Hepatocyte growth factor alleviates hepatic insulin resistance and lipid accumulation in high-fat diet-fed mice

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ABSTRACT

Aims/Introduction: Type 2 diabetes mellitus is frequently accompanied by fatty liver disease. Lipid accumulation within the liver is considered as one of the risk factors for insulin resistance. Hepatocyte growth factor (HGF) is used to treat liver dysfunction; however, the effect and mechanism of HGF on hepatic lipid metabolism are still not fully understood.

Materials and Methods: Male C57BL/6 mice were induced with a high-fat diet for 12 weeks, followed by a 4-week treatment of HGF or vehicle saline. The levels of fasting blood glucose, fasting insulin and homeostatic model assessment of insulin resistance were calculated for insulin sensitivity. Biochemical plasma parameters were also measured to assess the effect of HGF on lipid accumulation. Additionally, genes in the lipid metabolism pathway were evaluated in palmitic acid-treated HepG2 cells and high-fat diet mice.

Results: HGF treatment significantly decreased the levels of fasting blood glucose, hepatic triglyceride and cholesterol contents. Additionally, HGF-regulated expression levels of sterol regulatory element-binding protein-1c/fatty acid synthase, peroxidase proliferator-activated receptor-α, and upstream nuclear receptors, such as farnesoid X receptor and small heterodimer partner. Furthermore, c-Met inhibitor could partially reverse the effects of HGF.

Conclusions: HGF treatment can ameliorate hepatic insulin resistance and steatosis through regulation of lipid metabolism. These effects might occur through farnesoid X receptor–small heterodimer partner axis-dependent transcriptional activity.

INTRODUCTION

Type 2 diabetes mellitus is becoming an important global public health issue characterized by hyperglycemia caused by insulin resistance (IR). IR is a common pathophysiological basis in patients with several abnormalities, such as type 2 diabetes mellitus, obesity, hyperlipidemia and non-alcoholic fatty liver disease. As the liver shows an essential role in gluconeogenesis and lipogenesis, hepatic insulin resistance is vital for the development of type 2 diabetes1. Therefore, potential adjustment of glucose, as well as lipid metabolism in the liver, might be an important pathway for prevention and treatment of type 2 diabetes.

Hepatocyte growth factor (HGF) was initially known as a mitogen of hepatocytes by showing pleiotropic characteristics in a series of cells and tissues2. HGF can stimulate the regeneration of hepatocytes, which is important for liver repair during injury3. HGF shows anti-fibrotic function in the liver and anti-apoptotic or cytoprotective effects on hepatocytes in different animal models4. In recent years, HGF has been suggested to be favorable in obesity, insulin resistance and metabolic syndrome, and was defined as an important regulator in type 2 diabetes5. Several clinical studies showed that serum levels of HGF were correlated with fasting blood glucose, triglycerides, blood pressure, progression of insulin resistance and the presence of type 2 diabetes6,7. In vivo studies, β-cells-Met−/− mice showed apparent hyperglycemia and an impaired response to insulin8. Furthermore, recent investigations suggested macrophage mineralocorticoid receptor deficiency could protect against...
hepatic steatosis and insulin resistance through ERalpha/HGF/ Met\(^{8}\).

Most research so far has focused on addressing the effect of HGF in the modulation of glucose metabolism including pancreatic \(\beta\)-cells, intestinal epithelial cells and adipocytes\(^{10}\). In the liver tissue, Falafios et al.\(^{11}\) proved that HGF/Met composed a hybrid complex with an insulin receptor that resulted in a robust signaling to modulate glucose metabolism in the liver. However, few studies focus on the effect of HGF in hepatic lipid metabolism. Although recent studies have shown that hepatocyte-Met\(^{+/–}\) mice exhibited severe hyperlipidemia and steatosis along with anomalous \(\beta\)-oxidation of free fatty acids, the precise role of HGF in regulating insulin resistance by lipid metabolism is still not fully clarified\(^{12}\).

In the present study, we used a high-fat diet (HFD) mouse model to examine the effect of HGF on bodyweight, glucose intolerance, insulin sensitivity and hepatic lipid accumulation, and to explore the potential mechanisms underlying the action of HGF in regulating lipid metabolism.

**METHODS**

**Animals and experimental protocol**

Adult male C57BL/6 mice aged 4 weeks (weight 18–22 g) were acquired by Nanjing Medical University Animal Center. Animals were individually housed in polycarbonate cages under a specific pathogen-free condition. All animals were fed adaptively for 7 days before use and kept in standard conditions: 50% relative humidity, 22°C ± 2°C temperature and a 12-h light–dark cycle. The animals were fed a high-fat diet or normal chow diet for 12 weeks. The 20% kcal from carbohydrates, 60% kcal from fat and 20% kcal from protein comprises a high-fat diet, and a normal chow diet included 70% kcal from carbohydrates, 10% kcal from fat and 20% kcal from protein, both obtained from Yangzhou Animal Experiment Center (Yangzhou, China). The HFD-fed mice were randomly assigned to four groups (6 mice per group): HFD1 group, HFD2 group, the normal chow diet-fed mice group, and saline group (six mice per group). All animals were fed adaptively for 7 days before use and kept in standard conditions: 50% relative humidity, 22°C ± 2°C temperature and a 12-h light–dark cycle. The animals were fed a high-fat diet or normal chow diet for 12 weeks. The 20% kcal from carbohydrates, 60% kcal from fat and 20% kcal from protein comprises a high-fat diet, and a normal chow diet included 70% kcal from carbohydrates, 10% kcal from fat and 20% kcal from protein, both obtained from Yangzhou Animal Experiment Center (Yangzhou, China). The HFD-fed mice were randomly assigned and intraperitoneally injected with 0.5 mg/kg/day recombinant HGF (rHGF; R&D Systems, Minneapolis, MN, USA) once a day for 4 weeks (HFD2 + HGF group, \(n = 6\)) or with saline (HFD1 group, \(n = 6\), respectively). The normal chow diet-fed control mice were treated with saline (\(n = 6\)). The weight and food intake were monitored weekly throughout the study. All groups of mice were killed under sodium pentobarbital anesthesia at the end of the trial. Their serum samples were gathered and stored at −80°C. The liver tissues were obtained, weighed and frozen in liquid nitrogen, and then stored at −80°C or in 10% buffered neutral formalin for histological tests. All animal experimental processes were based on Jiangsu Provincial Experimental Animal Management Committee under Contract 2011-0069.

**Real-time quantitative polymerase chain reaction analysis**

Total ribonucleic acid (RNA) was isolated from cells and tissues using the Trizol reagent (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA), and 1 μg of total RNA synthesized complementary deoxyribonucleic acid using Moloney murine leukemia virus reverse transcriptase (New England Biolabs Inc., Beijing, China). All polymerase chain reaction (PCR) primers (listed in Table S1) were designed with the PRIMER3 software (http://frodo.wi.mit.edu/) using published sequence data obtained from the National Center for Biotechnology Information database. The PCR products were analyzed by electrophoresis on 1.5% agarose gel. Quantitative real-time PCR analyses were carried out using the SYBR Premix (DRR041A; Takara, Osaka, Japan) and analyzed with ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s specifications. The PCR program was 3 min at 95°C for enzyme activation and denaturation for 15 s at 95°C, annealing 30 s at 58°C and extension 30 s at 72°C for 40 cycles. The endogenous control was 18s ribosomal RNA for each sample. All samples were analyzed in triplicates.

**Biochemical and histopathological analysis**

Serum concentrations of transaminase, cholesterol and triglyceride were measured by clinical automatic analyzer (Hitachi 7170; Hitachi Ltd., Tokyo, Japan). Lipid content of the liver homogenate was determined using a specific kit according to the manufacturer’s instructions (Jiancheng Bioengineering Institute, Nanjing, China)\(^{13}\). For histological evaluation, hepatic tissues were fixed in 10% neutralized formaldehyde, embedded in paraffin and stained using the Oil Red O method.

**Cell culture**

HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO\(_2\) incubator, which were obtained from the American Type Culture Collection (Manassas, VA, USA). When reaching 65% confluence, cells were incubated in serum-free medium for 12 h and stimulated using 300 μmol/L palmitic acid (PA) for 24 h. For HGF stimulation, 40 ng/mL HGF was used at different time-points in PA-treated cells. Where indicated, the c-MET inhibitor, SU11274 (10 μmol/L; Selleck Chemicals Inc., Houston, TX, USA), was pretreated with the cells for 3 h before incubation with HGF. The appropriate vehicle was considered as the control.

**Western blot analysis**

Hepatic tissues from individual mice were lysed in radio immune precipitation buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L MgCl\(_2\), 2 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L NaF, 1% NP40 and 0.1% sodium dodecyl sulfate). Western blotting was measured as previously described\(^{13}\) and was evaluated with antibodies against farnesoid X receptor (FXR; FXR: 1:3000, ab126602; Abcam Inc., Cambridge, MA, USA), short heterodimer partner (SHP; 1:1000, sc-30169; Santa Cruz Biotech, Santa Cruz, CA, USA), fatty acid synthase (FAS; 1:1000, 610962; BD Inc., San Jose, CA, USA), steroid regulatory element-binding factor-1c (SREBP-1c; 1:1000, sc-8984;...
Santa Cruz Biotech), peroxidase proliferator-activated receptor-α (PPARα; 1:1000, ab89394; Abcam Inc.), Phospho-Met (Tyr1234/1235; #3126, Cell Signaling Technology Inc.), Met (C-28; 1:1000, sc-161; Santa Cruz Biotech) and GAPDH (Cell Signaling Technology Inc.). Protein expression levels were quantitated using the Image J software (https://imagej.nih.gov/ij/).

Enzyme-linked immunosorbent assay
For preparing plasma or serum samples, blood samples of individual mice were centrifuged at 1,500 g for 15 min. The concentrations of serum insulin and free fatty acid (FFA) were measured using the mouse insulin and FFA enzyme-linked immunosorbent assay kit according to the manufacturers’ instructions (Anda Biological Technology Co., LTD, Beijing, China). Similarly, HGF serum concentrations in individual mice were carried out using the mouse HGF enzyme-linked immunosorbent assay kit (R&D Systems) in duplicate following the manufacturer’s instructions.

Intraperitoneal glucose tolerance test and insulin tolerance test
After treatment with HGF or vehicle for 4 weeks, the intraperitoneal glucose tolerance test and the insulin tolerance test were carried out. The mice were fasted for 8 h and injected intraperitoneally with glucose (1 g/kg bodyweight) or regular human insulin (0.5 U/kg bodyweight). The levels of tail venous blood glucose in individual mice were measured at 0, 15, 30, 60 and 120 min after glucose/insulin injection. The plasma glucose meter (Johnson & Johnson, New Brunswick, NJ, USA).

Statistical analysis
All the data are presented as the mean ± standard error of the mean. Statistical significance was analyzed by one-way ANOVA with least significant difference post-hoc test for multiple comparisons. GraphPad Prism version 5.03 (GraphPad, San Diego, CA, USA) was used for plotting and statistical analyses. P-values <0.05 were considered statistically significant.

RESULTS
Effect of HGF on food intake, bodyweight, liver weight and liver index
First, bodyweight was measured every week in the normal chow diet-fed control mice group, HFD1 group and HFD2 + HGF group. After 2 weeks, bodyweight in the HFD2 + HGF mice was significantly lower than that in the HFD1 mice (Figure 1a). There were no differences in food intake among mice at any time (Figure 1b). Subsequently, we measured the serum HGF concentrations after 4 weeks’ treatment with rHGF in HFD-fed mice. The result showed the levels of HGF were significantly elevated compared with the untreated group (Figure 1c), suggesting that rHGF has biological activity in vivo. After 4-week treatment, liver weight in the HFD2 + HGF group was significantly less than that in the HFD1 group, and liver index (liver index = [liver weight / bodyweight] × 100) decreased in the HFD2 + HGF group compared with the HFD1 group, but there was no statistically significant difference. (Figure 1d,e).

Effect of HGF on glucose metabolism and insulin sensitivity
After 4-week treatment, the levels of fasting blood glucose and fasting serum insulin in the HFD2 + HGF group were significantly lower compared with the HFD1 group, although they were higher compared with the normal chow diet-fed control mice group (Figure 2a,b). Furthermore, HOMA-IR, an index of insulin resistance, gave lower scores for the HFD2 + HGF group than for the HFD1 group (7.0 ± 0.81 vs. 12.17 ± 1.67), showing that HGF treatment improved insulin sensitivity (Figure 2c). We next carried out an intraperitoneal glucose tolerance test as well as an insulin tolerance test. After glucose or insulin delivery, the HFD2 + HGF group had obviously lower levels of plasma glucose than control HFD1 mice (Figure 2d,e). Together, these results showed that treatment with HGF could normalize glucose and insulin plasma levels, and improve HFD-induced insulin resistance.

Effect of HGF on hepatic lipid accumulation
We then explored the effect of HGF on hepatic steatosis. Examples of the liver appearance are as shown in Figure 2f. In order to confirm the severity of the liver steatosis, we carried out Oil Red O staining, which is a measure of hepatic lipid content. Histopathological analysis of Oil Red O stained liver sections showed marked microvesicular steatosis in the HFD1 group. A notably decreased accumulation of lipid droplets in the liver could be observed in the HFD2 + HGF group. In agreement with the histological findings, the levels of hepatic triglyceride (TG) and total cholesterol contents were substantially reduced in the HFD2 + HGF group (Figure 2g). Obviously, HGF could markedly reduce hepatic lipid accumulation and alleviate the hepatic steatosis.

Effect of HGF on biochemical plasma parameters
The results of biochemical plasma parameters are shown in Figure 3. Analyses of serum transaminases activity showed lower levels of serum aspartate aminotransferase and alanine aminotransferase in the HFD2 + HGF group when compared with the HFD1 group. To determine whether HGF could influence serum lipid concentration, we next analyzed serum lipid concentrations. Levels of serum TG, total cholesterol, low-density lipoprotein and FFA in the HFD2 + HGF group were decreased compared with the HFD1 group. In addition, the HFD2 + HGF group also showed an increased level of HDL compared with the HFD1 group (Figure 3h). Thus, treatment with HGF could effectively reduce HFD-induced hypertriglyceride and improve the liver function.
Effect of HGF on lipogenic genes in the liver

To elucidate the detailed mechanism of hepatic lipid reduction by HGF, we first analyzed critical hepatic genes involved in lipid metabolism. Quantitative PCR showed that SREBP-1c and its downstream target gene, Fas, were decreased in the livers of the HFD2 + HGF group, and western blotting analysis also suggested protein levels of Fas were significantly inhibited in the HFD2 + HGF group (Figure 4a,b). There was no statistically significant difference in hepatic gene expression of SREBP-1c among the groups. Furthermore, we examined the effect of HGF on PPARs, which are transcription factors that modulate the expression of genes that participate in lipid metabolism. The result showed no statistically significant difference in hepatic gene expression of PPARα among the groups. However, messenger RNA and protein expression levels of PPARα were significantly higher in the livers from the HFD2 + HGF group of mice than from the HFD1 group of mice (Figure 4a,b). These results suggested that HGF attenuated hepatic lipid accumulation by affecting the expression levels of Fas and PPARα.

Subsequently, we analyzed the upstream nuclear receptors of SREBP-1c and PPARα. FXR, as a member of the nuclear receptor superfamily, has been found to modify several genes involved in triglyceride synthesis and lipid metabolism; for example, FXR-short heterodimer partner (SHP) that drives negative regulation of SREBP-1c expression14. Other mechanisms suggest that FXR might mediate hypertriglyceridemia by activating the expression of PPARα15. The present results also provided appropriate evidence of FXR directly influencing signaling molecules FAS, SREBP-1C and PPARα (Figure S1). As shown in Figure 4, the gene expression levels of FXR and SHP were significantly higher in the livers of the HFD2 + HGF mice than the HFD1 mice. In a similar way, western blotting showed that the levels of FXR and SHP protein in the livers were also increased (Figure 4).

Effect of HGF on lipogenic genes in PA-induced HepG2 cells

In order to further verify the role of HGF in hepatic lipid metabolism, human HepG2 cells were induced with PA as an insulin resistance model. We found that 40 ng/mL HGF could
significantly activate Phospho-Met in 12 h, and elevated levels of FXR and SHP were observed after a 6 h stimulation (Figure 5a). As shown in Figure 5b, protein levels of FXR and SHP were increased significantly by HGF treatment after 12 h in PA-induced HepG2 cells, and the opposite change was observed in SREBP-1c and Fas. However, there was no difference in levels of PPARα. Additionally, we used SU11274 (a specific inhibitor of c-Met) to block the HGF–c-Met axis, and found that SU11274 partially reversed the effect of HGF. The result showed that HGF might activate c-Met ligand to alleviate hepatic lipid accumulation. On the whole, these results indicated that HGF inhibited TG synthesis, at least to some extent, through the FXR–SHP axis-dependent transcriptional activity.

**DISCUSSION**

Recent basic and clinical studies have addressed HGF as a therapeutic option for the development and progression of insulin resistance. In our experiment, HFD-fed mice showed hyperglycemia, hypertriglyceridemia, hypercholesterolemia, hyperinsulinemia and insulin resistance with liver accumulation of fat. We found that HGF treatment significantly decreased the body weight and ameliorated glucose intolerance in the HFD-fed mice. Furthermore, HGF reduced liver weight and fat content.
in both serum and liver with attenuation of hepatic steatosis in HFD-fed mice. In addition, the latest study also suggested that HGF prevented HFD-induced obesity and improved insulin resistance in mice. The main experimental results were similar to our conclusion; however, our mechanism showed its effect through FXR–SHP-dependent regulated SREBP-1c/FAS and PPARα in the livers. These data showed that HGF treatment prevented hyperglycemia, and hyperlipidemia might be mediated by inhibiting lipogenesis in the livers of HFD-fed mice. Of note, HGF exerts a pleiotropic action on metabolic disorder. HGF increases glucose uptake in skeletal muscle, and skeletal muscle-specific HGF overexpression improved whole-body glucose tolerance independently of changes in bodyweight. Therefore, we speculated that HGF-decreased hepatic lipogenesis and improved glucose metabolism were mainly due to a direct effect on the insulin target organ, such as the liver, skeletal muscle and adipose tissue.

Previous research found that HGF could increase glucose transporters in pancreatic β-cells, adipocytes and intestinal epithelia. Furthermore, HGF directly facilitates glucose uptake in mouse skeletal muscle, and upregulates glucose transporters 1 and 4 (Glut-1 and Glut-4) levels in L6 myotubes. A
recent article by Fafalios et al.\textsuperscript{11} has insights into the correlation between HGF and glycometabolism. They showed that Met and insulin receptor combined to create a hybrid complex by recruiting and activating insulin receptor in hepatocytes. Furthermore, they found that HGF/Met participated in hepatic glucose, and the importance of Met in glucose homoeostasis was confirmed in vivo. Our experimental results also proved a similar conclusion, and we further observed the chronic effect of HGF in regulating glucose homoeostasis, which seems to be closer to the physiological mechanism.

However, the regulatory role of HGF–c-Met in lipid metabolism is complex and unclear. In our experiment, HGF significantly decreased plasma TG, total cholesterol, low-density lipoprotein and FFA levels in HFD C57BL/6 mice. HGF could also reduce liver enzymes, such as alanine aminotransferase and aspartate aminotransferase, and improved liver function. Hepatic triglyceride and cholesterol contents were significantly reduced by treatment with HGF, as well as the lipogenic-related genes, including SREBP-1c/FAS and PPAR\textsubscript{\textalpha}. In previous in vivo studies, HGF treatment resulted in recovery of fatty liver by increasing the apolipoprotein B levels and microsomal triglyceride transfer protein expression\textsuperscript{19}. One study suggested that HGF induced expression of the low-density lipoprotein receptor in HepG2 cells\textsuperscript{20}. Based on these results, it is probable that HGF exerts a positive effect on lipid metabolism.

Cumulative evidence has shown that HGF can significantly lower lipids levels; nevertheless, the precise mechanism of HGF on lipogenesis is still not fully clarified. A recent study suggested HGF treatment downregulated SREBP-1c and SCD-2 expression in primary hepatocytes, showing well-defined effects of HGF in lipogenic genes\textsuperscript{21}. In the present study, we also found that HGF could significantly decrease SREBP-1c and FAS expression levels, and increase expression of PPAR\textsubscript{\textalpha} in HFD-fed mice. SREBP-1c, a member of the SREBP family of transcription factors, is involved in the transcriptional effects of insulin and glucose\textsuperscript{22}. Abundant studies show that the inhibition of SREBP-1c and its usual target genes, such as FAS, could suppress the expression of lipogenic enzymes, decrease the accumulation of triglycerides and finally overturn hepatic steatosis in mice\textsuperscript{23,24}. PPAR\textsubscript{\textalpha} is a class II nuclear receptor that

![Figure 4](http://onlinelibrary.wiley.com/journal/jdi)

**Figure 4** | Effect of hepatocyte growth factor (HGF) on hepatic gene expression and relative protein levels involved in the lipid metabolism. The relative expression levels of and sterol regulatory element-binding factor-1c (SREBP-1c)/fatty acid synthase (FAS), peroxidase proliferator-activated receptor-\textalpha (PPAR\textsubscript{\textalpha}), farnesoid X receptor (FXR) and short heterodimer partner (SHP) in the livers of individual mice were determined by quantitative real-time reverse transcription polymerase chain reaction and western blot assays. The results represent the mean ± standard error of the mean of six mice. *P < 0.05 versus the normal chow diet-fed control mice (NC) group, \#P < 0.05 versus the high-fat diet injected with saline (HFD1) group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HFD2 + HGF, mice fed a high-fat diet for 12 weeks and treated with hepatocyte growth factor; mRNA, messenger ribonucleic acid.
Figure 5 | Effect of hepatocyte growth factor (HGF) on lipogenic genes in PA-induced HepG2 cells. (a) Palmitic acid (PA)-induced HepG2 cells were incubated with or without HGF for 3, 6, 12 and 24 h. (b,c) PA pretreated HepG2 cells and control cells were seeded in six-well plates; then, cells were treated with HGF for 12 h alone or with c-Met inhibitor SU11274. Total protein lysates were analyzed by immunoblotting. Membranes were blotted with farnesoid X receptor (FXR), short heterodimer partner (SHP), p-Met, c-Met fatty acid synthase (FAS), sterol regulatory element-binding factor-1c (SREBP-1c), proliferator-activated receptor-α (PPARα), β-actin and antibodies. Data represent means ± standard error of the mean, n = 4. *P < 0.05 versus the 0 h group.
drives transcription to match a series of lipid ligands. PPAR activation also facilitates fatty acid oxidation in the skeletal muscle.

To determine the upstream nuclear receptors of SREBP-1c and PPAR, we investigated FXR and SHP transcription in HGF-treated HFD mice and PA-induced HepG2 cells. As shown in Figures 4 and 5, the expression levels of FXR and SHP were significantly higher in the liver from the HGF treatment group than the vehicle saline group. FXR could activate several genes that regulate levels of TG, including the atypical nuclear receptor SHP, PPAR and apolipoprotein C-II. A recent study showed that activated FXR, by bile acids or FXR agonists GW4064, could increase SHP expression levels of FXR and HGF-treated HFD mice and PA-induced HepG2 cells. As reviewed present results combined with previous studies suggested that HGF can regulate FXR–a key role in insulin resistance–associated compensatory mechanisms.

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DISCLOSURE

The authors declare no conflict of interest.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1** | Primer sequences for real-time polymerase chain reaction analysis.

**Figure S1** | Farnesoid X receptor directly modulate genes involved in triglyceride synthesis and lipid metabolism.