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Alleviative effects of fibroblast growth factor 21 on fibrogenic genes expression in the fructose activated hepatic stellate cells, an *in vitro* study

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Abstract

Introduction: Liver fibrosis occurs through hepatic stellate cells (HSCs) activation during chronic liver injury. Cytokines especially transforming growth factor-β (TGF-β), activate HSCs by an autocrine effect. The activated HSCs generate more α-smooth muscle actin (α-SMA) and Collagen1. The activation of human HSCs by high fructose concentrations has been confirmed. Fibroblast growth factor 21 (FGF21) as a hepatic hormone possesses favorable metabolic effects, but its effect on activated HSCs and the process of fibrogenesis have not been studied yet.

Objectives: In this study, we evaluated the effect of FGF21 on suppressing the fructose-activated human HSCs and its effect on the TGF-β/ Smad3 signaling pathway.

Materials and Methods: The LX-2 cells were grown in the presence of high fructose concentrations (25 and 30 mM) for 48 hours for activation and then were treated with FGF21. Quantitative real-time polymerase chain reaction (real-time PCR) was performed to measure the expression of fibrogenic genes and western blotting to analyze the TGF-β signaling.

Results: Our results showed increased levels of mRNA expression of αSMA, collagen1, and TGF-β genes and smad3 phosphorylation in fructose-activated HSCs. FGF21 treatment significantly attenuated the induced fibrogenic gene expression and smad3 phosphorylation.

Conclusion: High fructose leads to fibrogenic pathways in the liver by activating HSCs and increasing TGF-β signaling. FGF21 is effective in reducing fibrogenesis in activated HSCs through downregulating the TGF-β/smad3 signaling and fibrogenic gene expression. Therefore, FGF21 may provide anti-fibrotic properties in liver fibrosis.

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Introduction

Liver fibrosis is a critical hepatic problem globally, mainly due to chronic viral or metabolic liver diseases. Liver fibrosis results from continued liver damage, further leading to hepatocellular carcinoma (1, 2). Liver fibrosis is characterized by progressive accumulation of extracellular matrix (ECM), which destroys the physiological structure of the liver (3). HSCs have a significant role in liver fibrosis by producing ECM (4). Toxic, metabolic, or viral factors can damage hepatocytes, leading to the activation of the hepatic stellate cells (HSCs) and their transformation into myofibroblasts. Physiologically, after a short-time injury, this action is balanced by counteracting anti-fibrotic mechanisms, i.e. inactivation and apoptosis of myofibroblasts for tissue repair. However, in chronic liver damages, an imbalance of pro-fibrogenic and anti-fibrogenic mechanisms causes excessive activation, proliferation, contraction and migration of myofibroblasts, leading to the excessive production of ECM (5, 6). Transforming growth factor-β (TGF-β), a hepatic cytokine, is produced in response to hepatocyte damage. It has a central role in regulating and activating HSCs and the progression of fibrosis (4). Smad3 is the principal intracellular mediator of the TGF-β receptor signaling, including type I receptor-mediated phosphorylation of Smad3 (7,8). It has been shown that blockers of TGF-β signaling through Smad3 could restrain

Key point

FGF21 reduces the mRNA expression of FGF21, TGF-β, α-SMA, and collagen1 genes in fructose-activated human hepatic stellate cells by the TGF-β/smad3 signaling pathway. According to our results, FGF21 possesses significant anti-fibrotic properties on hepatic stellate cells.

fibrogenesis with minimum side effects in some animal fibrosis models (9). Collagen1 is the main ECM protein in liver fibrosis. Activated HSC shows a myofibroblast-like feature that expresses α-smooth muscle actin (α-SMA), a cytoskeletal element in the cytoplasm. Activated HSCs express high levels of collagen and α-SMA (9, 10). Recognizing the factors that trigger the activation of HSCs and promote hepatic fibrosis is of great importance.

High fructose intake has detrimental effects on human health. Excessive hepatic fructose has pro-inflammatory effects. It can induce liver damage by generating saturated fatty acids, reactive oxygen species, endoplasmic reticulum stress, and mitochondrial injury. Increased fructose intake triggers the hepatic stress response by activating the JNK signaling pathway, which disrupts and reduces the hepatic insulin signaling pathway (11). Increased fructose intake also reduces antioxidants, increases HSP70, HSP27, and protein 62, which lead to the production of abnormal intracellular proteins, and ultimately lead to liver cell death and fibrosis (12). A fructose-rich diet disrupts metabolism by disrupting the production of adipokines, giving rise to oxidative stress, decreasing antioxidants, and developing insulin resistance. This phenomena, then prompts various metabolic diseases such as non-alcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH), which subsequently lead to liver fibrosis and inflammation (13). It has been shown that high fructose intake in rats causes overweight and fatty liver (14). In this study, we used high concentrations of fructose to activate HSCs.

Fibroblast growth factor 21 (FGF21) is related to the FGF superfamily, which has favorable metabolic effects. The liver is the primary source of FGF-21 secretion in the body. Adipose tissue and the pancreas are the other important sources. Starvation augments the expression of FGF-21in the liver, which increases hepatic gluconeogenesis and fatty acid oxidation. It has been shown that FGF21 treatment reduces body weight, decreases triglyceride levels in serum and liver, and alleviates fatty liver in obese mice (10). Therefore, FGF21 can halt the progression of NAFLD and reverse its effects through improvement in obesity and insulin resistance.

Objectives

The ability of FGF-21 to inhibit the activated HSCs and prevent hepatic fibrogenesis has not been thoroughly studied. To investigate the effects of FGF-21 against fructose-induced hepatic fibrogenesis and its possible underlying mechanism, we studied the effect of FGF21 on the expression levels of α-SMA, collagen1, and TGF-β (fibrogenic genes) in fructose-activated LX-2 cells. We also evaluated the effect of FGF-21 on the TGF-β/smad3 signaling pathway in the activated HSCs.

Materials and Methods

Materials

Cell culture grade fructose and FGF-21 were provided from Sigma-Aldrich (USA) and Abnova (Taiwan). FBS (fetal bovine serum), Dulbecco's modified Eagle's medium (DMEM), and antibiotics (Penicillin-Streptomycin) were purchased from Gibco (USA). Fructose was prepared as a sterile solution in water (30mM stock solution). FGF-21 was dissolved in PBS (1 μM) and was used in cell culture treatments.

Cell culture

The LX-2 cell line (an immortalized human hepatic stellate cell line) was gifted by Professor Scott Friedman (Mount Sinai School of Medicine, New York. NY, USA). The cells were seeded in DMEM containing 10% FBS and antibiotics (streptomycin and penicillin, 1%) in a 5% CO2 incubator at 37°C.

Treatment of LX-2 cells with different concentrations of fructose

For fructose treatment, the cells were seeded in six-well plates at a density of 2×10^5 cells/well and were grown in a 5% CO2 incubator at 37°C until 80% confluency. The cells were subject to serum starvation for 24 hours before treatments. Then the cells were treated with high fructose concentrations, including 25 and 30 mM of fructose for 48 in the same conditions. According to our previous study, the cells will be activated in these concentrations, and the expression of fibrogenic genes (TGF-β, α-SMA and collagen1) will be increased (15).

Treatment of fructose activated hepatic stellate cells with FGF-21

In the next step, to investigate the inhibitory effects of FGF-21 on activated stellate cells, fructose-activated HSCs (25 and 30 mM) were treated by FGF21 (1 μM) for 24 hours. Real-time polymerase chain reaction (real-time PCR) was employed to evaluate the expression of genes involved in liver fibrogenesis followed by western blot analysis to detect the phosphorylation level of Smad3 in cells exposed to 25 mM fructose and FGF21.

Evaluation of gene expression by real-time PCR

Total RNA extraction was carried out following the treatments using the QIAGEN RNA extraction kit (Germany). Then, complementary DNA (cDNA) was synthesized using the cDNA synthesis Kit (Yekta Tajhiz, Iran) with random hexamer and oligo-dT primers. Real-time PCR was conducted with the ABI Applied Biosystems machine with the following specific primers:

TGF-β: forward 5'-GTGGACATCAACGGGTTCACT-3',
reverse: 5'-CTCCGTGGAGCTGAAGCAATA-3';
α-SMA: forward 5'-CCGGGACTAAGACGGGAATC- 3',
reverse 5'-CCATCACCCCCTGATGTCTG-3';
Collagen1α:
forward 5'-GGAATGAAGGGACACAGAGGTT-3',
reverse 5'-AGTAGCACCATCATTTCCACGA-3';
GAPDH: forward 5'-GACAGTCAGCCGCATCTTCT-3',

reverse 5'-GCCCAATACGACCAAATCCGT-3'.

The relative expression of each target gene was determined against GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the internal control by applying the ΔΔCt method.

Western blotting

Cells were lysed in radioimmunoprecipitation assay buffer together with a protease inhibitor for total protein extraction (Sigma Aldrich, USA). Nuclear proteins were extracted by a BCA protein assay kit (Abcam, UK). SDS polyacrylamide gel electrophoresis was conducted, followed by electroblotting onto polyvinylidene difluoride membranes (Millipore, USA) to separate proteins. Membranes blocking was carried out using 5% nonfat dry milk in TBS (times with tris-buffered saline) blocking buffer for two hours and then were incubated with antibodies against collagen 1, α-SMA, TGF-β, and Smad3. After antibody incubation, the membranes were washed with TBST (Tween-20) three times for 10 minutes and were incubated with the secondary antibody diluted in TBS blocking buffer for 1 hour. Finally, band detection was performed on a ChemiDoc system using the ECL detection reagents (Sigma-Aldrich). The Image J software was applied to analyze the band densities. The phosphorylated Smad3 was measured in cells that received 25 mM fructose following 1, 2, 4, 8 and 16 hours incubation.

Statistical analysis

All experiments were performed with three replications. The data were presented as the means ± SEM of the variables. The data in the experimental groups were analyzed using ANOVA and the Tukey post hoc test using the GraphPad Prism 9 software. The significance level was considered at $P < 0.05$.

Results

FGF-21 reduces the fructose-induced fibrogenic genes expression in HSCs

Modulation of TGF-β, α-SMA, and collagen I genes expression by 1 μM FGF21 in HSCs was evaluated following their activation with 25 and 30 mM fructose. Real-time PCR results showed that the mRNA expression of TGF-β, α-SMA, and collagen I was increased as much as 3.8, 3.4, and 2.1 folds respectively in response to 30 mM FGF21 compared to the control group ($P < 0.01$). Following treatment with FGF-21 downregulated this increase to 2.5, 2.2, and 1.3 folds respectively compared to the control group ($P < 0.05$). These data showed that FGF-21 attenuated the fructose-induced fibrogenic gene expression in HSCs (Figure 1).

Effects of fructose treatment on Smad3C phosphorylation

We evaluated the phosphorylation of Smad3C protein to investigate the TGF-β/Smad3C signaling pathway in human HSCs in response to fructose. The western blotting technique was conducted at different incubation times to assess the best time point of Smad3C phosphorylation. After 8 hours of fructose treatment (final concentration of 25 μM), the highest phosphorylated Smad3C protein level was seen. Following 8 hours fructose challenge, the Smad3C phosphorylation was markedly increased in HSCs (3.1-fold change, $^{**}P<0.05$; Figure 2).

Effect of FGF21 on the fructose-induced Smad3C phosphorylation

The phosphorylation of Smad3C was measured to study the modulation of the TGF-β/Smad3C signaling pathway in HSCs in response to FGF-21 (Figure 3). We investigated whether FGF-21 can attenuate the fructose-induced TGF-β/Smad3 signaling pathway to inhibit hepatic fibrogenic gene expression in human HSCs. Western blotting data showed that 1 μM FGF21 treatment significantly suppressed the phosphorylated Smad3 level compared to the activated pathway in response to 25 mM fructose (3.5 versus 1.5 fold-changes, $^{\#}P < 0.05$). These results showed the modulation of the TGF-β/Smad3 signaling pathway by FGF21.

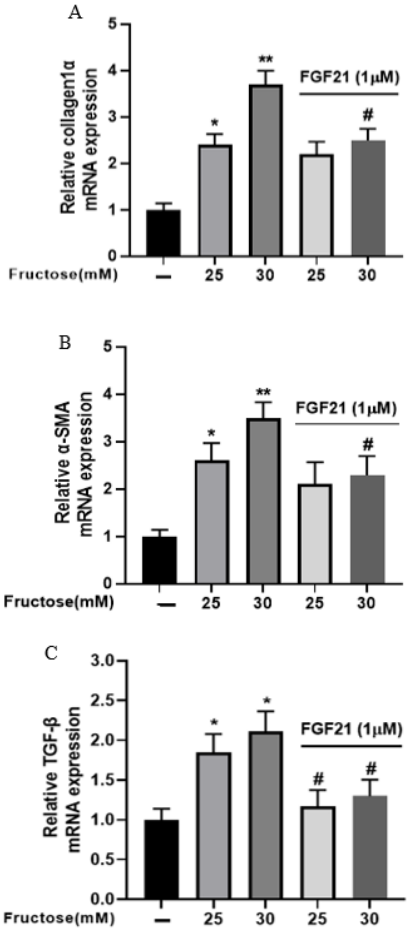

Figure 1. The effects of FGF21 on mRNA expression of TGF-β, α SMA and Collagen1genes in the activated hepatic stellate cells treated by 25- and 30-mM fructose after 48 hours of incubation * $P<0.05$ for fructose-treated groups compared to the control group and #$P < 0.05$ for FGF21 treatment vs. 30 mM fructose.

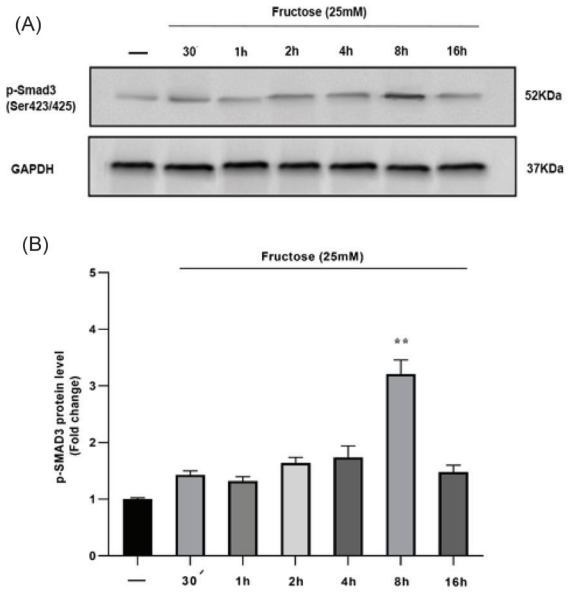

Figure 2. Western blot analysis of the phosphorylated level of Smad 3 in the human hepatic stellate cells treated with 25 mM fructose. The highest level of Smad3C protein was obtained following 8 hours of treatment (**P<0.05).

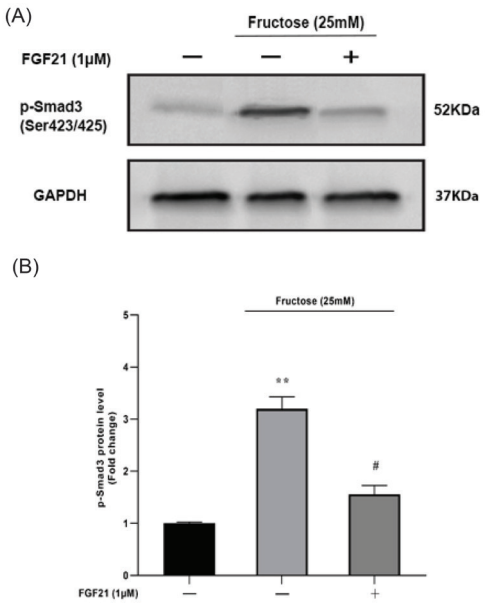

Figure 3. Western blot analysis of the phosphorylated level of Smad 3 in fructose activated human hepatic stellate cells following FGF21 treatment. **P<0.05 for fructose treatment vs. control and #P < 0.05 for FGF21 treatment vs. fructose.

Discussion

In the present study, we showed that FGF-21 modulates fibrogenic phenotype in the fructose-activated LX-2 human HSC line through down-regulation of the TGF-β/smad3 signaling pathway. The initial stage of liver fibrosis is reversible, but it may progress to liver cirrhosis and cancer if left untreated (16-18). HSCs have a central role in the initiation and progression of liver fibrosis (16). Therefore, it is necessary to recognize the agents that prevent the activation of HSCs and reduce the fibrogenic genes expression to prevent the development and progression of hepatic fibrosis. Transplantation of all or part of the liver tissue is the only solution to treat advanced liver fibrosis. This treatment is invasive and has its difficulties and complications; therefore, it is essential to find other treatments and pharmacologic interventions to interrupt the process of fibrogenesis (19, 20).

Fructose is used as a sweetener in various food industries (18) and is widely used in food and beverages, so fructose consumption is high in the community (21). In this study, fructose-activated stellate cells were treated with FGF21 to inhibit fibrogenic genes expression (α-SMA, collagen1, and TGF-β). We found a significant decrease in the mRNA expression levels of these genes in activated HSCs treated with FGF21. The level of smad3 phosphorylation was significantly reduced in the activated HSCs treated with FGF21. Thus, FGF21 was able to reduce the activation of HSCs in vitro. Our data suggested that FGF21 exerts its effect in part by disrupting the TGF-β signaling pathway and downregulating Smad3 phosphorylation. Decreased TGF-β signaling pathway and Smad3 phosphorylation, prevent further HSCs activation and reduce αSMA and collagen1 genes expression. Previous studies have shown that FGF21 has anti-diabetic and anti-fat effects, increases energy expenditure, and provides favorable metabolic effects (22). In line with our findings, it has been reported that FGF-21 injection normalized features of liver fibrosis using an animal model of liver fibrosis by inhibiting inflammatory cytokines such as IL-6, TNF-α, IL-β as well as suppressing the NF-κB (nuclear factor kappa B) and TGF-β signaling pathways (23). In a previous study investigating the genes involved in liver fibrosis induced by TGF-β in mice, the increased expression of αSMA and collagen1 genes were down-regulated by Ly2405319, an analog of FGF21 (24).

Based on these primary findings, FGF21 and its analogs can be suggested as an effective therapy in the prevention and therapy of liver fibrosis, but their clinical application warrants further in vivo trial studies. However, our study is subject to some limitations, and the results have to be seen in light of these limitations. All experiments in this study were on cultured cells and treated LX-2 cell line in vitro, and the results may not be fully applied to the whole organism. Therefore, it is recommended to evaluate these findings on living animal models or humans to confirm their usefulness in clinical practice and management of the disease. Liver fibrosis is a dynamic process that results from the cooperation of HSC with other liver cell types that warrants further examination on the effect of fructose and FGF21 on all hepatic cells interaction. However, the FGF21 level in living organisms may vary depending on the metabolic state and conditions of the metabolic organs. FGF21resistance mainly should be addressed in this context to evaluate the FGF21 effects on the whole

organism.

Conclusion

According to our results, FGF-21 effectively modulates fibrogenic phenotype in HSCs treated with high fructose concentrations through down-regulation of TGF-β/smad3 signaling pathway and fibrogenic genes. Therefore, FGF21 possesses significant anti-fibrotic potentials in liver fibrosis and can be suggested for the pharmacologic intervention of liver fibrosis and drug development in this case.

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Authors' contribution

NM, and ES were the principal investigators. HY and ES wrote the draft of the manuscript. NM and ES were included in data collection. HY and AK analyzed data. NM, and HY supervised research and reviewed the manuscript. All authors read and approved the content of the manuscript and confirmed the content and integrity of any part of the work.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical issues

The research followed the tenets of the Declaration of Helsinki. The Ethics Committee of Ahvaz Jundishapur University of Medical Sciences approved this study (Ethical code #IR.AJUMS.MEDICINE.REC.1398.041). This study was extracted from a research project was conducted at Ahvaz Jundishapur University of Medical Sciences. Additionally, the authors have entirely observed ethical issues (including plagiarism, data fabrication, and double publication).

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