Neurophysiol
Kriebel ME, Keller B. The unitary evoked potential at the frog nerve-muscle junction. Results from synchronous gating of functional pores at docked vesicles. J Cell Biol 1999; 147: 1203-1213.

Smogorzewski MJ. Central nervous dysfunction in uremia. Am J Kidney Dis 2001; 38(4 Suppl 1): S122-S128.

Satin LS. Localized calcium influx in pancreatic beta-cells: its significance for Ca2+-dependent insulin secretion from the islets of Langerhans. Endocrine 2000; 13: 251-262.

Murthy KS, Zhou H. Selective phosphorylation of the IP3R-1 in vitro by cGMP-dependent protein kinase in smooth muscle. Am J Physiol Gastrointest Liver Physiol 2003; 284: G221-230.

Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, Masaki T. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc Natl Acad Sci U S A 1989; 86: 2863-2867.

Xu D, Emoto N, Giaid A, Slaughter C, Kaw S, deWit D, Yanagisawa M. ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. Cell 1994; 78: 473-485.

Clozel M, Gray GA, Breu V, Loffler BM, Osterwalder R. The endothelin ETB receptor mediates both vasodilation and vasoconstriction in vivo. Biochem Biophys Res Commun 1992; 186: 867-872.

Sakurai T, Yanagisawa M, Masaki T. Molecular characterization of endothelin receptors. Trends Pharmacol Sci 1992; 13: 103-108.

Higuchi H, Satoh T. Endothelin-1 induces vasoconstriction and nitric oxide release via endothelin ET(B) receptors in isolated perfused rat liver. Eur J Pharmacol 1997; 328: 175-182.

Jouenoux C, Mallatt A, Serradell-Le Gal C, Goldsmith P, Hanoune J, Lortet-Terry S. Coupling of endothelin B receptors to the calcium pump and phospholipase C via Gs and Gq in rat liver. J Biol Chem 1994; 269: 1845-1851.

Kuddus RH, Nalesnik MA, Subbotin VM, Rao AS, Gandhi CR. Enhanced synthesis and reduced metabolism of endothelin-1 (ET-1) by hepatocytes—an important mechanism of increased endogenous levels of ET-1 in liver cirrhosis. J Hepatol 2001; 35: 725-732.

Leivas A, Jimenez W, Bruix J, Boix L, Bosch J, Arroyo V, Rivero F. Rodes J. Gene expression of endothelin-1 and ET(A) and ET(B) receptors in human cirrhosis: relationship with hepatic hemodynamics. J Hepatol 1998; 30: 186-193.

Gerbes AL, Moller S, Gubler V, Heinrich J. Enhanced expression of endothelin-1 and ET(B) in patients with cirrhosis: role of splanchic and renal passage and liver function. Hepatology 1995; 21: 735-739.

Uchiyama I, Izu M, Nishi T, Marumo F. Clinical significance of elevated plasma endothelin concentration in patients with cirrhosis. Hepatology 1992; 16: 95-99.

Asbert M, Gines A, Gines P, Jimenez W, Claria J, Sala J, Arroyo V, Rivero F, Rodes J. Circulating levels of endothelin in cirrhosis. Gastroenterology 1993; 104: 1485-1491.

Matsumoto H, Uematsu J, Kitano M, Kawasaki H. Clinical significance of plasma endothelin-1 in patients with chronic liver disease. Dig Dis Sci 1994; 39: 2665-2670.

Moller S, Gubler V, Heinrich JH, Gerbes AL. Endothelin-1 and ET-3 plasma concentrations in patients with cirrhosis: role of splanchic and renal passage and liver function. Hepatology 1995; 21: 735-739.

Tsai YT, Lin HC, Yang MC, Lee FY, Hou MC, Chen LS, Lee SD. Plasma endothelin levels in patients with cirrhosis and their relationships to the severity of cirrhosis and renal function. J Hepatol 1995; 23: 681-688.

Martinet JP, Legault L, Cernacek P, Roy L, Dufresne MP, Spahr L, Fenyes D, Pomier-Layrargues G. Changes in plasma endothelin-1 and Big endothelin-1 induced by transjugular intraperitoneal portosystemic shunts in patients with cirrhosis and refractory ascites. J Hepatol 1996; 25: 700-706.

Bernardi M, Gubler V, Colantoni A, Trevisani F, Gasbarrini A, Gerbes AL. Plasma endothelin-1 and -3 in cirrhosis: relationship with systemic hemodynamics, renal function and neurohumoral systems. J Hepatol 1996; 24: 161-168.

Nagasue N, Dhar DK, Yamanoi A, Emy I, Udagawa J, Yamamoto A, Tachibana M, Kubota H, Kohno H, Harada T. Production and release of endothelin-1 from the gut and spleen in portal hypertension due to cirrhosis. Hepatol 2000; 31: 1107-1114.

Rieder H, Ramadori G, Meyer zum Buschenfelde KH. Sinusoidal endothelial liver cells in vitro release endothelin: augmentation by transforming growth factor beta and Kupffer cell-conditioned media. Klin Wochenschr 1991; 69: 387-391.

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