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Octreotide decreases portal pressure: Hepatic stellate cells may play a pivotal role

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The aim of this study is to elucidate the effects of different dosages of octreotide on portal pressure in cirrhotic patients and to investigate the mechanism of activated human hepatic stellate cells (HSCs) on octreotide. Thirty-one (31) hepatitis B-related cirrhotic patients were randomly assigned to receive treatment with a 50 (group A, n = 12) or a 25 µg/h infusion of octreotide for 72 h (group B, n = 14); the control group C (n = 5) received conventional treatment. Dynamic portal pressure was directly measured via a portal vein catheter. To study the cellular mechanism of octreotide, the expression of SSTRs 1-5 in LX-2, an HSC line, was examined by immunostaining and RT-PCR. Intracellular Ca^{2+} in LX2 was measured by laser scanning confocal microscopy (LSCM). The protein and mRNA levels in all five subtypes of SSTRs were positively expressed in LX-2. Octreotide led to an immediate two-fold drop in intracellular Ca^{2+} (P < 0.01). Portal pressure, in both groups A and B, decreased significantly (mean, -20.6%) after octreotide infusion. Octreotide decreased portal pressure in cirrhotic patients by inhibiting HSC contractility by decreasing intracellular Ca^{2+} concentration via stimulation of all SSTRs on HSCs.

Key words: Hepatic stellate cell, somatostatin receptor subtype, octreotide, portal hypertension, liver cirrhosis.

INTRODUCTION

Chronic liver diseases, especially liver cirrhosis, are often complicated with portal hypertension, which is characterized by increased intrahepatic vascular resistance and hyperdynamic states (Blei, 2007; Sanyal et al., 2008). Complications associated with portal hypertension occurs when the portal pressure exceeds 12 mmHg (Sharara and Rockey, 2001). The most frequent and severe complication of portal hypertension, gastro-esophageal varices bleeding (EVB), has a high mortality rate in patients with cirrhosis (Dragoteanu et al., 2008; Garcia-Tsao, 2003). Over the last 20 years, marked progress in the understanding of the pathophysiology of portal hypertension has opened doors to pharmacologic treatments. It is assumed that sustained reduction in portal pressure by long-term drug administration may prevent and treat the complications of portal hypertension (Garcia-Tsao et al., 2008; Minor and Grace, 2006). Therefore, a major focus of management of patients with portal hypertension is to reduce functional portal venous flow and portal pressure.

The growth hormone release inhibitors, native somato-statin (SST) and its octapeptide analog "octreotide". They are important in the treatment of liver cirrhosis portal hypertension complicated by acute EVB, although the mechanism of action has not been completely elucidated

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Abbreviations: RT-PCR, Reverse transcription polymerase chain reaction; **LSCM,** laser scanning confocal microscopy; **SSTR,** somatostatin receptor; **HSC,** hepatic stellate cell; **EVB,** gastroesophageal varices bleeding; **GAPDH,** glyceraldehyde phosphate dehydrogenase; **DMEM,** dulbecco's modified eagle medium; **GTPase,** guanosine triphosphatase; **IgG,** immunoglobulin G.

Table 1. Baseline characteristics of the study subjects.

Available indexes	Group A (n = 12)	Group B (n = 14)	Group C (n = 5)
Age	47.8 ± 4.2	48.9 ± 5.2	48.2 ± 2.5
Gender (M/F)	9/3	9/5	4/1
Child A	7	9	4
Child B	5	5	1
Ascites (yes)	0	0	0
Encepahalopathy (yes)	0	0	0
EVB history (yes/no)	11/1	14/1	5/0

Baseline characteristics of the study subjects were comparable between group A, B and C (P > 0.05).

(Gonzalez et al., 2006; Triantos and Burroughs, 2007). A recent study showed that hepatic stellate cells (HSCs), the perisinusoidal pericytes, may play an important role in the regulation of intrahepatic resistance and micro-hemodynamics because of their smooth muscular characteristics, namely contractility (Reynaert et al., 2004; Song et al., 2004; Xu et al., 2005). HSC contractility is regulated by intracellular Ca^{2+} and certain vaso-active factors (Ding et al., 2004; Reynaert et al., 2002). Although the precise contractile and regulatory mechanisms of HSCs are not as well studied as smooth muscle cells, many studies have described intracellular Ca^{2+} signaling pathways and proteins involved in HSC contraction (Ding et al., 2004; Reynaert et al., 2002; Reynaert et al., 2001; Xu et al., 2005). Therefore, HSC-targeted therapy may play an important role in reducing portal venous pressure and improving a hyperdynamic circulatory state.

SST and its analogues are now the preferred medication in the treatment of liver cirrhosis portal hypertension complicated by EVB, while octreotide is used more widely in clinical practice (Sharara and Rockey, 2001). The mechanism of SST and octreotide in reducing portal pressure is still under debate. Song et al. (2004) showed that there is strong mRNA expression of SSTR subtypes-SSTR2, STR3 and SSTR5, but not SSTR1 and SSTR4 in rat HSCs. It is thought that octreotide may affect HSCs via SSTR and thereby exert its biological activity. However, other authors have reported strong staining of SSTR1, SSTR2 and SSTR3 in activated rat HSCs, but not in normal liver. SST causes significant partial inhibition of ET-1-induced contraction of HSC, mainly via stimulation of SSTR1 (Reynaert et al., 2001). The purpose of this study was to determine the patterns of expression of five SSTR subtypes in human activated HSCs, while also studying the effects of octreotide on intracellular Ca^{2+} in the regulation of HSC contractility. The effects of two dosages of octreotide on portal pressure in cirrhotic patients with portal hypertension were determined. This study may provide important guidance for the rational clinical use of octreotide and assessment of new drugs in the treatment of portal hypertension.

MATERIALS AND METHODS

Patients

Thirty-one (31) hepatitis B-related cirrhotic patients with portal hypertension were enrolled in a randomized, open study. Twenty-two (22) male and 9 female patients participated with a mean age of 48.6 years. A diagnostic work-up was performed that included a physical examination, laboratory tests and B-ultrasonography or computerized tomography (CT) scan, according to the criteria suggested by the Chinese Medical Association for Liver Diseases in 2000. A gastrointestinal endoscopic examination demonstrated that the number of patients with severe and moderate esopha-geal/gastric varices were 29 and 2, respectively. Of these patients, 29 patients had undergone surgery because of portal hypertension with a history of variceal bleeding. The other patients had voluntarily undergone surgery because of severe esophageal varices with hypersplenia. The number of patients with Child-Pugh grade A and B cirrhosis were 20 and 11, respectively.

In addition to the splenectomy, paraesophagogastric devascular-isations were routinely performed, which, in brief, entails making an oblique incision under the left costal border. During the surgery, the extremity of a single lumen venous catheter (7F SPECATH, Beijing, China) was inserted into 5 cm of the main stem of the portal vein through the right gastro-omental vein and the proximity was fixed on the right abdominal wall. The purpose of the portal vein catheter was to prevent portal vein thrombosis after splenectomy and devas-culation and to monitor the portal pressure (Lin et al., 2007). The catheter was irrigated with heparinized saline (heparin, 25 unit/ml), connected to a three-way tube and the heparin cap was prepared.

The patients were randomly divided into three groups using the lot-drawing method after 24 h of surgery. In group A, the patients (n = 12) received a bolus of octreotide (100 μg) followed by a continuous infusion of 50 μg/h and conventional treatment for 3 days. Patients in group B (n = 14) received a bolus of octreotide (100 μg) followed by a continuous infusion of 25 μg/h and conventional treatment for 3 days. Patients in group C (n = 5) received conventional treatment, which was regarded as placebo control. The baseline characteristics of the study subjects, including mean age, gender and Child-Pugh grades and conventional treatment in groups A, B and C were comparable as shown in Table 1. The study was explained to the patients and/or their relatives and consent was obtained from all patients. The study protocol was approved by the Ethical Committee of Beijing You'an Hospital of Capital Medical University.

Table 2. Primer sequences for amplification of SSTR subtypes 1-5 and reference gene GAPDH.

Receptor	Upstream	Downstream	Amplified fragment (bp)
SSTR1	5'-AGCCGGTTGACTATTACGCC-3'	5'-GCTCTCACTTCTACCATTGTC-3'	334
SSTR2	5'-GGTGAAGTCCTCTGGAATCC-3'	5'-CCATTGCCAGTAGACAGAGC-3'	461
SSTR3	5'-TCATCTGCCTCTGCTACCTG-3'	5'-GAGCCCAAAGAAGGCAGGCT-3'	221
SSTR4	5'-CGGCAGTCTTCGTGGTCTAC-3'	5'-GCATCAAGGCTGGTCACGAC-3'	247
SSTR5	5'-AACACGCTGGTCATCTACGTGGT-3'	5'-AGACACTGGTGAACTGGTTGAC-3'	211
GAPDH	5'-CCCTTCATTGACCTCAAC-3'	5'-TTCACACCCATCACAAAC-3'	301

Dynamic measurement of portal pressure

The catheter was irrigated with heparinized saline (heparin, 25 unit/ml) and the three-way tube and heparin cap were connected. The pre-devascularization portal venous pressure was measured and the zero point was defined using the mid-axillary line level. The proximity of the single lumen venous catheter was connected to the monitor (Hp M3) through a pressure transducer. After 24 h when the patient's vital signs were stabilized, the portal venous pressures were measured before drug administration (regarded as baseline), 5 min, 15 min, 60 min, 2 h, 24 h, 48 h and 72 h after drug administration and 15 min, 30 min, 60 min, 24 h and 48 h after drug withdrawal. The change in portal pressure was calculated as: portal pressure (%) = (baseline-portal pressure)/ baseline × 100. The catheter was safely removed after the investigation at 7 days. No complications related to insertion of the catheter were observed. All patients recovered from their surgery.

Human HSC culture

The activated human HSC cell line, LX-2, which was established from healthy HSCs transfected with SV40 and selectively cultured with medium containing a low concentration of fetal bovine serum (FBS), was donated by Professor JD Jia (Liver disease center, Beijing Friendship Hospital). The phenotype was shown to be activated HSCs (Sanyal et al., 2008). Frozen cells were thawed at 37°C, cultured with Dulbecco's Modified Eagle Medium (DMEM, Gibco BRC, Grand Island, NY ,USA) containing 10% FBS and prepared as a HSC single cell suspensions of 8×10^4 cells. LX-2 was plated on 20 × 20 mm glass coverslips in 35-mm culture dishes at a density of 8×10^4 cells/ml and routinely incubated in 2 ml DMEM media, supplemented with penicillin, streptomycin and 10% FBS for 24 h in a 95% O_2/5% CO_2 mixture at 37°C. Each experiment was repeated 3 times.

Measurement of intracellular Ca^{2+}

Cultured LX-2 cells were observed using a TE300-F70 inverted microscope (NIKON, Tokyo, Japan). After the cells were completely adherent, the medium was switched to 2 ml of serum-free DMEM media. Intracellular Ca^{2+}, loaded by a dosage of 5 μl Flu-3/AM (1:50 dilution; Biotium, Hayward, CA, USA) for 30 min under 95% air and 5% carbon dioxide at 37°C, was measured by MRC-1024 type laser scanning confocal microscopy (LSCM; Bio-Rad, HERCULES,CA ,USA) at a 488 nm excitation wavelength and 526 nm emission wavelength. Dynamic fluorescence scans of Flu-3 labeled 10 cells were randomly selected for detection of intracellular fluorescence intensity (FI) using LSCM at a different treatment stage. The concentration of intracellular Ca^{2+} was represented using FI. The dynamic changes in intracellular Ca^{2+} stimulated by octreotide (final concentration, 5 μg/ml), endothelin-1 (ET-1, 2 μg/ml) and KCl (2

mmol/ml) were recorded by LSCM. The effects of octreotide on intracellular Ca^{2+} stimulated by ET-1 or KCl were also observed.

Immunocytochemistry

After 24 h of culture of LX-2, the coverslips were washed with PBS 3 times, fixed in 4% paraformaldehyde for 1 h and then incubated in 3% H_2O_2-deionized water at room temperature for 10 min to block endogenous peroxidase. A 1:200 ratio of rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to mouse antigens (SSTR2/SSTR5) was incubated with the cells at 37°C for 2 h. After that, cells were incubated with goat anti-rabbit IgG antibody-HRP polymer (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) at 37°C for 30 min and then treated with AEC developing solution. After approximately 7 min, when the coloration was good, coverslips were mounted with gelatin. Expression of SSTR2 and SSTR5 was examined by a cytology specialist. The expression of SSTR1, 3 and 4 of HSCs was not carried out because commercial antibodies to mouse antigens for SSTR1/SSTR3/SSTR4 are not available. Therefore, we designed the following detection methods in order to observe the SSTR1/SSTR3/SSTR4 expression of HSCs.

RT-PCR

After 24 h of culture of LX-2, total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA ,USA) and reverse transcribed to cDNA using the Reverse Transcription System (Applied Biosystems, Framingham, MA , USA) according to the manufacturer's instructions. SSTR 1-5 primers were designed as shown in Table 2, confirmed for accuracy in GenBank and mismatched 10 - 11 basic group TC at the downstream primer of SSTR4 was changed to CT. In parallel, glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal negative control and its primers were designed as shown in Table 2. Diethylpyrocarbonate (DEPC) water used in our laboratory was used as negative control. RT-PCR was performed as recommended by the manufacturer (Invitrogen). In brief, RT-PCR was carried out in a total volume of 25 μl reactions consisting of ddH_2O (13.9 μl), 10× buffer (2.5 μl), 25 mmol/l $MgCl2$ (25 mM; 1.5 μl), 10 mM dNTP (1 μl) and Taq polymerase (0.1 μl; Promega, Madison, WI, USA), 10 μM upstream and downstream primers (2 μl each), cDNA template (2 μl) and 2 μl of parogen. RT-PCR amplification was conventionally carried out. 10 μl of PCR reaction was conventional in 20 mg/g agarose gel with 100-bp DNA markers. Densitometric analysis of PCR products was performed by computer software (Quantity One, Furi Company, Shanghai, China) and standardized by the GAPDH.

Statistical analysis

Statistical analysis was performed using the Student's t-test as
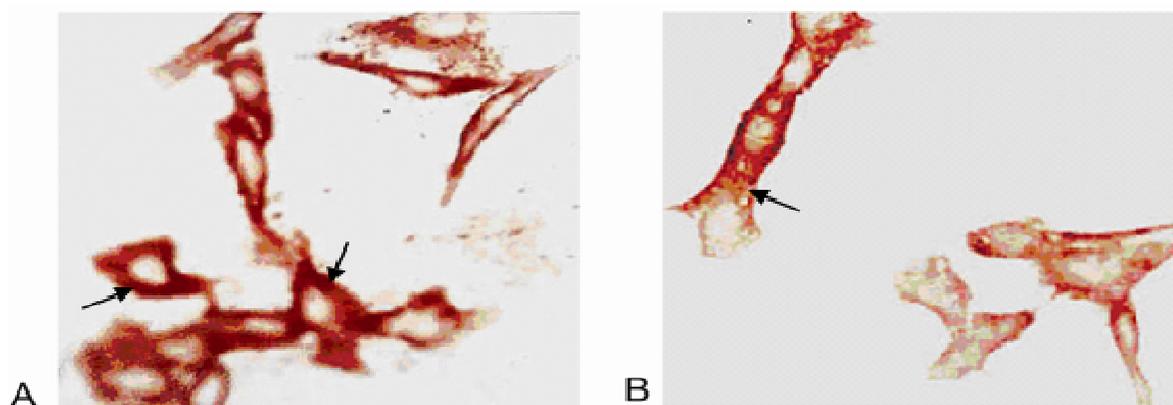
Figure 1. The immunocytochemical staining showed that SSTR2 (A), SSTR5 (B), labeled with reddish brown (arrows), in LX-2 cells were positive (40 × 10).
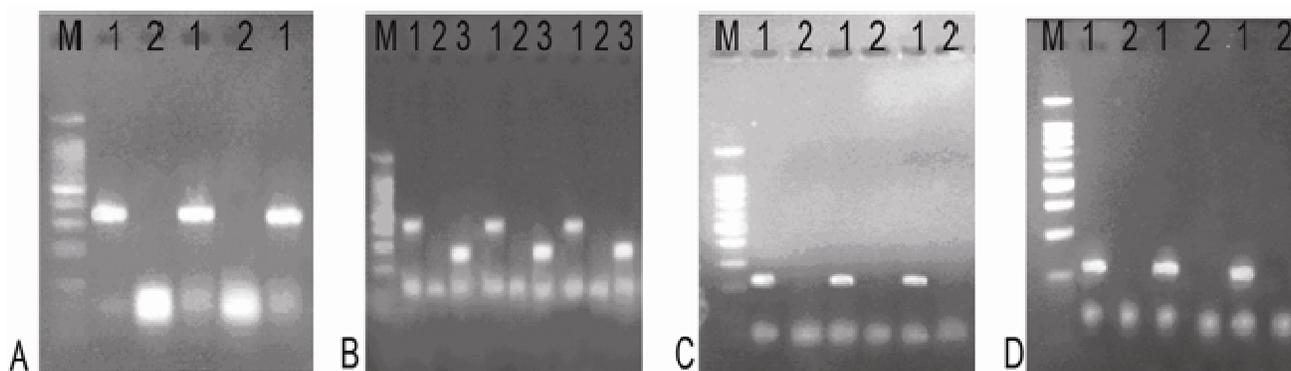

Figure 2. The results of RT-PCR showed that the mRNA of SSTR subtypes (SSTR1, 2, 3, 4 and 5) in LX-2 cells in A-D. Figure 2A shows expression of SSTR1(1) and negative control (2); Figure 2B shows expression of SSTR2(1), SSTR4(3) and negative control (2); Figure 2C shows expression of SSTR3(1) and negative control (2); while Figure 2D shows expression of SSTR5(1) and negative control (2). M = marker.

given in the Statistical Package for Social Sciences (SPSS11.5). FI represented intracellular Ca^{2+} concentration. Quantitative data were expressed as the mean ± SD and analyzed with a paired t-tests and Wilcoxon tests, while the chi-squared test was used for semi-quantitative data. Statistical significance was established at P < 0.05.

RESULTS

Expression of SSTR 1-5 subtypes in LX-2

Immunocytochemical staining showed that LX-2 cells were positive for SSTR2 and SSTR5, which were mainly localized on the cell membrane and in the cytoplasm (Figure 1). RT-PCR detected mRNA expression of SSTRs 1-5 in LX-2 cells (Figure 2). Results of gel electrophoresis were analyzed and semi-quantified with software Quantity One and the relative levels of expression of SSTR subtypes 1 - 5 were found to be SSTR 1, 4 > SSTR 2, 5 > SSTR 3 (P < 0.05).

Effect of octreotide on intracellular Ca^{2+}

Octreotide lowered the intracellular Ca^{2+} and ET-1 significantly increased intracellular Ca^{2+} concentration in LX-2 cells (Table 3). KCl, an L-type voltage-dependent calcium channel agonist, also increased the intracellular Ca^{2+} concentration in LX-2 cells. Moreover, we also showed that octreotide did not inhibit the up-regulation of intracellular Ca^{2+} concentration stimulated by KCl or ET-1.

Effect of octreotide on portal pressure in cirrhotic patients

Following splenectomy with paraesophagogastric devas-cularization, the portal venous pressure decreased by an average of 15.4%. Portal venous pressure significantly decreased after 5 min in both groups A and B and octreotide achieved a maximum effect after 24 h, with an average decrease in portal venous pressure of 20.6% (Figure 3). In the patients who received a 50 μg/h infusion

Table 3. The results of intracellular Ca^{2+} in LX2 cell line stimulated by octreotide or ET-1.

FI	Octreotide treatment (n = 10)	ET-1 treatment (n = 10)
Before	8.62 ± 1.32	8.62 ± 1.32
After	4.31 ± 1.35*	17.6 ± 2.31**

Results are given using mean ± SD; compared with before * P < 0.01,** P < 0.001.

of octreotide, the portal venous pressure decreased by 23.1% on average in the 48 h following drug withdrawal. However, patients in the 25 µg/h octreotide infusion group had a tendency for increased portal venous pressure 2 h following drug withdrawal and the portal venous pressure decreased by 11.6% on average. The portal pressure was not significantly changed after surgery in group C and the average portal pressure in group C was higher compared to either group A or B (P < 0.05). The degree of change in the portal venous pressure and the percent of portal venous pressure at 24 and 48 h were significantly different between groups A and B (P < 0.05). The heart rate and mean arterial blood pressure before and after octreotide infusion showed no significant differences between the groups.

DISCUSSION

SST and its analogues, particularly the synthetic analogue, octreotide, are widely used as first-line treatment for liver cirrhosis portal hypertension compli-cated by variceal bleeding (Masyuk et al., 2007). This study demonstrated that a bolus of octreotide (100 µg) followed by a continuous infusion of octreotide (25 or 50 µg/h) can significantly reduce the portal venous pressure in these patients. The maximum effect of octreotide was observed after 24 h. However, the portal venous pressure of patients receiving a 50 µg/h infusion remained at a low level 48 h after drug withdrawal, while portal venous pressure in the patients who received a 25 µg/h infusion increased 2 h after drug withdrawal. This may be the pharmacologic basis of the lower re-bleeding rates in patients receiving 50 µg/h of octreotide after drug withdrawal (Association 2005). Nevertheless, the mecha-nism of SST/octreotide in reducing portal venous pre-ssure is not clear (Minor and Grace, 2006; Yang et al., 2005). Initial studies suggest that the inhibitory effect of SST/octreotide on the release of glucagon is the principal mechanism by which SST reduces portal venous pressure (Yang et al., 2005). Another study indicated that a bolus injection of SST caused a maximal decrease in variceal and portal pressure within 0.5 - 2 min. Indeed, it takes 10 min for maximal inhibition of glucagon secretion and the half-life of glucagon is 25 min in the cirrhotic model (Song et al., 2004). Some investigators have

suggested that octreotide reduces portal venous pressure by selectively contracting the superior mesenteric artery and causing a reduction of blood flow in the liver (Sharara and Rockey 2001). In our previous study, we demon-strated that octreotide had no direct effect on the contraction of vascular smooth muscles (Ding et al., 2004).

Intrahepatic vascular resistance can be reduced by 20 - 30% with drugs (Gonzalez et al., 2006; Sanyal et al., 2008; Triantos and Burroughs, 2007). Drug acting sites must be targeted to factors that reversibly increase intrahepatic vascular pressure and/or portal venous blood flow. HSCs are located in the perisinusoidal space of disse close to sinusoidal endothelia and spread many long synapses surrounding the hepatic sinusoids, which are in contact with numerous hepatic cells, adjacent HSCs, nerve terminals and extend among the hepatic cells to adjacent hepatic sinusoids (Sharara and Rockey, 2001). Activated HSCs have characteristics that are similar to smooth muscle cells and the contraction and relaxation of HSCs are important regulatory factors of intrahepatic resistance and sinusoidal blood flow (Ding et al., 2004; Reynaert et al., 2002; Sharara and Rockey, 2001). Therefore, it is possible that SSTs reduce portal venous pressure partly through their action on HSCs.

Another study indicated that the intracellular Ca^{2+}-mediated signal system plays an important role in regulating contraction and relaxation of vascular smooth muscle cells, while the Rho system of low-molecular-weight GTPase plays an important role in regulating the functions of non-smooth muscle cells (Ding et al., 2004). Recent studies have demonstrated that the intracellular Ca^{2+}-mediated signal system also plays an important role in regulating HSC contraction and relaxation (Ding et al., 2004; Reynaert et al., 2002). We reported that octreotide could significantly and immediately down-regulate Ca^{2+} concentration in HSCs-T6 cells, a cell line established from SD rat HSCs transfected with SV40 (Ding et al., 2004). In the current study, we found that, while octreotide could significantly and immediately down-regu-late the Ca^{2+} concentration in LX-2 cells, it did not inhibit an increase in Ca^{2+} concentration in HSCs caused by KCL-stimulated L-type voltage-operated calcium channels (L-VOCC) opening. Nor could it inhibit an increase in Ca^{2+} concentration in HSCs caused by ET-1 stimulation. We speculate that octreotide down-regulates Ca^{2+} concentration in HSCs, which inhibits HSC contraction, thereby reducing intrahepatic resistance and portal venous pressure.

The mechanism of octreotide action may lay in the binding of SSTRs on HSCs. SSTR subtypes 1-5 have been cloned successfully from human, rat, mice, cow and pig and induce many biological effects (Masyuk et al.,2007; Reynaert et al., 2004; Reynaert et al., 2001; Song et al., 2004). Studies of HSCs expressing an SSTR, especially in human HSCs, are rare (Xu et al. 2005). Reynaert et al. (Reynaert et al. 2001) demonstrated that

SSTRs 1, 2 and 3 are expressed at the protein and mRNA levels in cultured activated rat HSCs, but not in normal rat liver. More recently, Reynaert et al. (2001) also demonstrated that in normal human liver, both hepatocytes and HSC are negative for SSTRs 1-5. However, cirrhotic liver and hepatocellular carcinoma as well as cultured HSCs, expressed all five SSTRs (Reynaert et al., 2004). In the current study, we demonstrated that the LX-2 HSC cell line can express all five SSTRs at the protein and mRNA levels, providing evidence for the possibility that HSCs may be a target site of SST in reducing portal venous pressure.

A pharmacologic study demonstrated that natural SST has a high affinity for all SSTRs, while long-acting analogues such as octreotide, only have a high affinity for SSTR2 and SSTR5, a medium affinity for SSTR3 and almost no affinity for SSTR1 and SSTR4 (Song et al., 2004; Yang et al., 2005). Other studies have indicated that natural SST is more effective than octreotide at reducing hepatic sinusoidal pressure and this effect might be due to an NO-independent mechanism (Gonzalez et al., 2006; Yang et al., 2005). In this study, we also indirectly demonstrated that HSCs play an important role in decreasing portal pressure from the SSTR1-5 aspect, suggesting that SST may have better effects in reducing hepatic sinusoidal pressure than octreotide. Therefore, we considered the possibility that octreotide down-regulates Ca^{2+} concentration in HSCs, thereby inhibiting the contraction of HSCs, leading to reduced intrahepatic resistance and portal venous pressure. The mechanism may be one of octreotide combining with specific SSTRs on HSCs. These results provide evidence for the possibility that HSCs may be the targeted site of action of SST and its analogues in reducing portal venous pressure. Clinical research evaluating SST or octreotide for the treatment of portal hypertension should take these findings into account. This project may provide important guidance for the rational clinical use of octreotide and assessment of the new drugs in the treatment of portal hypertension.

Conclusion

These results provide evidence for the possibility that HSCs may be the targeted site of action of SST and its analogues in reducing portal venous pressure. Clinical research evaluating SST or octreotide for the treatment of portal hypertension should take these findings into account.

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