Promotion of apoptosis in high glucose-activated hepatic stellate cells by GLP-1 receptor agonist and its potential mechanism

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ABSTRACT. This study aimed to investigate the stimulatory effect of glucagon-like peptide 1 (GLP-1) receptor agonist (GLP-1RA) on the apoptosis of hepatic stellate cells (HSCs) activated by high glucose, and to explore the underlying molecular mechanism with a focus on the c-Jun N-terminal kinase (JNK) and extracellular signal-related kinase (ERK) signaling pathways. Human HSCs were cultured in-vitro and their morphological features were identified. HSC samples were randomly collected and divided into a control group, GLP-1RA group, GLP-1RA+JNK blockade group, and ERK blockade group. The apoptosis of HSCs in each group were analyzed by fluorescence-activated cell sorting (FACS) after 120 h of culture. Phosphorylated JNK and ERK (p-JNK and p-ERK)
proteins were detected by western blotting. p-JNK expression was higher in the GLP-1RA group compared with that of the control group and the GLP-1RA+JNK blockade group (both P<0.01). The apoptosis rate was higher in the GLP-1RA group compared with that of the control group and the GLP-1RA+JNK blockade group (both P<0.01). p-ERK expression was lower in the GLP-1RA group and ERK blockade group than in the control group (both P<0.01). The apoptosis rates in the GLP-1RA group and ERK blockade group were higher than that of the control group (both P<0.01). GLP-1RA can promote the apoptosis of high glucose-activated HSCs through activation of the JNK signal pathway and through blocking the ERK signal pathway.

**Key words:** Hepatic stellate cells; Apoptosis; JNK signal pathway; ERK signal pathway.

**Abbreviations:**
HSCs: Hepatic Stellate Cells
JNK: c-Jun N-Terminal Kinase
ERK: Extracellular Signal-Related Kinase
FACS, FITC: Fluorescence-Activated Cell Sorting
Annexin V-Fluorescent Isothiocyanate, GLP-1: Glucagon-like peptide 1
GLP-1RA: Glucagon-Like Peptide 1 Receptor agonist
IL: Interleukin
MAPK: Mitogen-Activated Protein Kinase
PI: Propidium Iodide
PI3K/AKT: Phosphoinositide 3-Kinase/ Serine/Threonine Kinase
PVDF: Polyvinylidene Fluoride
TBS: Tris-Buffered Saline

**INTRODUCTION**

Recent studies have shown that glucagon-like peptide 1 receptor agonist (GLP-1RA) has an anti-hepatic fibrosis effect (Jendle J (2009), et al. Park CW (2007), et al. Hillman M (2012), et al. Tang X (2008), et al. Zhang H (2011), et al. The critical step of hepatic fibrosis is production of a large amount of extracellular matrix by activated hepatic stellate cells (HSCs), and the apoptosis of activated HSCs is a determining factor for the development of hepatic fibrosis. The mechanism of apoptosis is extremely complicated, and is modulated by different mechanisms through multiple intracellular signaling pathways. Two families have been proven to participate in the process of apoptosis, namely, the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase/ serine/threonine kinase (PI3K/AKT) families. The MAPK family, including the c-Jun N-terminal kinase (JNK), p38 MAPK, and extracellular signal-related kinase (ERK) subfamilies, is a group of kinases playing vital roles in apoptosis. We speculated that these pathways might be involved in the anti-hepatic fibrosis mechanism of GLP-1RA. Therefore, in this study, the stimulatory effect of GLP-1RA on HSC apoptosis was investigated, and the working mechanism was explored with a focus on the involvement of the JNK and ERK pathways.

**MATERIAL AND METHODS**

**Main materials and reagents**

The human HSC line IL-90 was obtained from the experimental animal facility of Guangzhou Zhongshan University. The liraglutide injection reagent (GLP-1RA) was obtained from Novo Nordisk A/S (Denmark). The JNK inhibitor SP600125 and the ERK inhibitor PD98059 were obtained from Sigma (USA). The anti-p-JNK, -p-ERK, and -GAPDH antibodies, and the biotinylated secondary antibody were purchased from New England Biolabs (USA). The sodium dodecyl sulfate-polyacrylamide gel electrophoresis preparation kit and ECL chemiluminescence kit were purchased from Wuhan Booster Biological Technology, Ltd. Annexin V-fluorescent isothiocyanate (FITC), propidium iodide (PI), and other fluorescence-activated cell sorting (FACS)-related consumables were obtained from Roche (USA).
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Grouping
The HSC samples were randomly collected and divided into a control group (n=20), GLP-1RA group (n=20), GLP-1RA+JNK blockade group (n=20), and ERK blockade group (n=20). HSCs in the control group were routinely cultured (see below) with the addition of a high glucose concentration (25 mmol/l). Cells of the GLP-1RA group were cultured in the same manner as those in the control group with the addition of the GLP-1RA liraglutide (final concentration of 10 mmol/l). Cells of the GLP-1RA+JNK blockade group were cultured in the same manner as those in the GLP-1RA group with the addition of the JNK inhibitor SP600125 (final concentration of 20 µmol/l). Cells of the ERK blockade group were cultured in the same manner as those in the control group with the addition of the ERK inhibitor PD98059 (final concentration of 50 mmol/l). The cells were cultured for 120 h before analysis.

Culture of human HSCs
The human HSCs were inoculated on the plastic culture plate after recovery from cryopreserved vials, and cultured in L-Dulbecco's modified Eagle medium containing 10% foetal calf serum at 37°C with 5% CO2. The culture medium was refreshed once every three days.

Morphological identification of human HSCs
The living HSCs appeared as flat cells lacking fat droplets under an inverted microscope (Olympus Corporation, Tokyo, Japan), with well-differentiated stress fibres and a large volume.

Protein sample extraction and concentration determination
The HSCs from each group were lysed with fresh prepared cell lysis buffer (containing 8 M urea, 4% CHAPS, 1% volume fraction of TATA-binding protein, 0.2% BioLyte, and 0.001% bromophenol blue). Cell breakage was performed by intermittent ultrasound sonication on an ice bath followed by centrifugation at 22,000 g in a high-speed centrifuge (A Ting Scientific Instrument, Shanghai, China). The supernatants were placed in a new tube for protein concentration determination by the Bradford method.

Western blotting
The phosphorylated protein products of the JNK and ERK signaling pathways (p-JNK and p-ERK) were detected by western blotting. The polyacrylamide gel was prepared and moved to the electrophoresis instrument for electrophoresis and protein sample separation. The gel was then shifted to the transfer plate and the proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane under a current of 350 mA for 1 h after electrophoresis. The PVDF membrane was rinsed with Tris-buffered saline (TBS) for 10 min. The PVDF membrane was then incubated at room temperature (20–28°C) with blocking buffer (TBS with Tween [TBS/T] with a final concentration of 5% bovine serum albumin) for 1 h, and then washed with TBS/T three times for 5–10 min each time.

The primary antibody was diluted with blocking buffer and incubated with the PVDF membrane at 4°C overnight, and then washed with TBS/T three times for 5–10 min each time. The horseradish peroxidase-labelled secondary antibody was diluted with blocking buffer (1:2000), incubated with the PVDF membrane at room temperature for 1 h, and then washed with TBS/T three times for 5–10 min each time. The membrane was washed once again with TBS. The PVDF membrane was developed with the ECL kit, exposed to X-ray film, fixed, and scanned by a gel imaging analysis system. GAPDH was used as the internal control for semi-quantitative analysis.

FACS analysis of the apoptosis of HSCs
HSCs in each group were harvested by trypsin without ethylenediaminetetraacetic acid, and collected with centrifugation at 1000 g for 5 min. The harvested cells were washed with phosphate buffered saline twice and centrifuged at 1000 g for 5 min.

Cells (1–5 × 10^5) were re-suspended with 500 µl of Binding Buffer. The re-suspended cells were mixed with 5 µl Annexin V-FITC and 5 µl PI at room temperature, mixed well in the dark, and subjected to FACS in a flow
cytometer (BD Corporation, NJ, USA) after incubation for 5–15 min. The excitation wavelength was 488 nm and the emission wavelength was 530 nm.

**Statistical analysis**

The software SPSS17.0 was utilized for statistical analysis. The data fulfilled the assumptions of the normal distribution and were therefore analyzed with the Student t-test for comparisons between two independent data samples.

**RESULTS**

The expression level of p-JNK was determined by western blotting with GAPDH as the internal control, and the results are shown in Figure 1. The expression level of p-JNK was higher in the GLP-1RA group compared with that of the control group and the GLP-1RA+JNK blockade group (both P<0.01, Table 1). Therefore, GLP-1RA can promote the phosphorylation of JNK to activate the JNK signaling pathway, which can be inhibited through abolishing JNK phosphorylation by the JNK inhibitor.

The apoptosis of HSCs was analyzed by FACS as shown in Figure 2. The apoptosis rate was higher in the GLP-1RA group compared with that of the control and GLP-1RA+JNK blockade groups (both P<0.01, Table 1). Therefore, the HSC apoptosis rate can be increased by GLP-1RA and then inhibited by the JNK inhibitor, indicating that activation of the JNK signaling pathway can promote the apoptosis of HSCs.

The expression level of p-ERK protein in HSCs of each group was determined by western blotting with GAPDH as the internal control, and the results are shown in Figure 1. The expression levels of p-ERK were lower in the GLP-1RA and ERK blockade groups compared with that of the control group (both P<0.01, Table 1). Therefore, GLP-1RA can block the ERK signaling pathway through inhibition of ERK phosphorylation.

Figure 1. Expression levels of p-JNK and p-ERK in HSCs from the control, GLP-1RA, GLP-1RA+JNK blockade, and ERK blockade groups determined by western blotting.

Figure 2. The apoptosis rates of HSCs from the control, GLP-1RA, GLP-1RA+JNK blockade, and ERK blockade groups analyzed by FACS.
The apoptosis of HSCs from each group was analyzed by FACS as shown in Figure 2. The apoptosis rates of HSCs in the GLP-1RA and ERK blockade groups were both higher than that in the control group (both P<0.01, Table 1). Therefore, both GLP-1RA and blocking the ERK signaling pathway can promote the apoptosis of HSCs.

### Table 1. The apoptosis rate and relative expression levels of p-JNK and p-ERK in HSCs from each group (mean ± SD).

<table>
<thead>
<tr>
<th>Group</th>
<th>p-JNK/GAPDH</th>
<th>p-ERK/GAPDH</th>
<th>Apoptosis rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.63 ± 4.95%</td>
<td>23.13 ± 5.05%</td>
<td>9.90 ± 5.92%</td>
</tr>
<tr>
<td>GLP-1RA</td>
<td>22.79 ± 3.80%**</td>
<td>14.65 ± 3.59%**</td>
<td>30.28 ± 3.18%**</td>
</tr>
<tr>
<td>GLP-1RA+JNK blockade</td>
<td>13.66 ± 4.15%**</td>
<td>14.12 ± 2.92%**</td>
<td>10.48 ± 4.41%**</td>
</tr>
<tr>
<td>ERK blockade</td>
<td>8.05 ± 3.66%</td>
<td>13.73 ± 3.85%**</td>
<td>31.59 ± 5.02%**</td>
</tr>
</tbody>
</table>

**P<0.01 compared with the control group; ΔΔP<0.01 compared with the GLP-1RA group.

### DISCUSSION

Accumulating studies have demonstrated that GLP-1RA has an anti-fibrosis effect on several organs, including the liver. HSCs are key cells involved in the process of hepatic fibrosis, and are important target cells in most studies of hepatic diseases. Our previous study showed that GLP-1 receptor can be detected on the surface of HSCs Wu LK, (2013), et al. indicating that the anti-hepatic fibrosis mechanism of GLP-1RA might also be related to HSCs. The apoptosis of HSCs is a crucial aspect to reverse or cure hepatic fibrosis Friedman SL (2008), De Minicis (2007), et al. and ‘activation’ is the precondition for the apoptosis of HSCs. There are two end points of activated HSCs: 1) conversion to the static status from the activated status, or 2) apoptosis and cell death. The elimination of activated HSCs through apoptosis is one of the main mechanisms of hepatic fibrosis regression. Li CX (2007), et al. Saile (1997) et al. observed the spontaneous apoptosis of activated HSCs in a rat model during the repair stage of acute liver injury, which was positively associated with the degree of activation; however, there was no apoptosis detected during the early stage of acute liver injury. Therefore, it is considered that the apoptosis and activation of HSCs occur in parallel, and there is no apoptosis in static HSCs. The precondition of HSC apoptosis is the transition from a static state to an activated state. Yang Qiao (2008), et al. and Sigala (2013), et al. showed that HSCs can be activated by noradrenaline norepinephrine in a dosage-dependent manner. In addition, Sugimoto (2005), et al. reported that HSCs can be activated by high glucose, which was also confirmed in our previous study in which HSCs were activated when cultured with a high glucose concentration of 25 mmol/l Wu L (2016), et al.

The apoptosis of activated HSCs is associated with complicated intracellular signaling pathways, and the involvement of MAPK signaling pathways has been analyzed in the greatest detail to date. The involvement of MAPKs in the regulation of cell apoptosis has been observed in multiple types of cell models Siegfried Z (2013), et al. Therefore, we speculated that these pathways might be responsible for the anti-hepatic fibrosis effect of GLP-1RA. There are three members in the MAPK family: JNK, p38 MAPK, and ERK. JNK is a major factor involved in apoptosis and can be activated by inflammatory cytokines such as tumor necrosis factor-alpha, interleukin (IL)-1, and IL-6. JNK can phosphorylate the serine residues at the N-terminal positions 63 and 73 of the transcription factor c-Jun to activate it and enhance its transcription ability, which ultimately conveys the signals from the endoplasmic reticulum membrane to downstream transcription factors, eventually promoting the expression of cytokines Poulsen KL (2014), et al. JNK participates in both the inflammation reaction and apoptosis Duo CC (2014), et al. Choi H (2014), et al. SP600125, as a selective JNK inhibitor, can inhibit the phosphorylation of JNK, decrease the expression of p-JNK, and therefore block the JNK signaling pathway. Accordingly, SP600125 is a commonly used reagent for studying the JNK signaling pathway.

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pathway. ERK, as a regulator of cell survival, is closely associated with the inhibition of apoptosis. The ERK signaling pathway has been shown to play vital roles in apoptosis, especially in the apoptosis of neurons and cancer cells Agrawal M (2015), et al. Esmaeili MA (2014), et al. PD98059, as a selective ERK inhibitor, can inhibit the phosphorylation of ERK to decrease the expression of p-ERK, eventually blocking the ERK signaling pathway. Therefore, PD98059 is a commonly used reagent for studying the ERK signaling pathway.

CONCLUSION

In this study, we obtained activated HSCs by culturing them in a high-glucose environment. We found that GLP-1RA could promote the apoptosis of activated HSCs. Therefore, we studied the mechanism by which GLP-1RA promotes HSC apoptosis with a focus on the JNK and ERK pathways. Finally, we found that GLP-1RA can activate the JNK signaling pathway through enhancing JNK phosphorylation, which in turn promotes the apoptosis of HSCs. Therefore, GLP-1RA can promote the apoptosis of high glucose-activated HSCs through the JNK signaling pathway. We also found that GLP-1RA can block the ERK signaling pathway through inhibition of ERK phosphorylation, and blocking the ERK signaling pathway can promote the apoptosis of HSCs. Therefore, GLP-1RA can promote the apoptosis of high glucose-activated HSCs through blocking the ERK signaling pathway. In short, GLP-1RA can promote the apoptosis of activated HSCs through the JNK signaling pathway and through blocking the ERK signaling pathway. These findings provide new clues for the in-depth investigation of the anti-hepatic fibrosis mechanism of GLP-1RA, which can help to promote its clinical application.

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DISCLOSURE OF CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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