Leptin Improves Fatty Liver Independently of Insulin Sensitization and Appetite Suppression in Hepatocyte-Specific Pten-Deficient Mice with Insulin Hypersensitivity

Horm Metab Res

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is recognized as the hepatic component of the metabolic syndrome. Although NAFLD is a major cause of cirrhosis and cancer of the liver of unknown cause, no established pharmacological treatment for NAFLD has been established yet. It has been reported that leptin treatment improved fatty liver dramatically as well as insulin resistance and hyperphagia in patients with lipodystrophy. However, it is unclear whether leptin improves fatty liver independently of these metabolic improvements. We investigated the liver effect of leptin independently of insulin sensitization and appetite suppression using hepatocyte-specific Pten-deficient (AlbCrePtenff) mouse, a model of severe fatty liver with insulin hypersensitivity. Male AlbCrePtenff mice were infused subcutaneously with leptin (20 ng/g/h) for 2 weeks using osmotic minipumps. Leptin infusion effectively reduced liver weight, liver triglyceride content, and glutamate pyruvate transaminase (GPT) concentrations as well as food intake and body weight without the change of plasma insulin concentration in AlbCrePtenff mice. Pair-feeding also reduced body weight but not liver triglyceride content. Pair-feeding reduced α1 and α2 AMP-activated protein kinase (AMPK) activities and PGC1α gene expression in the liver, while leptin infusion unchanged them. The present study clearly demonstrated that leptin improve fatty liver independently of insulin sensitization and suppression of food intake. It was suggested that leptin improves fatty liver by stimulation of β-oxidation in the liver. The present study might provide a further understanding on the mechanism of metabolic effect of leptin.

Introduction

Metabolic syndrome, which is a multiplex risk factor for cardiovascular disease, predicts the development of nonalcoholic fatty liver disease (NAFLD) [1,2]. NAFLD is now recognized as the hepatic component of the metabolic syndrome [3]. In addition, metabolic syndrome with its individual components is also the major risk factor for the development of nonalcoholic steatohepatitis (NASH), the most severe form of NAFLD [4]. NASH may progress to cirrhosis, hepatocellular carcinoma, and liver failure. However, at the present time, there is no established pharmacological treatment for NAFLD. Leptin is an adipocyte-derived hormone that regulates energy homeostasis mainly through the hypothalamus [5–7]. In addition to food intake and energy expenditure, leptin regulates glucose and lipid metabolism [8–10]. Given the anti-diabetic action of leptin, the usefulness of leptin treatment has been demonstrated in various types of diabetes including type 1, type 2, and lipoatrophic diabetes in rodent models [11–13]. Although there has been no clinical report on the efficacy of leptin treatment in type 1 and type 2 diabetes, the clinical application of leptin treatment in lipodystrophic diabetes that develops with lipodystrophy has already started [14–16]. Lipodystrophy is a disease characterized by a paucity of adipose tissue that leads to leptin deficiency. Patients with lipodystrophy generally suffer severe insulin-resistant diabetes, hyperphagia, and NAFLD. We and others have demonstrated that leptin effectively improves these metabolic disorders including NAFLD in patients with lipodystrophy [14–16]. Leptin improves hepatomegaly, reduces liver fat content, and decreases transaminase concentrations. Histological analysis also shows significant reductions of steatosis and hepatocellular ballooning injury [17].
While leptin reduces liver triglyceride content, the details on the liver effect of leptin remain unclear. In patients with lipodystrophy, leptin improves not only fatty liver but also insulin resistance and hyperphagia, which are major risk factors for NAFLD. Hyperinsulinemia due to insulin resistance stimulates de novo lipid synthesis in the liver and hyperphagia causes metabolic overflow, which leads to intrahepatic lipid accumulation [18]. It is unknown whether leptin improves fatty liver independently of insulin sensitization and appetite suppression. The improvement of fatty liver by leptin has also been reported in many rodent models but leptin administration generally suppresses food intake and improves insulin resistance in these animal models [19]. Thus, it is difficult to separate the liver effect of leptin from insulin sensitization and appetite suppression, which may contribute to the improvement of fatty liver. The aim of this study is to examine whether leptin improves fatty liver independently of insulin sensitization and appetite suppression. In order to approach this question, we used AlbCrePtenff mice, which have a hepatocyte-specific null mutation of Pten with the Cre-lox P system [20]. Because of Pten deficiency, the phosphatidylinositol 3-kinase (PI3K)-Akt/PIK signaling pathway is always upregulated in hepatocytes of this mouse, which causes constitutive lipogenesis and insulin signaling activation. Therefore, AlbCrePtenff mice have severe fatty liver with triglyceride accumulation and insulin hypersensitivity at the same time. In addition, plasma triglyceride concentration in AlbCrePtenff mice does not change as compared to AlbCre control mice. We infused leptin subcutaneously in AlbCrePtenff mice for 2 weeks and examined the change of liver weight, triglyceride content, history, and other associated metabolic parameters. Furthermore, to assess the effect of food intake suppression by leptin, we also performed the pair feeding experiment in which mice were fed at the same amount of food consumed by leptin treated mice in AlbCrePtenff mice. The present study might provide a further understanding on the mechanism of metabolic effect of leptin.

Materials and Methods

Animals

AlbCre and AlbCrePtenff mice were provided from Division of Cancer Genetics, Medical Institute of Bioregulation, Kyusyu University (Fukuoka, Japan) [20]. All mice were caged individually and kept under a 12-h light/dark cycle (light on at 09:00 AM) with free access to water and standard diet (NMF, 14.6kJ/g, 13% of energy as fat; Oriental Yeast, Co., Ltd., Tokyo, Japan). Animal care and all experiments were conducted in accordance with the Guidelines for Animal Experiments of Kyoto University and were approved by the Animal Research Committee, Kyoto University Graduate School of Medicine.

Leptin infusion and pair-feeding experiments

Thirty-six weeks old male AlbCrePtenff mice, which are old enough to develop apparent fatty liver based on previous report [21], were divided into 3 treatment groups (saline, leptin, saline plus pair fed to leptin infused mice) not to make differences in body weight among groups. Pair feeding experiment was performed in order to assess the influence of food intake reduction. On day 0, a mini-osmotic pump (Alzet model 2002; Durect, Cupertino, CA, USA) was implanted subcutaneously in the midscapular region in each mouse. The pump delivered either saline (0.5μl/h) or recombinant murine leptin (Amgen, Thousand Oaks, CA, USA) (20ng/g/h) subcutaneously for 14 days. Thirty-six weeks old male AlbCre mice were also infused with saline. For pair fed mice, food of the same amount consumed by leptin infused mice on the previous day was placed once daily around 09:00 AM for 14 days. Food intake and body weight were measured every day and every other day, respectively, for 14 days of the leptin infusion experiment.

Metabolic variables

Blood was obtained from mice between 13:00 and 15:00h after 4-h fasting at the end of the experiment by cardiophuncture. Plasma leptin concentrations were determined using an ELISA kit for mouse leptin (Millipore, Billerica, MA, USA). Blood glucose concentrations were determined by the glucose oxidase method using a reflectance glucometer (MS-GR102; Terumo, Tokyo, Japan). Plasma insulin concentrations were measured by enzyme immunoassay with an insulin-EIA kit (Morinaga, Tokyo, Japan). Plasma triglyceride, free fatty acid (FFA), total cholesterol, glutamate oxaloacetate transaminase (GOT), and glutamate pyruvate transaminase (GPT) concentrations were measured using enzymatic kits (Triglyceride E-test Wako, NEFA C-test Wako, Cholesterol E-test Wako, and transaminase C II-test Wako respectively; Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Liver histology

Livers were sampled from mice at the end of the experiment. Their pictures were taken with a camera. They were fixed in 10% neutrally buffered formalin and subsequently embedded in paraffin. Histological sections of 5-μm thickness were stained with hematoxylin and eosin, and examined by light microscopy.

Liver triglyceride contents

Livers were sampled from mice at the end of the experiment and immediately frozen in liquid nitrogen. Lipids were extracted with isopropyl alcohol/heptane (1:1 vol/vol). After evaporating the solvent, lipids were resuspended in 99.5% (vol/vol) ethanol and triglyceride content was measured by an enzymatic kit (Triglyceride E-test Wako; Wako Pure Chemical Industries, Ltd., Japan).

Isoform-specific AMPK activities

AMPK activities in the liver were determined as described previously [21]. Briefly, frozen liver was homogenized in Hepes-Tris-based lysis buffer and then centrifuged. The supernatants were immunoprecipitated with protein A-Sepharose beads and isoform-specific antibodies against AMPK a1 or 2 (Millipore). Kinase activities in the immune complex were determined by the phosphorylation of the SAMS peptide using [γ-32P]ATP.

mRNA expression analysis

Total RNA was extracted from the liver using TRIzol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. Total RNA (1μg) was reverse-transcribed with iScript™cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, US). Quantitative real-time PCR was performed with StepOnePlus Real-Time PCR System using TaqMan (Life Technologies). Using quantitative real-time PCR, we evaluated gene expressions of sterol regulatory element binding protein-1c (SREBP1c), fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD1), peroxisome proliferator-activated receptor gamma (PPARγ), farnesoid...
X Receptor (FXR), peroxisome proliferator-activated receptor alpha (PPARα), caffeine palmitoyl transferase 1-alpha (CPT1α) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α). Specific primers used in the present study were as follows: Sreb1pIC (5'-GGAGGCCATGGATTGCACAT-3'/5'-CCTTGTCAACCCCCACAGATA-3'), Fas (5'-GGGATCATTTGGGACTCCCT-3'/5'-CTCCCGCAGCTGTCATT-3'), Sod1 (5'-ATGTCCTCAAGAGATCTCAGTCTCT-3'/5'-CTCCACCTTCTCCTCCATTCC-3'), Pparα (5'-CTCCATCACGGAGTAGTCCACAG-3'/5'-AGACAGTGCTCCTGGTGCC-3'), Fxr (5'-TGGGCTTCCCAATCTCCCTAGA-3'/5'-TGGTCCTCCTGAGATTACGGTT-3'), Ppara (5'-AGGATCCTGCTCTCCTTATTG-3'/5'-GTTGCTCCGAATCCTCTTAGA-3'), Pgc1α (5'-GAAGAGATCTCCAGTTCT-3'/5'-CTTCACCTTCTCTCGTTCATTTCC-3'), Cpt1α (5'-CTCAGTGGGAGCGACTCTTCAA-3'/5'-GGCCTCTGTGGTAACTCAGGC-3'), and 18S (5'-GGGCTCCGAATCCTCTTAGA-3'/5'-TGGTCCTCCTGAGATTACGGTT-3'). Relative amounts of mRNAs were normalized with the ribosomal 18S RNA.

Statistical analyses

Data are expressed as means±SEM. Comparison between or among groups was by two-sided Student's t-test or one-way ANOVA followed by Tukey-Kramer test. A p-value of <0.05 was considered statistically significant.

Results

Metabolic characteristics of AlbCrePtenff mice

Table 1. The body weight of AlbCrePtenff mice was lower than that of AlbCre mice. It was mainly due to the amount of adipose tissue because the weight of epididymal fat of AlbCrePtenff mice was less than one-fourth of that of AlbCre mice. In addition, plasma leptin concentration in AlbCrePtenff mice was less than 5% of that of AlbCre mice. Cumulative food intake in AlbCrePtenff mice was increased compared with AlbCre mice. The blood glucose and plasma insulin concentrations were decreased in AlbCrePtenff mice by 31% and 80% as compared to those of AlbCre mice. On the other hand, plasma total cholesterol concentration in AlbCrePtenff mice was 1.9 times higher than that in AlbCre mice, although plasma total triglyceride concentration and plasma free fatty acid concentration in AlbCrePtenff mice were not significantly different from those in AlbCre mice. Liver weight in AlbCrePtenff mice was approximately 3 times higher than that in AlbCre mice. Furthermore, liver triglyceride content in AlbCrePtenff mice was approximately 6 times as much as that in AlbCre mice. Although plasma GOT concentration did not increase significantly, plasma GPT concentration increased in AlbCrePtenff mice 4.7 times as high as that in AlbCre mice. Consistent with these results, macroscopically, the livers of saline-infused AlbCrePtenff mice were pale and enlarged compared with saline-infused AlbCre mice and liver histology of AlbCrePtenff mice showed severe fatty liver.

Effect of leptin infusion on body weight, food intake, and metabolic parameters

Leptin infusion decreased body weight by 10.4% (Fig. 1a). Pair feeding also decreased body weights to the same extent as the leptin infused AlbCrePtenff mice. The weights of epididymal fat in leptin infused AlbCrePtenff mice decreased as compared to saline group by 32.4%, while those in pair fed AlbCrePtenff mice did not decrease significantly (Fig. 1b). Continuous leptin infusion effectively increased plasma leptin levels by around 10 ng/ml (Fig. 1c). Leptin infusion suppressed food intake by 28.2% compared with saline-infused AlbCrePtenff mice (Fig. 1d). Leptin infusion significantly decreased plasma glucose concentrations in AlbCrePtenff mice by 73%, while it did not significantly change plasma insulin concentrations (Fig. 2a, b). Pair feeding did not change either blood glucose or plasma insulin concentrations significantly as compared to saline infused AlbCrePtenff mice. In addition, leptin infusion effectively decreased plasma triglyceride, free fatty acid, and total cholesterol concentrations in AlbCrePtenff mice while pair feeding did not affect these lipid parameters (Fig. 2c, d, e).

Effect of leptin infusion on fatty liver

Macroscopically, the livers of saline infused AlbCrePtenff mice were pale and enlarged (Fig. 3a). In liver histological assessments with hematoxylin-eosin stain, most of hepatocytes in saline infused AlbCrePtenff mice had weakly eosinophilic cytoplasm and contained numerous microvesicular vacuoles (Fig. 3b). Leptin infusion for 14 days shrank the liver size and the livers looked more reddish in AlbCrePtenff mice (Fig. 3a). Liver histology showed that leptin infusion remarkably improved fatty liver in AlbCrePtenff mice with less microvesicular vacuoles (Fig. 3b). Consistent with this, leptin infusion significantly decreased liver weight, liver triglyceride content, and plasma GPT concentrations by 53%, 47%, and 56%, respectively (Fig. 3c, d, f), although plasma GOT concentrations were not decreased significantly in the leptin infused AlbCrePtenff mice (Fig. 3e). On the other hand, pair feeding did not affect the liver histology, liver weight, liver triglyceride content, plasma GOT concentrations, or plasma GPT concentrations significantly.
Effect of leptin infusion on AMPK activities and gene expressions in the liver

To investigate how leptin improved fatty liver, we examined AMPK activities and expressions of genes associated with lipid metabolism in the liver. Compared with saline infused AlbCre mice, α1 isoform-specific AMPK (AMPK α1) activity was decreased in the saline infused AlbCrePtenff mice, though α2 isoform-specific AMPK (AMPK α2) activity was not significantly decreased. Leptin infusion tended to increase AMPK α1 and α2 activities in the liver although statistical significance was not achieved (Fig. 4a, b). However, compared with the pair fed group, AMPK α1 and α2 activities were increased in the leptin infused AlbCrePtenff group by 1.8 and 2.8 times, respectively. On gene expression analysis, mRNA expressions of lipogenic genes such as Fas, Scd1 and Pparg, and β-oxidative genes such as Ppara, Cpt1a and Pgc1a were upregulated in saline infused AlbCrePtenff mice, compared with saline infused AlbCre mice. These gene expressions were unchanged by leptin treatment. On the other hand, pair feeding decreased gene expressions of β-oxidative genes. Gene expressions of Ppara, Cpt1a, and Pgc1a in pair fed AlbCrePtenff mice decreased by 71%, 56%, and 72%, respectively, as compared to saline infused AlbCrePtenff mice though statistical significance was not achieved in Ppara and Cpt1a.

Discussion and Conclusions

To examine whether leptin has an improvement effect on fatty liver independently of insulin sensitization and appetite suppression, we used AlbCrePtenff mice with hepatocyte-specific Pten deficiency with the Cre-loxP system. Pten deficiency leads to constitutive activation of PI3K-Akt/PKB signaling pathway, a downstream of insulin signaling [20, 22]. Activation of insulin signaling enhances lipogenesis in the liver. Thus, AlbCrePtenff mice developed severe fatty liver in spite of low plasma insulin concentration (Table 1). Constitutive activation of insulin signaling in the liver of AlbCrePtenff mice also suppresses hepatic gluconeogenesis, which leads to hypoglycemia, hypoinsulinemia and hyperphagia. AlbCrePtenff mice showed lower body weight and lower fat weight when compared to AlbCre control mice, which may be due to the low plasma insulin concentration. Consistent with low fat weight, plasma leptin concentration was also decreased in AlbCrePtenff mice.

In the present study, 14-days of leptin infusion effectively reduced liver triglyceride content in AlbCrePtenff mice, which showed insulin hypersensitivity and low plasma insulin concentration. Leptin infusion did not significantly change plasma insulin concentration but reduced blood glucose concentration, suggesting leptin still enhanced whole body insulin sensitivity in AlbCrePtenff mice. Generally, hyperinsulinemia facilitates lipogenesis in the liver. Therefore, insulin sensitization suppresses lipogenesis as a consequence of decrease in plasma insulin concentration. However, in AlbCrePtenff mice, enhancement of insulin sensitivity by leptin could increase, but would not decrease lipogenesis because the change of plasma insulin concentration was minimum, if any, under the condition of constitutive activation in PI3K-Akt/PKB signaling pathway in their hepatocytes. Thus, the improvement of fatty liver by leptin in AlbCrePtenff mice indicates that leptin has an improvement effect on fatty liver independently of insulin sensitizing action or insulin lowering effect. In addition, pair feeding by which mice were fed the same amount of food consumed by leptin infused mice did not reduce liver weight or liver triglyceride content significantly, although it reduced body weight to the
same extent as leptin infusion. These results indicate that leptin also has an improvement effect on fatty liver independently of food intake suppression or body weight reduction. Taken together, the present study clearly demonstrates that leptin has an improvement effect on fatty liver independently of insulin sensitization and appetite suppression.

It has been demonstrated that leptin treatment reduces lipid content in the skeletal muscle in patients with lipodystrophy [23, 24]. Using rodent models, it was demonstrated that leptin activates AMPK in the skeletal muscle [25–27]. AMPK is a heterotrimeric enzyme that is conserved from yeast to humans and functions as a ‘fuel gauge’ to monitor the status of cellular energy. AMPK potently stimulates fatty acid oxidation by inhibiting the activity of acetyl CoA carboxylase [28]. Thus, AMPK activation by leptin is a plausible mechanism by which leptin reduces lipid accumulation in the skeletal muscle. In addition to the skeletal muscle, recent studies have shown the physiological significance of AMPK activation by leptin in the liver [29]. It was demonstrated that AMPK activation by leptin in the liver is mainly through the CNS and that leptin has no direct AMPK-activating effect on hepatocytes [29]. In the present study, leptin infusion showed a tendency to activate both AMPK α1 and α2, while pair-feeding showed a tendency to suppress them in the liver of AlbCrePtenff mice (Fig. 4a, b). Hepatic AMPK activation is one of the potential mechanisms by which leptin reduces lipid content in the liver.

In AlbCrePtenff mice, not only lipogenic gene but also β-oxidative gene expressions in the liver were upregulated when compared to those in AlbCre control mice, supporting the concept that fatty liver in AlbCrePtenff mice is due to the activation of lipol-
genesis but not the suppression of β-oxidation in the liver [21,22]. In the present study, pair feeding suppressed Scd1 gene expression but also decreased expressions of β-oxidative genes such as Ppara, Cpt1a, and Pgc1a in the liver in AlbCrePtenff mice. On the other hand, leptin infusion did not decrease β-oxidative gene expressions, although it also did not change lipogenic gene expressions. These results suggest that leptin improves fatty liver by stimulation of β-oxidation rather than suppression of lipogenesis in the liver. It was reported that leptin suppressed Scd1 gene expression in the liver in ob/ob mice [30,31]. In the present study, leptin infusion did not significantly decrease Scd1 gene expression in AlbCrePtenff mice in which insulin signaling and lipogenesis in the liver were constitutively activated.

There are some limitations in the present study. AlbCrePtenff mice show hyperphagia that may be needed to maintain normal blood glucose concentrations. Under this condition, leptin treatment suppressed hyperphagia and lowered blood glucose concentrations. Thus, it is possible that long-term leptin treatment may not lead to improvement of prognosis in AlbCrePtenff mice although it improves fatty liver. The present study demonstrated the action pathway of improvement effect of leptin on fatty liver but not the usefulness of leptin treatment in AlbCrePtenff mice. It is also possible that unphysiological hypoglycemia caused by leptin treatment may induce unknown compensatory mechanism to reduce triglyceride content in the liver in AlbCrePtenff mice.
mice. If so, the effect of leptin on fatty liver observed in the present study cannot be generalized simply to more common disease state.

In conclusion, using the AlbCrePtenff mice, the present study clearly demonstrated that leptin has a liver-specific effect on fatty liver independently of insulin sensitizing or insulin lowering effect as well as anorectic or antiobese effect. The present study suggest that leptin reduced liver triglyceride content by stimulation of β-oxidation rather than suppression of lipogenesis in the liver. The present study might provide a further understanding on the mechanism of metabolic effect of leptin.

Acknowledgements

We thank Keiko Hayashi for technical assistance. The authors also acknowledge Yoko Koyama for secretarial assistance. This work was supported by research grants from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, the Japanese Ministry of Health, Labor and Welfare, and Uehara Memorial Foundation.

Conflict of Interest

The authors declare that they have no conflicts of interest in the authorship or publication of this contribution.

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Fig. 4 Effect of leptin administration or pair feeding on isoform-specific AMPK activities in the liver of AlbCrePtenff mice and on gene expressions in the liver of AlbCrePtenff mice. a AMPK α1 activity and b AMPK α2 activity on day 14 of the experiment. c mRNA expression levels of Srebp1c, Fas, Scd1, Pparγ, Fxr, Ppara, Cpt1α, and Pgc1α on day 14 of the experiment were normalized by 18S and expressed relative to those in saline-infused AlbCre mice. Values are means ± SEM (AlbCre: n = 6, Saline: n = 6, Leptin: n = 6, Pair fed: n = 5). * p < 0.05, ** p < 0.01 (Student’s t-test).

Affiliations

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