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• BASIC RESEARCH •

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

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Abstract

AIM: To explore the effects of endothelin-1(ET-1) on hepatic stellate cells (HSCs) DNA uptake, DNA synthesis, collagen synthesis and secretion, inward whole-cell calcium concentration ($[Ca^{2+}]_i$) as well as the blocking effect of verapamil on ET-1-stimulated release of inward calcium (Ca^{2+}) of HSC *in vitro*.

METHODS: Rat hepatic stellate cells (HSCs) were isolated and cultivated. ^{3}H-TdR and ^{3}H-proline incorporation used for testing DNA uptake and synthesis, collagen synthesis and secretion of HSCs cultured *in vitro*; Fluorescent calcium indicator Fura-2/AM was used to measure $[Ca^{2+}]_i$ inward HSCs.

RESULTS: ET-1 at the concentration of 5×10^{-8} mol/L, caused significant increase both in HSC DNA synthesis (2 247±344 cpm, $P<0.05$) and DNA uptake ($P<0.05$) when compared with the control group. ET-1 could also increase collagen synthesis ($P<0.05$ *vs* control group) and collagen secretion ($P<0.05$ *vs* control group). Besides, inward HSC $[Ca^{2+}]_i$ reached a peak concentration (422±98 mol/L, $P<0.001$) at 2 min and then went down slowly to165±51 mol/L ($P<0.01$) at 25 min from resting state (39±4 mol/L) after treated with ET-1. Verapamil (5 mol/L) blocked ET-1-activated $[Ca^{2+}]_i$ inward HSCs compared with control group ($P<0.05$). Fura-2/AM loaded HSC was suspended in no Ca^{2+} buffer containing 1 mol/L EGTA, 5 min later, 10^{-8} mol/L of ET-1 was added, $[Ca^{2+}]_i$ inward HSCs rose from resting state to peak 399±123 mol/L, then began to come down by the time of 20 min. It could also raise $[Ca^{2+}]_i$ inward HSCs even without Ca^{2+} in extracellular fluid, and had a remarkable dose-effect relationship($P<0.05$). Meanwhile, verapamil could restrain the action of ET-1($P<0.05$).

CONCLUSION: Actions of ET-1 on collagen metabolism of HSCs may depend on the transportation of inward whole-cell calcium.

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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]. 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[2-6]. 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 $[Ca^{2+}]_i$ 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, pronase, trypsin, DMEM, DAB-H_2O_2 were from Sigma; verapamil from Knoll; collagenase from Medical Industry Academy of Shanghai; RPMI 1640 from Gibco; HEPES from EMK; ^{3}H-L-proline from Academy of Atomic Energy in China (66.6 GBq/mmoL, radioactivity purity>90%). ^{3}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 pronase E, 0.5 g/L collagenase and 10 mmoL 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 pronase E, 0.25 g/L collagenase and 10 mmol/L 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^{-8}/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. ^{3}H-TdR and ^{3}H-proline were used to assay the incorporation.

HSC ingestion of DNA ^{3}H-TdR 18.5 GBq/mmoL was added at 10, 20, 30 and 60 min respectively, washed 3 times with PBS of 1×10^5 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 added 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 ^{3}H-TdR, before transferred to F_{49} 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, l, 1.2 mol/L trichloroacetic acid was fixed for 2 h, transferred to F_{4a} 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.

$[Ca^{2+}]_i$ 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 nmol/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 10^6/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 excited 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 (F min).

Calculation of $[Ca^{2+}]_i$ Intracellular $[Ca^{2+}]_i$ (nmol/L) = kd (F-F min)/(F max-F). Kd is a dissociation constant to Fura-2/Ca^{2+} compound which equals to 224 nmol/L.

Statistical analysis Variance homogeneity tests were used to make comparisons.

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 ^{3}H-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 ^{3}H-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 ^{3}H-Proline incorporation into HSCs and collagen synthesis at the concentration of 5×10^{-8} mol/L (P<0.05), in a concentration-dependent manner.

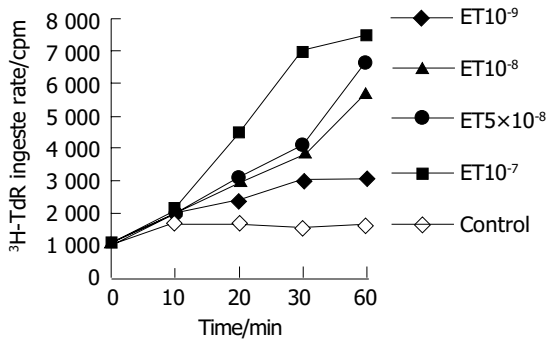

Figure 1 Effect of ET-1 on HSC ingeste ^{3}H-TdR.

Table 1 Effects of ET-1 on HSC DNA synthesis, collagen synthesis and secretion (cpm, mean±SD)

Group	DNA synthesis	Collagen Synthesis	Collagen secretion
Control	1 370±113	2 167±454	1 431±389
ET 10^{-9} mol/L	1 489±305	2 206±725	1 528±242
ET 5×10^{-8} mol/L	1 986±457[a]	2 698±304[a]	1 903±552[a]
ET 10^{-8} mol/L	2 247±344[a]	2 876±396[a]	2 087±128[a]
ET 10^{-7} mol/L	4 015±102[a]	3 056±401[a]	2 794±397[b]

[a]P<0.05, [b]P<0.01 *vs* control group.

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 $[Ca^{2+}]_i$

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), $[Ca^{2+}]_i$ in HSCs rose from (39±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 $[Ca^{2+}]_i$ (P<0.01). It suggested that the effect of ET-1 on $[Ca^{2+}]_i$ in HSCs could be divided into 2 phases, a fast phase (I P) and a slow phase (II P).

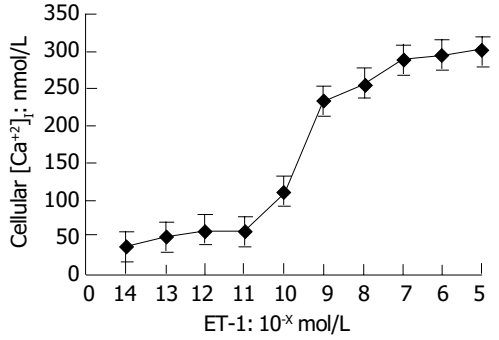

Figure 2 Effect of ET-1 on [Ca^{2+}]i peak value in HSCs.

Effect of ET-1 on peak concentration of $[Ca^{2+}]_i$ in HSCs

As shown in Figure 3, $[Ca^{2+}]_i$ in HSCs was in a ET-1 concentration-dependent manner. No change of $[Ca^{2+}]_i$ occurred in HSCs when ET-1 was less than 10^{-11} mol/L. $[Ca^{2+}]_i$ reached its peak in a ET-1-dose-dependent manner when ET-1 was greater than 10^{-9} mol/L.

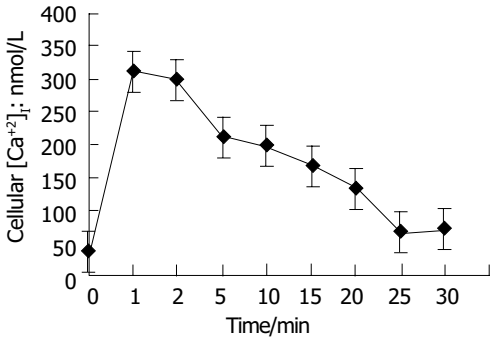

Figure 3 Effect of ET-1 on [Ca^{2+}]i in HSCs (10^{-10}mol/L).

Blocking effect of verapamil

As shown in Figure 4, calcium channel blocking agent verapamil (5 μmol/L) could significantly restrain I P and II P effects on [Ca^{2+}]$_i$ in HSCs excited by ET-1 compared with control group (P<0.05). Fura-2/AM loaded HSCs suspended in Ca^{2+}-free buffer containing 1 mol/L EGTA made [Ca^{2+}]$_i$ in HSCs raise from resting state to peak (399±123) mol/L, then go down to (49±17) mol/L at the time of 20 min when first treated with 10^{-8} mol/L of ET-1, suggesting that Ca^{2+}-free buffer had no remarkable effect on I P of [Ca^{2+}]$_i$ in HSCs excited by ET-1 but completely blocked II P.

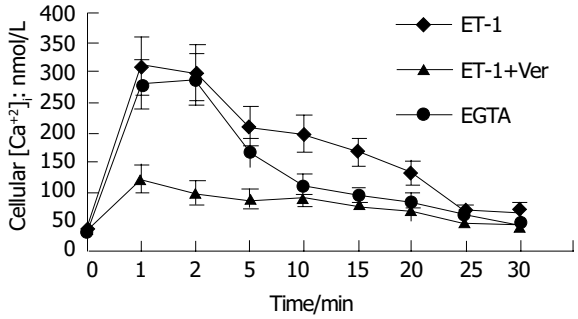

Figure 4 Effect of ET-1 increase [Ca^{2+}]i in HSCs.

DISCUSSION

HSCs were first detected by Ito and Nemoto in 1952, which provided a new way to study episode mechanism of hepatic fibrosis and deepened the cognition of hepatic fibrosis from an angle of source cells of collagen production[2-6,15,16]. HSCs is also named Ito cell, VitA storing cell, liver antrum around cell, fat-storing cell, and is one of the liver interstitial cells. The main function of HSC is to store and metabolize VitA. It has been found to be able to synthesize and secrete ECM and synthesize collagenase[2-6]. When hepatic fibrosis occurred, HSC turned into fibroblasts or myofibroblasts that were the cause of liver synthesis of ECM. This change of HSC was called activation or conversion[2,3]. It has been certificated that interstitial cells especially HSCs are the main cells which producte collagen when hepatic fibrosis occurs. So it has become a central link in hepatic fibrosis occurrence mechanism.

ET distributes widely in liver and portal vein system, and has important biological effects on liver[18-24]. This experiment showed that ET could remarkably accelerate HSC proliferation, DNA synthesis, collagen synthesis and secretion. It is thus clear that ET-1 had double roles during hepatic fibrosis, accelerating not only HSC synthesis of collagen but also selective excretion of collagen. Besides[25,26], endothelial cells in hepatic sinusoid secrete endothelins that can activate HSCs. It has been reported that ET could raise [Ca^{2+}]$_i$ in smooth muscle cells[27-29]. This study showed that ET-1 could raise [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 Ca^{2+}, the [Ca^{2+}]$_i$ in HSCs will be higher than that without extracellular Ca^{2+}. ET-1 can remarkably raise [Ca^{2+}]$_i$ in HSC with or without extracellular Ca^{2+}. It implies that ET-1 can accelerate HSC release of intracellular Ca^{2+}.

Three different ways have been found to elevate [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 Ca^{2+} out of cells; Ca^{2+}storing systems such as mitochondrion and endoplasm increase Ca^{2+}. We used Ca^{2+}-free buffer and found it had no effect on [Ca^{2+}]$_I$ in I P in HSC excited by ET-1 but could block [Ca^{2+}]$_I$ in II P. It implies that elevated [Ca^{2+}]$_I$ in I P is caused by increased Ca^{2+} stored in cells, while elevated [Ca^{2+}]$_I$ in II P is caused by Ca^{2+} flowing out of cells. It has been currently accepted by some of scholars that the raise of Ca^{2+} in HSC is through the way of phospholipase C (PLC)-inositol triphosphate (IP_3)- diacylgcerol (DAG)[34-51]. 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 Ca^{2+} in cell release to cytoplasm and increase free [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 I P lasts for a very short time, but IP_4 can accelerate the opening of 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 [Ca^{2+}]i in cells.

Physiological and pathological significance of elevated free 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 [Ca^{2+}]$_i$ in HSC and of release of Ca^{2+} in cells, thus accelerating proliferation of fibrous tissues and repair of injury tissues.

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