

Liraglutide reduces fatty degeneration in hepatic cells via the AMPK/SREBP1 pathway

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Abstract. Recent studies have suggested that liraglutide could have a potential function in improving non-alcoholic fatty liver disease (NAFLD); however, the underlying molecular mechanism remains unclear. The aim of the present study was to investigate the role of the AMP-activated protein kinase (AMPK)/sterol regulatory element binding protein 1 (SREBP1) pathway in mediating the effect of liraglutide in reducing fatty degeneration in an *in vitro* NAFLD model. To resemble the NAFLD condition *in vitro*, L-02 cells were treated with 0.5 mM free fatty acids (FFAs) for 24 h. Liraglutide could affect the expression of AMPK α 1, phosphorylated AMPK α 1 and SREBP1 in a dose-dependent manner in FFA-exposed L-02 cells, as demonstrated by western blot analysis. The intracellular lipid accumulation was significantly decreased, as shown by oil red O staining. A significant decrease in the content of triglyceride and total cholesterol was observed when the FFA-exposed L-02 cells were incubated with liraglutide. In addition, the increased expression of liver-type fatty acid-binding protein in FFA-exposed L-02 cells was suppressed by liraglutide. These effects were reversed by compound C, an AMPK inhibitor. In conclusion, this study has demonstrated that liraglutide can reduce fatty degeneration induced by FFAs in hepatocytes, and this effect may be partially mediated by the AMPK/SREBP1 pathway.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a clinical syndrome characterized by the accumulation of excess fat in the liver of individuals that drink little or no alcohol. The prevalence of NAFLD is 15-30% in Western populations (1-4) and 6-25%

in Asian populations (5). NAFLD is associated with metabolic syndrome, obesity, diabetes and cardiovascular disease (6). Currently, no standard for the treatment of NAFLD exists, and treating risk factors remains the focus of managing NAFLD (7).

Liraglutide is a potent, long-acting synthetic analogue of the human glucagon-like peptide 1 (GLP-1) molecule. It shares 97% sequence identity with human GLP-1. Liraglutide has previously been used for the treatment of diabetes (8); however, a recent study reported that liraglutide could improve oxidative stress and lipid peroxidation and decrease transforming growth factor- β 1 and tumor necrosis factor- α expression in rats with NAFLD (9). In a study by Olaywi *et al* (10), it was revealed that liraglutide induced improvements in transaminases, as well as histology, in patients with non-alcoholic steatohepatitis. These studies have suggested the potential function of liraglutide in improving NAFLD.

The AMP-activated protein kinase (AMPK) signaling pathway plays a key role in regulating hepatic lipid metabolism. It has been revealed that AMPK coordinates the long-term adaptation of lipid metabolism by downregulating the transcriptional factor sterol regulatory element binding protein 1 (SREBP1) (11,12).

To the best of our knowledge, studies on the molecular mechanisms underlying the liraglutide-induced improvement in NAFLD are limited (13). Whether liraglutide reduces fatty degeneration in hepatic cells via the AMPK/SREBP1 pathway remains unclear; therefore, a well-described model of *in vitro* NAFLD was established in the present study using a normal human hepatocyte-derived cell line, with the aim of investigating the role of the AMPK/SREBP1 pathway in mediating the liraglutide-induced reduction in fatty degeneration.

Materials and methods

Cell culture. The L-02 human normal liver cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco-BRL) at 37°C with 5% CO₂. Oleic acid (OA) and palmitic acid (PA) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and added to the culture medium at the concentration of 0.5 mM in a 2:1 molar ratio. Liraglutide was provided by Novo Nordisk (Copenhagen, Denmark) and diluted to concentrations of

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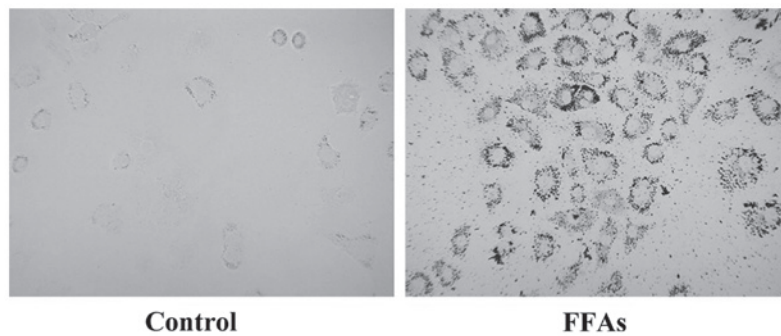


Figure 1. Oil red O staining of L-02 cells following FFA treatment (magnification, x400). FFA, free fatty acid.

0.1, 0.5, 1.0 and 2.0 nM. Compound C was obtained from Calbiochem (San Diego, CA, USA) and used at a concentration of 20 μ M. Cells were incubated with free fatty acids (FFAs), liraglutide or compound C alone or in combination.

Oil red O staining. The cells were fixed with 10% formaldehyde for 10 min and then washed with phosphate-buffered saline (PBS). Oil red O solution (60% oil red O dye and 40% water) (Sigma-Aldrich) was filtered and added to stain the cells at room temperature for 15 min. PBS was then used to remove the unbound dye, and the cells were observed under the microscope (TE200; Nikon Corp., Tokyo, Japan).

Measurement of triglyceride (TG) and total cholesterol (TC). The TG quantification and cholesterol assay kits were purchased from Abcam (Cambridge, MA, USA). The levels of TG and TC were determined according to the manufacturer's instructions. Briefly, a standard curve was prepared, and the cells were lysed. For the measurement of TG, 2 μ l lipase was added to each well and incubated at room temperature for 20 min. A total of 50 μ l reaction mix, including 46 μ l TG assay buffer, 2 μ l TG probe and 2 μ l TG enzyme mix, was then added and incubated at room temperature for 30-60 min. For the measurement of TC, 50 μ l reaction mix, containing 44 μ l cholesterol assay buffer, 2 μ l cholesterol probe, 2 μ l enzyme mix and 2 μ l cholesterol esterase, was added to each well and incubated at 37°C for 60 min. The optical density at 570 nm was measured using a microtiter plate reader (ELx800NB; BioTek Instruments, Inc., Winooski, VT, USA).

Western blot analysis. The cells were lysed in radioimmunoprecipitation assay buffer (Sangon Biotech, Shanghai, China), and protein concentrations were quantified via the bicinchoninic acid (BCA) assay method using a BCA Protein Assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Proteins were separated using 12% sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated in Tris-buffered saline containing 5% non-fat milk at 37°C for 2 h. The membranes were then incubated at 37°C for 1 h with the following primary antibodies: Rabbit monoclonal to AMPK α 1 (1:1000; ab32047), rabbit polyclonal to AMPK α 1 (phospho S487) (1:1,000; ab131357), rabbit monoclonal to AMPK α 1 (phospho S496) (1:1,000; ab92701), rabbit polyclonal to SREBP1 (1:500; ab193318), rabbit polyclonal to SREBP1 (phospho S372) (1:500; ab140483), rabbit

polyclonal to SREBP1 (phospho S439) (1:500; ab138663), rabbit monoclonal to liver-type fatty acid-binding protein (L-FABP) (1:1,000; ab129203) (all Abcam) and mouse monoclonal to β -actin (1:1,000; sc-47778; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After washing with PBS, the membranes were incubated with the secondary antibodies [goat anti-rabbit IgG/horseradish peroxidase (HRP) and goat anti-mouse IgG/HRP; Santa Cruz Biotechnology, Inc.] at 37°C for 1 h. The signals were detected using enhanced chemiluminescence (ECL) (ECL western blotting kit; Pierce, Rockford, IL, USA).

Statistical analysis. Data were analyzed using SPSS software, version 19.0 (IBM SPSS, Armonk, NY, USA). Data are expressed as the mean \pm standard deviation. The differences between two groups were analyzed using the Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

FFA treatment induces lipid accumulation in L-02 cells. L-02 cells were treated with 0.5 mM FFAs for 24 h, and oil red O staining was then performed. As shown in Fig. 1, no lipid droplets were detected in the L-02 cells in the control group; however, a large quantity of red lipid droplets was observed in the L-02 cells in the FFA group. In addition, the intracellular levels of TG and TC were significantly increased by treatment with FFAs ($P < 0.01$; Fig. 2).

FFA treatment induces L-FABP expression in L-02 cells. L-02 cells were treated with 0.5 mM FFAs for 24 h, and the expression of L-FABP in the L-02 cells was then analyzed using western blot analysis. It was found that the L-FABP expression was significantly different between the control and the FFA group. The protein expression of L-FABP was significantly upregulated following FFA treatment ($P < 0.01$; Fig. 3).

FFA treatment affects the AMPK/SREBP1 pathway in L-02 cells. As shown in Fig. 4, incubation of the L-02 cells with FFAs for 24 h resulted in the decreased expression of AMPK α 1 and a reduction in the phosphorylation of AMPK α 1 ($P < 0.01$). The expression of SREBP1 was increased significantly by treatment with FFAs ($P < 0.01$).

Liraglutide activates the AMPK/SREBP1 pathway in FFA-exposed L-02 cells. To determine the effect of liraglutide

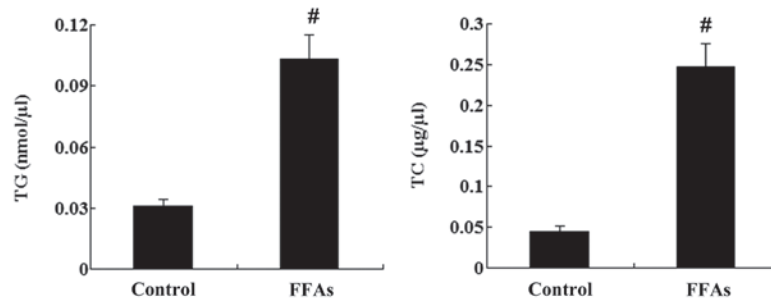


Figure 2. Intracellular levels of TG and TC in L-02 cells following FFA treatment. [#]P<0.01 vs. control group. TG, triglyceride; TC, total cholesterol; FFA, free fatty acid.

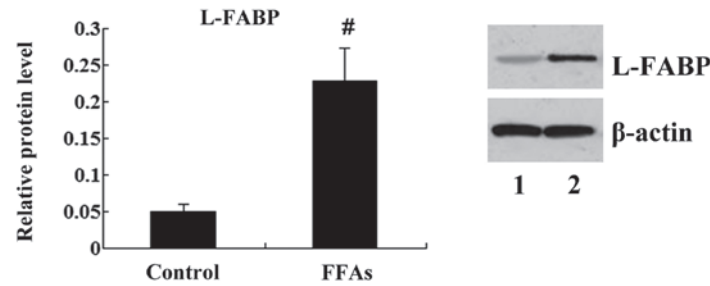


Figure 3. Expression of L-FABP protein in L-02 cells following FFA treatment. β -actin expression was used as an internal control. Lane 1, control group; lane 2, FFA group. [#]P<0.01 vs. control group. L-FABP, liver-type fatty acid-binding protein; FFA, free fatty acid.

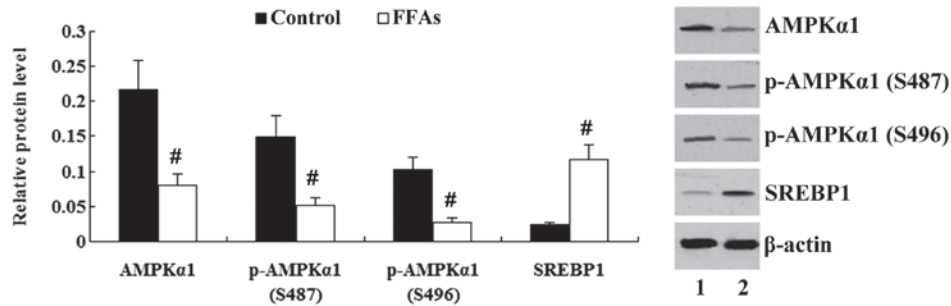


Figure 4. Expression of AMPK α 1, p-AMPK α 1 and SREBP1 proteins in L-02 cells following FFA treatment. β -actin expression was used as an internal control. Lane 1, control group; lane 2, FFA group. [#]P<0.01 vs. control group. p-AMPK α 1, phosphorylated AMP-activated protein kinase α 1; SREBP1, sterol regulatory element binding protein 1; FFA, free fatty acid.

on the AMPK/SREBP1 pathway, FFA-exposed L-02 cells were incubated with liraglutide at different concentrations (0.1, 0.5, 1.0 and 2.0 nM). The expression of AMPK α 1 and SREBP1 was determined using western blot analysis. As shown in Fig. 5, the expression of AMPK α 1 and phosphorylated (p-)AMPK α 1 (S496) was significantly increased in the FFA-exposed L-02 cells following treatment with liraglutide (0.5-2.0 nM) (P<0.05). Liraglutide at the concentrations of 1.0 and 2.0 nM significantly upregulated p-AMPK α 1 (S487) expression (P<0.01); however, the expression of SREBP1 in FFA-exposed L-02 cells was significantly decreased by treatment with 1.0 and 2.0 nM liraglutide (P<0.01) (Fig. 5).

Liraglutide reduces lipid accumulation and L-FABP expression in FFA-exposed L-02 cells. Next, the effect of liraglutide on lipid accumulation and L-FABP expression in FFA-exposed L-02 cells was investigated. FFA-exposed L-02 cells were

incubated with liraglutide (1.0 nM), and oil red O staining was then performed. As shown in Fig. 6, liraglutide decreased the number of lipid droplets in the FFA-exposed L-02 cells. The intracellular levels of TG and TC were also decreased significantly in the FFA-exposed L-02 cells following treatment with liraglutide (1.0 nM) (P<0.01; Fig. 7). Western blotting results demonstrated that the incubation of FFA-exposed L-02 cells with liraglutide resulted in a decreased expression of L-FABP protein (P<0.05; Fig. 8).

AMPK/SREBP1 pathway is involved in the effect of liraglutide on lipid accumulation and L-FABP expression in FFA-exposed L-02 cells. To investigate whether the AMPK/SREBP1 pathway was involved in the effect of liraglutide on lipid accumulation and L-FABP expression in the FFA-exposed L-02 cells, compound C, an AMPK inhibitor was added to treat the cells. As indicated in Fig. 9, AMPK α 1

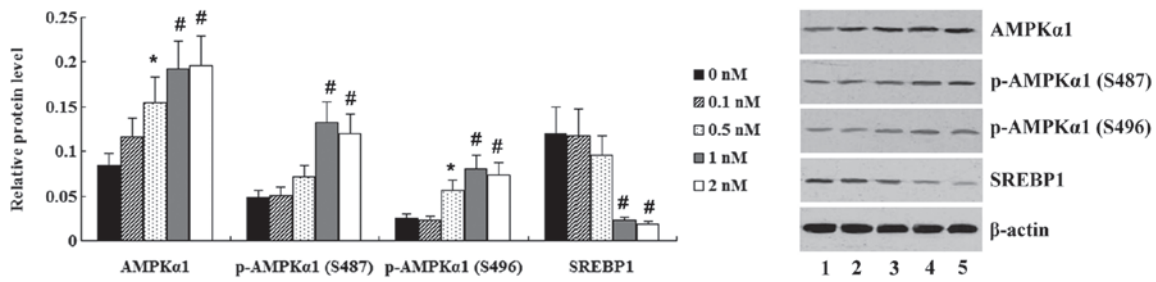


Figure 5. Expression of AMPKα1, p-AMPKα1 and SREBP1 proteins in FFA-exposed L-02 cells following treatment with different concentrations of liraglutide. β-actin expression was used as an internal control. Lanes 1-5 represent cells that were treated with 0, 0.1, 0.5, 1.0 and 2.0 nM liraglutide, respectively. *P<0.05 and #P<0.01 vs. 0 nM group. p-AMPKα1, phosphorylated AMP-activated protein kinase α1; SREBP1, sterol regulatory element binding protein 1; FFA, free fatty acid.

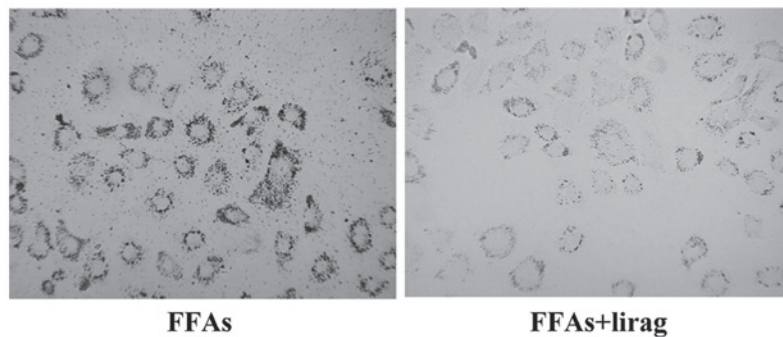


Figure 6. Oil red O staining of FFA-exposed L-02 cells following treatment with liraglutide (magnification, x400). FFA, free fatty acid; lirag, liraglutide.

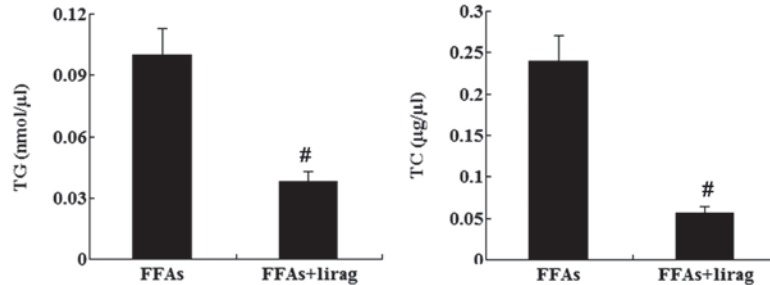


Figure 7. Intracellular levels of TG and TC in FFA-exposed L-02 cells following treatment with liraglutide. #P<0.01 vs. FFA group. TG, triglyceride; TC, total cholesterol; FFA, free fatty acid; lirag, liraglutide.

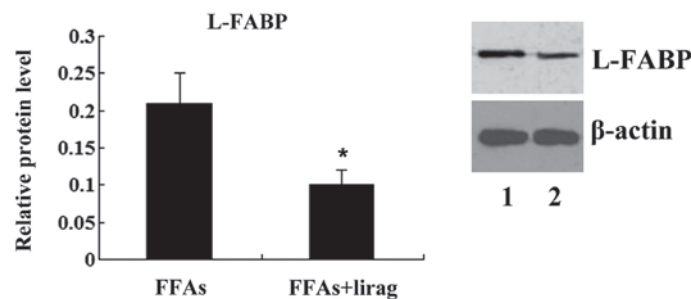


Figure 8. Expression of L-FABP protein in FFA-exposed L-02 cells following treatment with liraglutide. β-actin expression was used as an internal control. Lane 1, FFA group; lane 2, FFAs + lirag group. *P<0.05 vs. FFA group. L-FABP, liver-type fatty acid-binding protein; FFA, free fatty acid; lirag, liraglutide.

and p-AMPKα1 protein levels were significantly decreased (P<0.05), while SREBP1 and p-SREBP1 protein levels were significantly increased (P<0.01), in the FFA-exposed L-02

cells in the liraglutide + compound C group compared with those in the liraglutide group. Oil red O staining showed that liraglutide reduced the number of lipid droplets in the

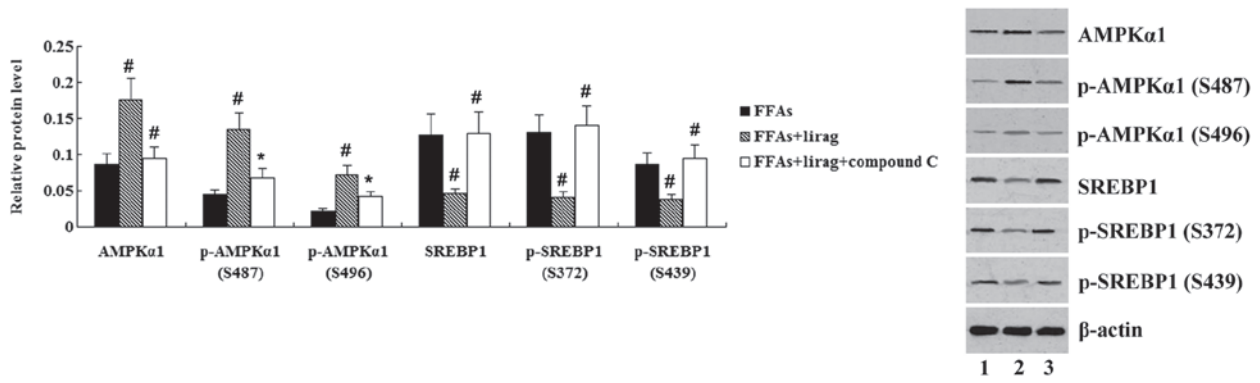


Figure 9. Expression of AMPKα1, p-AMPKα1, SREBP1 and p-SREBP1 proteins in FFA-exposed L-02 cells following treatment with liraglutide and compound C. β-actin expression was used as an internal control. Lane 1, FFA group; lane 2, FFAs + lirag group; lane 3, FFAs + lirag + compound C group. ^{*}P<0.05 and [#]P<0.01 vs. FFA group. p-AMPKα1, phosphorylated AMP-activated protein kinase α1; SREBP1, sterol regulatory element binding protein 1; FFA, free fatty acid; lirag, liraglutide.

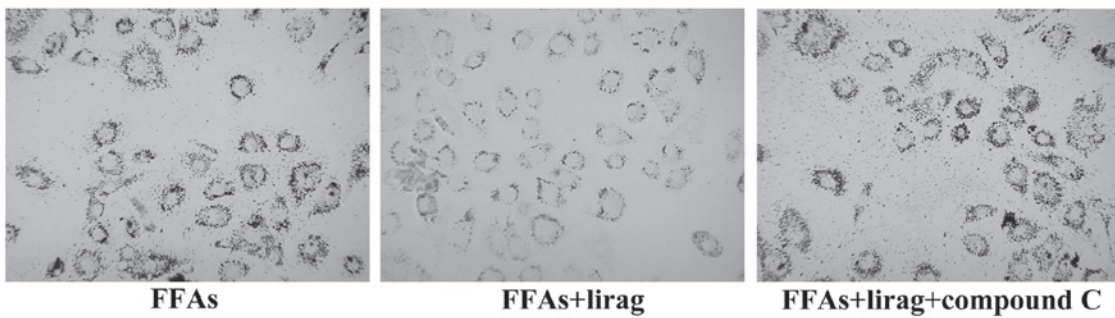


Figure 10. Oil red O staining of FFA-exposed L-02 cells following treatment with liraglutide and compound C (magnification, x400). FFA, free fatty acid; lirag, liraglutide.

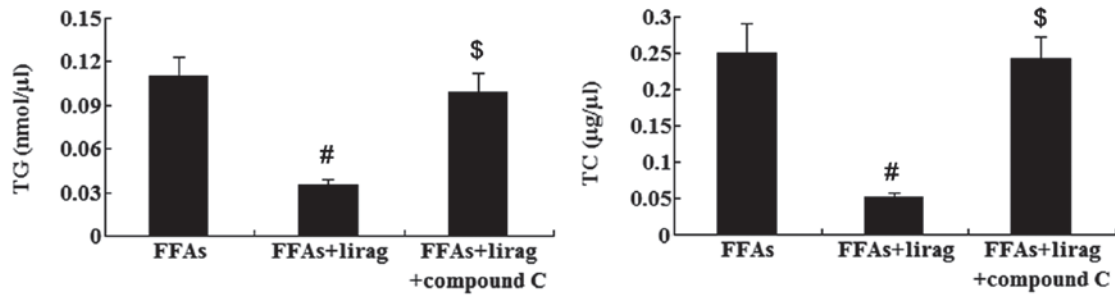


Figure 11. Intracellular levels of TG and TC in FFA-exposed L-02 cells following treatment with liraglutide and compound C. [#]P<0.01 vs. FFA group; ^{\$}P<0.01 vs. FFAs + lirag group. TG, triglyceride; TC, total cholesterol; FFA, free fatty acid; lirag, liraglutide.

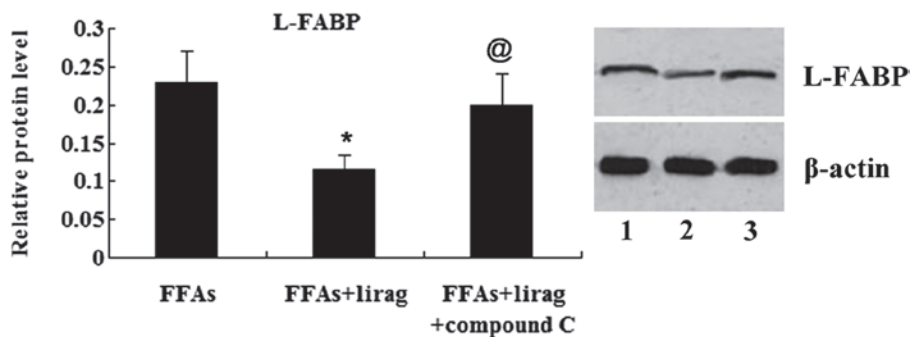


Figure 12. Expression of L-FABP protein in FFA-exposed L-02 cells following treatment with liraglutide and compound C. β-actin expression was used as an internal control. Lane 1, FFA group; lane 2, FFAs + lirag group; lane 3, FFAs + lirag + compound C group. ^{*}P<0.05 vs. FFA group; [@]P<0.05 vs. FFAs + lirag group. L-FABP, liver-type fatty acid-binding protein; FFA, free fatty acid; lirag, liraglutide.

FFA-exposed L-02 cells; however, this effect was attenuated by treatment with 20 μ M compound C (Fig. 10). Furthermore, it was found that, compared with the liraglutide group, treatment with 20 μ M compound C induced elevated levels of TG and TC in the FFA-exposed L-02 cells ($P < 0.01$) (Fig. 11). The results from the western blot analysis revealed that the expression of L-FABP was suppressed by liraglutide; however, this effect was abolished by the treatment of the FFA-exposed L-02 cells with compound C (Fig. 12).

Discussion

To simulate the NAFLD condition *in vitro*, L-02 normal human hepatocyte-derived cells were treated with 0.5 mM FFAs (PA and OA in a 1:2 molar ratio) for 24 h. The effect of liraglutide on the development of NAFLD was then examined *in vitro*.

The results from liraglutide clinical trials have demonstrated the effect of liraglutide on reductions in TG, low-density lipoprotein cholesterol and FFAs (14-19). Consistent with the previous *in vivo* studies, oil red O staining in the present study demonstrated that liraglutide attenuated the significant increase in intracellular lipid accumulation. In addition, a significant decrease in the content of TG and TC was observed when the FFA-exposed L-02 cells were incubated with liraglutide, as indicated.

L-FABP plays a key role in the fatty acid metabolism of the liver. It has recently been reported that L-FABP constitutes a novel diagnostic marker for detecting NAFLD, and the serum and hepatic expression levels of L-FABP were significantly upregulated in NAFLD patients compared with those in control subjects (20,21). In the present study, it was found that the increased expression of L-FABP in FFA-exposed L-02 cells could be suppressed by liraglutide. These findings together demonstrate that liraglutide has a potential function in improving NAFLD *in vitro*.

The AMPK/SREBP1 pathway plays an important role in the development of orotic acid-induced fatty liver (22). AMPK is a heterotrimeric enzyme complex that is involved in a variety of biological activities that normalize glucose, lipid and energy imbalances. SREBP1 is a transcription factor responsible for fatty acid synthesis (23). It has been demonstrated that AMPK phosphorylation can inhibit the expression of SREBP1 (12,24,25), whereas the inhibition of AMPK augments the expression of SREBP1, thus leading to the activation of lipogenesis (26). In the present study, FFA-exposed L-02 cells were treated with various concentrations of liraglutide, and the results from the western blot analysis revealed that liraglutide affected the AMPK/SREBP1 pathway in a dose-dependent manner. The aim of the next part of the study was to examine whether the AMPK/SREBP1 pathway mediated the liraglutide-induced reduction in fatty degeneration.

The AMPK-mediated mechanism could be suppressed by compound C, an inhibitor of AMPK (27,28). In the present study, it was found that compound C blocked the inhibitory effect of liraglutide on the increases in intracellular lipid accumulation, TG and TC content, as well as the elevated expression of L-FABP induced by FFAs. These results indicate that the effect of liraglutide on reducing fatty degeneration was mediated by the AMPK/SREBP1 pathway.

In conclusion, the present study has demonstrated that liraglutide can reduce the fatty degeneration induced by FFAs in hepatocytes. This effect may be partially mediated by the AMPK/SREBP1 pathway.

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