Effects of the Combination of Evogliptin and Leucine on Insulin Resistance and Hepatic Steatosis in High-Fat Diet-Fed Mice

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Abstract

In this study, we aimed to investigate the effects of 8 weeks of treatment with a combination of evogliptin and leucine, a branched-chain amino acid, in mice with high-fat diet (HFD)-induced diabetes. Treatment with evogliptin alone or in combination with leucine reduced the body weight of the mice, compared to the case for those from the HFD control group. Long-term treatment with evogliptin alone or in combination with leucine resulted in a significant reduction in glucose intolerance; however, leucine alone did not affect postprandial glucose control, compared to the case for the mice from the HFD control group. Furthermore, the combination of evogliptin and leucine prevented HFD-induced insulin resistance, which was associated with improved homeostasis model assessment for insulin resistance, accompanied by markedly reduced liver fat deposition, hepatic triglyceride content, and plasma alanine aminotransferase levels. The combination of evogliptin and leucine increased the gene expression levels of hepatic peroxisome proliferator-activated receptor alpha, whereas those of the sterol regulatory element-binding protein 1 and stearoyl-CoA desaturase 1 were not altered, compared to the case in the HFD-fed mice ($p<0.05$). Thus, our results suggest that the combination of evogliptin and leucine may be beneficial for treating patients with type 2 diabetes and hepatic steatosis; however, further studies are needed to delineate the molecular mechanisms underlying the action of this combination.

Key Words: Evogliptin, Leucine, High-fat diet, Insulin resistance

INTRODUCTION

Mice with high-fat diet (HFD)-induced diabetes are characterized by insulin resistance and hepatic steatosis (Kim *et al*., 2012; Kim *et al*., 2017a); insulin resistance can aggravate hepatic steatosis (Browning and Horton, 2004; Perry *et al*., 2014). Prolonged hyperglycemia and obesity can result in insulin resistance, thereby leading to enhanced lipogenesis and fatty acid uptake with reduced fatty acid oxidation and hepatic triglyceride secretion, which consequently leads to the worsening of hepatic steatosis (Koo, 2013; Aroor *et al*., 2015).

Dipeptidyl peptidase 4 (DPP4) is a serine protease that inactivates various endogenous peptide substrates. Increased hepatic DPP4 expression is pathophysiologically linked to insulin resistance and non-alcoholic fatty liver disease (NAFLD) progression (Balaban *et al*., 2007; Lamers *et al*., 2011; Miyazaki *et al*., 2012; Williams *et al*., 2015). DPP4 inhibitors block the degradation of biologically active glucagon-like peptide-1 (GLP-1). Thus, although DPP4 inhibitors might show therapeutic potential for the treatment of NAFLD (Armstrong *et al*., 2013), it remains unclear where there is a direct causal relationship between DPP4 inhibitors and NAFLD.

Branched-chain amino acids (BCAAs), particularly, leucine, have been suggested to play roles in obesity and metabolic syndrome (Layman and Walker, 2006). Leucine is an essential BCAA that cannot be produced by the human body and can only be obtained from the diet (Zhang *et al*., 2007). Previous studies have suggested that leucine stimulates GLP-1 secretion (Chen and Reimer, 2009) and reduces the increase in blood glucose levels when co-ingested with glucose (Kalogeropoulou *et al*., 2008).

Potential pharmacodynamic interactions between DPP4 inhibitors and BCAAs have not yet been evaluated; thus, the mechanisms underlying the combined effects of DPP4 inhibitors and leucine on the blood glucose levels remain unclear. Leucine has been shown to promote GLP-1 secretion from the intestine (Chen and Reimer, 2009), and evogliptin can prolong the action of GLP-1 via the inhibition of DPP4 activity (Kim *et al*., 2012). Therefore, the combination of evogliptin and leucine may improve glucose metabolism, thereby ameliorating

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insulin resistance and NAFLD.

Therefore, the aim of this study was to evaluate the pharmacodynamic interactions between evogliptin and leucine in mice with HFD-induced diabetes.

MATERIALS AND METHODS

Materials

Evogliptin ((R)-4-[(R)-3-amino-4-(2,4,5-trifluorophenyl)butanoyl]-3-(t-butoxymethyl) piperazin-2-one) l-tartrate salt was synthesized by Dong-A ST (Yongin, Korea), and l-leucine was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were purchased from Sigma-Aldrich, unless otherwise stated.

Animals

All animal experiments were performed in accordance with, and approved by, the Institutional Animal Care and Use Committee of the Chung-Ang University (Seoul, Korea; IACUC-201700056). Male ICR mice were obtained from RaonBio (Yongin, Korea).

HFD-induced diabetes mouse model

At 6 weeks of age, ICR mice were fed an HFD (20% protein, 35.5% fat, and 36.3% carbohydrates, w/w; Research Diets, New Brunswick, NJ, USA) *ad libitum* for 10 weeks. They were maintained at 21°C with a 12:12-h light/dark cycle at the animal facilities of the College of Pharmacy at the Chung-Ang University.

The HFD-fed mice (16-weeks-old) were housed (two mice/cage). They were evenly divided on the basis of their blood glucose levels and body weights. The mice were treated with evogliptin as a diet admixture (0.1%, w/w), leucine in drinking water (1%, w/w), or a combination of evogliptin (0.1%) and leucine (1%) for 8 weeks (n=8/group). The target free-form doses were approximately 100 and 1,000 mg/kg/day for 0.1% (w/w) evogliptin and 1% (w/v) leucine, respectively. The mean administered doses were 40.6 ± 1.9 and 771.7 ± 54.5 mg/kg/day for 0.1% (w/w) evogliptin and 1% (w/v) leucine, respectively. The non-fasting blood glucose levels, body weight, diet, and water consumption were measured on a weekly basis.

After eight weeks of treatment, the mice were fasted overnight, and blood was collected from the orbital venous sinus into heparin-filled tubes. The plasma was separated by centrifugation at 5,000 *g* for 5 min.

Oral glucose tolerance test (OGTT)

An OGTT was conducted after 4 weeks of treatment with evogliptin, leucine, or their combination. The mice were fasted overnight, and basal blood glucose levels (t=–30 min) were measured in the tail vein blood using a glucometer (AccuChek Active, Roche, Ireland). The mice were then challenged orally with a glucose solution (2 g/10 mL/kg), and blood glucose levels in the tail vein blood were measured at the indicated time points for 2 h after the glucose challenge. The blood glucose excursions are represented as the area under the curve (AUC_{0-2h}).

Insulin tolerance test (ITT)

An ITT was conducted after six weeks of treatment with evogliptin, leucine, or their combination. The mice were fasted

for 6 h, and 0.75 U/kg human insulin (Sigma-Aldrich) was intraperitoneally injected. Blood glucose levels were measured in tail vein blood samples 0, 15, 30, 60, 90, and 120 min after insulin injection. Data were processed and are expressed as percentages of the basal fasting glucose level, and the area under the normalized blood glucose-time curve is presented as AUC_{0-2h} (% min).

Histological examination

Formalin-fixed liver tissue samples were processed, and 5-μm-thick paraffin sections were stained with hematoxylin and eosin (H&E). The slides were examined under an IX71 microscope and photographed using a Cool Snap color digital camera (Olympus, Tokyo, Japan). All sections were examined by the same person, who was blinded to the treatment. At least three different liver sections were examined from eight mice from each treatment group. The scoring of lipid droplet accumulation in liver sections was conducted in a blinded manner using a scoring scale from 1 to 4 (minimal, mild, moderate, and severe). The relative area of the hepatic lipid droplets was assessed using Image-Pro Plus software ver. 5.1 (Media Cybernetics, Silver Spring, MD, USA).

Biochemical parameters

Plasma glucose, triglyceride (TG), total cholesterol, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were measured by enzymatic methods using a Model 20i® Analyzer (Konelab, Waltham, MA, USA). Plasma insulin levels were measured using an insulin enzyme-linked immunosorbent assay (ELISA) kit (Shibayagi, Gunma, Japan). Plasma total GLP-1 (KT-876) levels were analyzed using an ELISA kit (Epitope Diagnostics Inc., San Diego, CA, USA). Plasma glucagon levels were determined using a mouse/rat glucagon ELISA kit (YK090, Eagle Biosciences, Amherst, NH, USA). Insulin-sensitizing effects after the treatments were assessed using the homeostasis model assessment for insulin resistance (HOMA-IR; [plasma insulin level (μU/mL)×plasma glucose level (mmol/L)]/22.5) (Matthews *et al*., 1985).

Real-time quantitative polymerase chain reaction (qPCR)

Total RNA (2 μg) was extracted from the tissues using a total RNA Mini Kit (W72070-150, Wizbiosolutions, Seongnam, Korea). The RNA was then reverse-transcribed using the RT Master system (W2203, Wizbiosolutions). Then, qPCR was performed using the QGreen 2X SYBR Green qPCR Master Mix (QG-05, Cellsafe, Yongin, Korea), following the manufacturer's instructions. The data were analyzed using the QIAGEN Rotor-Gene Q Series software. The primers used are listed in Table 1.

Data analysis

The data are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using SigmaStat 2.0 (SPSS, Chicago, IL, USA). Two-group comparisons were performed using the Student's *t*-test. For multigroup comparisons, one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls multiple comparison test, was used. Statistical significance was set at $p < 0.05$.

RESULTS

Effects of evogliptin, leucine, and their combination on body weight gain

We investigated the effects of eight weeks of treatment with evogliptin, leucine, and their combination on body weight gain on HFD-fed mice. Evogliptin alone and in combination with leucine continuously reduced HFD-induced body weight gain by 38% and 45%, respectively, compared with that in HFD-fed control mice (Fig. 1A). However, leucine alone did not induce body weight reduction (+6% *vs.* HFD-fed control mice). There were no significant differences in food and water consumption among the groups (Fig. 1B, 1C). Non-fasting blood glucose levels did not differ among the different treatment groups (Fig. 1D). These results showed that evogliptin or a combination of evogliptin and leucine prevented HFD-induced body weight gain without altering the food and water consumption.

Effects of evogliptin, leucine, and their combination on postprandial glycemic control in HFD-fed mice

To confirm the beneficial effects of the combination treatment on glycemic control in HFD-fed mice, we performed an OGTT four weeks after the drug treatments. The HFD-fed control mice exhibited a significantly higher glucose AUC than the normal control mice, which indicates severe glucose intolerance in the HFD-fed mice (Fig. 2A, 2B). Mice treated with evogliptin showed a 19.6% reduction in the glucose AUC; however, the leucine-treated mice did not show a significant change in glucose AUC (Fig. 2B). The addition of leucine augmented the glucose level-lowering effects of evogliptin (−30.6% *vs.* HFD-fed control mice; $p < 0.05$). These results showed that long-term combination treatment with evogliptin and leucine improved glucose tolerance in mice with HFD-induced diabetes.

Effects of evogliptin, leucine, and their combination on insulin resistance in HFD-fed mice

To confirm the beneficial effects of the combination therapy on insulin resistance in HFD-fed mice, we performed an ITT six weeks after the drug treatments. The HFD-fed control mice exhibited a significantly higher glucose AUC than the normal control mice, following the normalization of the glucose levels to the basal blood glucose level (Fig. 3A). Mice treated
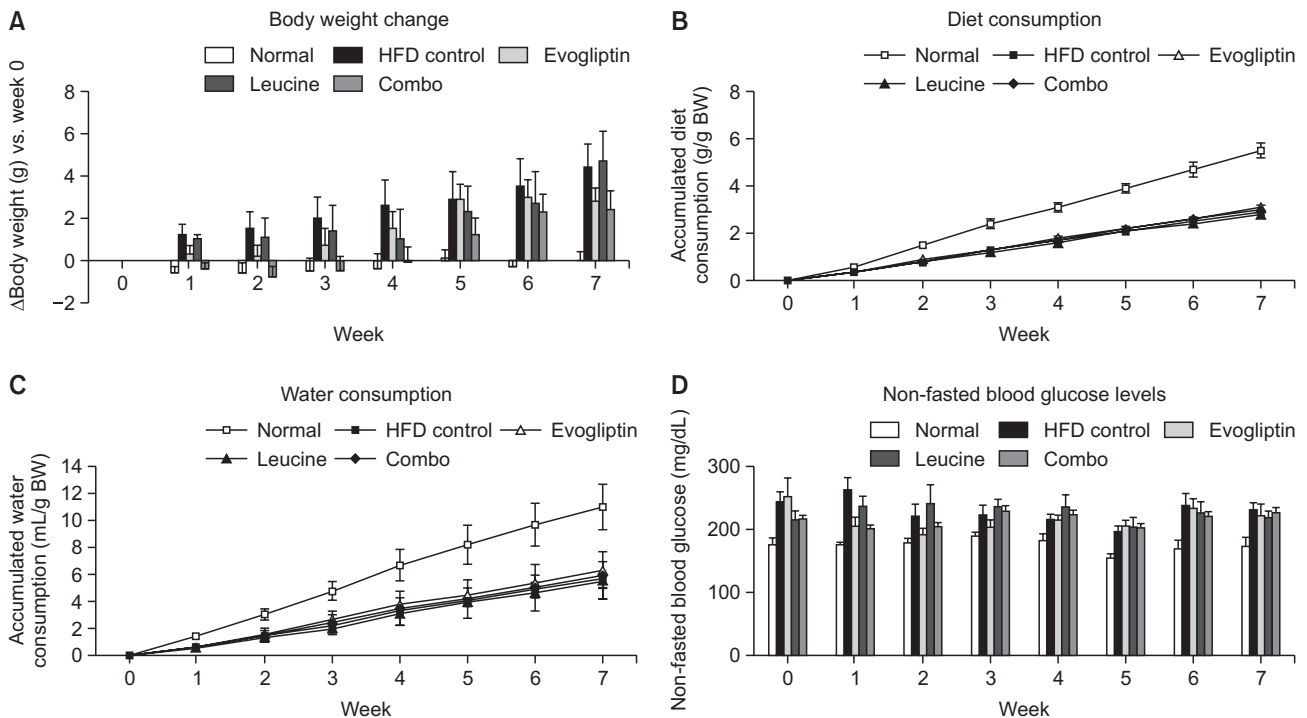
Table 1. Primers used for quantitative real-time PCR

Target gene	Forward/reverse primers
β-actin	Forward: 5′-CGCCACCAGTTCGCCATGGA-3′
	Reverse: 5′-TACAGCCCGGGGAGCATCGT-3′
SREBP-1c	Forward: 5′-CAAGGCCATCGACTACATCCG-3′
	Reverse: 5′-CACCACTTCGGGTTTCATGC-3′
SCD-1	Forward: 5′-TTCTTGCGATACACTCTGGTGC-3′
	Reverse: 5′-CGGGATTGAATGTTCTTGTCGT-3′
PPAR-α	Forward: 5′-ATTCTTACCTGTGAACACGACCTG-3′
	Reverse: 5′-GGGTTGTTGCTGGTCTTTCC-3′
PEPCK	Forward: 5′-CTGCATAACGGTCTGGACTTC-3′
	Reverse: 5′-CAGCAACTGCCCGTACTCC-3′
G6Pase	Forward: 5′-AAAAAGCCAACGTATGGATTCCG-3′
	Reverse: 5′-CAGCAAGGTAGATCCGGGA-3′

Fig. 1. Effects of eight weeks of treatment with evogliptin, leucine, and the combination of these two agents in HFD-fed mice. Body weight changes (A), diet intake (B), water intake (C), and non-fasting blood glucose levels (D) were measured weekly. Data are expressed as the means ± SEM. $p < 0.05$ *vs.* the baseline (paired *t*-test).

with 0.1% evogliptin or 1% leucine did not show significant improvements in insulin resistance despite 11.3% and 4.7% decreases in the glucose AUC, respectively, compared with the HFD-fed control mice. However, the combination of evogliptin and leucine resulted in a significant decrease in insulin resistance, with a 22.1% decrease in the glucose AUC, compared with that in the HFD-fed control mice (Fig. 3B).

After eight weeks of treatment, the plasma glucose levels in the mice treated with the combination of evogliptin and leucine did not significantly differ from those in the mice from the HFD-fed control group (Fig. 3C). Plasma insulin levels tended to decrease in the evogliptin-treated mice, whereas leucine did not notably affect the plasma insulin levels (Fig. 3D). The combination treatment resulted in markedly decreased plasma insulin levels and improved HOMA-IR compared with those in

the HFD-fed control mice (Fig. 3E). These results showed that long-term combination treatment with evogliptin and leucine prevented HFD-induced insulin resistance.

Effects of evogliptin, leucine, and their combination on tissue weight and biochemical parameters

We next examined the effects of eight weeks of treatment with evogliptin, leucine, and their combination on lipid dysregulation. The weights of the liver and epididymal fat were not significantly altered by evogliptin or leucine treatment (Table 2). The HFD-fed control mice showed notable changes in the plasma total cholesterol, glucagon, and ALT levels compared with the normal mice. The HFD-fed mice showed markedly elevated plasma ALT levels, which indicated hepatocyte damage owing to lipid accumulation. However, the HFD-fed control mice did not show a significant change in the plasma TG levels compared with the normal mice (Table 3). Evogliptin and leucine alone did not affect the plasma ALT levels, which was consistent with the insulin resistance not being affected by treatment with each of the agents alone. However, the combination treatment resulted in a significant decrease in the plasma ALT levels (Table 3). Collectively, these results suggest that the combination of evogliptin and leucine prevents HFD-induced disturbances in lipid metabolism.
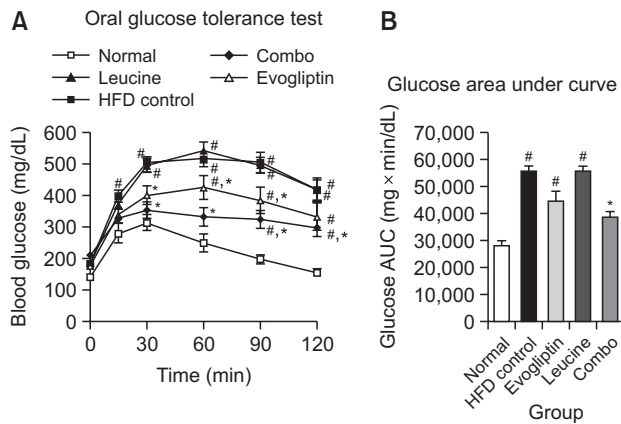

Fig. 2. Effects of evogliptin, leucine, and the combination of these two agents on postprandial glycemic control in HFD-fed mice. Blood glucose levels were measured 0, 15, 30, 60, 90, and 120 min after glucose challenge (A). Blood glucose AUC$_{0-2h}$ was determined, and the percentage inhibition values were determined (B). Data are expressed as the means ± SEM. $^{\#}p<0.05$ *vs.* normal control mice, $^{*}p<0.05$ *vs.* HFD-fed control mice.

Table 2. Effects of various treatments on liver weight in HFD-fed mice

Group	BW (g)	Liver (g)	Liver/BW
Normal control	38.3 ± 0.3	1.74 ± 0.04	0.0454 ± 0.0013
HFD control	57.5 ± 1$^{\#}$	2.56 ± 0.15	0.0445 ± 0.0027
Evogliptin	56.5 ± 0.8$^{\#}$	2.66 ± 0.15	0.0469 ± 0.0021
Leucine	57.5 ± 1.1$^{\#}$	2.8 ± 0.21	0.0484 ± 0.0032
Combination	54.6 ± 1.5$^{\#}$	2.31 ± 0.13	0.0422 ± 0.0016

Data are expressed as the means ± SEM. $^{\#}p<0.05$ *vs.* normal control mice.
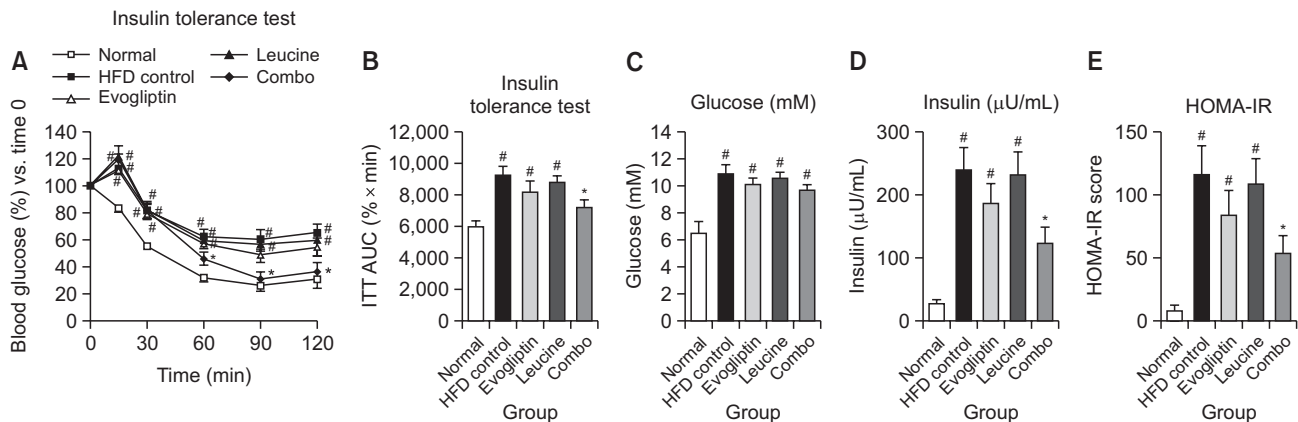

Fig. 3. Effects of evogliptin, leucine, and the combination of these two agents on insulin resistance and postprandial glycemic control in HFD-fed mice. Blood glucose changes were determined for 2 h (A), and the blood glucose excursion over 2 h is presented (B). At week 8, the animals were euthanized, and both fasting plasma glucose (C) and plasma insulin (D) levels were measured. Homeostasis model assessment for insulin resistance (HOMA-IR) was improved by the combination treatment (E). Data are expressed as the means ± SEM. $^{\#}p<0.05$ *vs.* normal control mice, $^{*}p<0.05$ *vs.* HFD-fed control mice.

Table 3. Effects of various treatments on biochemical parameters in HFD-fed mice

Group	ALT (U/L)	AST (U/L)	Triglycerides (mg/dL)	Total cholesterol (mg/dL)	Total GLP-1 (pM)	Glucagon (pg/mL)
Lean	43.7 ± 4.8	122.3 ± 5.8	154.1 ± 27.6	129 ±5.5	2.74 ± 0	414.3 ± 14.5
HFD control	80.2 ± 11.8[#]	127.3 ± 8	130.5 ± 9.8	246.2 ± 19[#]	2.71 ± 0.01	901.4 ± 82[#]
Evogliptin	67.5 ± 7.3[#]	126.4 ± 4.7	114.1 ± 7.7	291.6 ± 11.2[#]	2.77 ± 0.01	753.7 ± 93.3[#]
Leucine	80.9 ± 8.3[#]	156.3 ± 9	114.8 ± 5.2	283.8 ± 12.5[#]	2.9 ± 0.02	870.2 ± 115.6[#]
Combination	46.5 ± 6.2*	134.6 ± 7.3	136.1 ± 12.5	287 ± 15.7[#]	3.25 ± 0.34	882 ± 74[#]

Data are expressed as the means ± SEM. [#]$p < 0.05$ *vs.* normal control mice, *$p < 0.05$ *vs.* HFD-fed control mice.
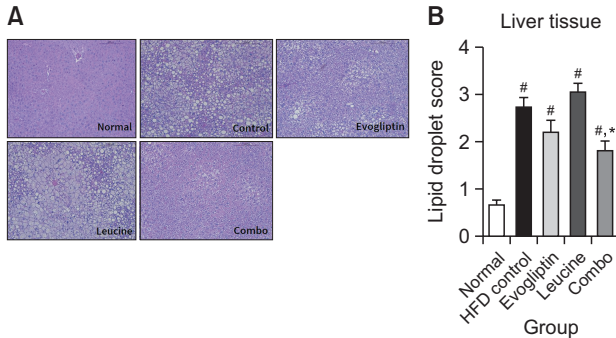
Fig. 4. Effects of evogliptin, leucine, and the combination of these two agents on hepatic steatosis in HFD-fed mice. After eight weeks of the administration of evogliptin, leucine, or their combination in HFD-fed mice, the degree of hepatic steatosis was assessed by examining hepatic lipid accumulation histologically (A) and biochemically (B). Data are expressed as the means ± SEM. [#]$p < 0.05$ *vs.* normal control mice, *$p < 0.05$ *vs.* HFD-fed control mice.

Histological examination of fat content in liver tissues from HFD-fed mice

A histological examination was performed to measure the fat contents in H&E-stained liver sections. The HFD-fed mice exhibited prominent adipocyte hypertrophy compared with the normal mice (Fig. 4). Evogliptin and leucine alone did not reduce the fat content; however, their combination markedly improved hepatic histology, as shown by quantitative histomorphometry analysis (Fig. 4).

Chronic effects of evogliptin, leucine, and their combination on hepatic gene expression and intracellular signaling in HFD-fed mice

Considering the importance of the liver in the regulation of systemic insulin sensitivity, we explored the effects of evogliptin, leucine, and their combination on the expression of genes involved in glucose and lipid metabolism. Sterol regulatory element-binding protein-1c (SREBP-1c) is a key transcription factor that regulates *de novo* lipogenesis under the control of insulin signaling (Yecies *et al.*, 2011). To determine whether the reduction of hepatic TG accumulation by evogliptin and leucine treatment was mediated by the suppression of lipogenesis, we assessed the gene expression levels of SREBP-1c and stearoyl-CoA desaturase-1 (SCD-1), a downstream gene. Evogliptin, leucine, and their combination did not affect the expression of SREBP-1c, compared with that in the control mice (Fig. 5A). Similarly, the expression of SCD-1 was not significantly affected by the drug treatment (Fig. 5B). In the liver, peroxisome prolif-

erator-activated receptor alpha (PPARα) is highly expressed, and its activation inhibits lipogenesis via both direct and indirect mechanisms. Next, we examined whether evogliptin, leucine, and their combination induce the upregulation of PPARα. Hepatic PPAR-α expression decreased by 60.4% in HFD-fed mice compared with the normal control mice. However, treatments with evogliptin and leucine alone normalized the PPARα expression. Furthermore, the combination treatment significantly increased the expression of PPARα, compared with that in the HFD-fed control mice (Fig. 5C). These data suggest that the restoration or activation of PPARα expression by the combination treatment partly contributes to the preventive effects of evogliptin and leucine against lipogenesis. We then examined whether evogliptin, leucine, and their combination suppress the expression of gluconeogenic genes. In the HFD-fed mice, the hepatic expression levels of glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), which are key genes involved in the regulation of hepatic gluconeogenesis, were not different from those in the normal control mice. Additionally, the drug treatments did not notably affect the mRNA expression of G6Pase and PEPCK; however, evogliptin alone and the combination treatment decreased the mRNA expression of G6Pase by 33.9% and 39%, respectively (Fig. 5D, 5E).

DISCUSSION

In this study, the combination of evogliptin and leucine exhibited synergistic therapeutic effects against hepatic steatosis by decreasing insulin resistance and suppressing lipogenesis.

Although most DPP4 inhibitors have been reported to have neutral effects on body weight gain in animals and humans (Kern *et al.*, 2012; Holst and Deacon, 2013; Omar *et al.*, 2013), several DPP4 inhibitors have been shown to exhibit anti-obesity effects in mice with HFD-induced obesity (Fukuda-Tsuru *et al.*, 2014; Chae *et al.*, 2015). In this study, there were no marked changes in body weight in mice treated with evogliptin or leucine alone, although their combination reduced the HFD-induced body weight gain. In a previous study (Kim *et al.*, 2017a), there was a significant difference in body weight between HFD-fed and evogliptin-treated (0.5%; 300 mg/kg) mice, but not between HFD-fed and evogliptin-treated (0.2%; 100 mg/kg) mice. Therefore, it seems that evogliptin (0.1%) affected the DPP4 activity, but not to an extent that was sufficient enough to reduce body weight, which is consistent with the findings of a previous report (Chae *et al.*, 2015). Zhang *et al.* (2007) reported that 1.5% leucine supplementation induces a 32% reduction in HFD-induced weight gain; however, in this study, 1% leucine supplementation did not affect body
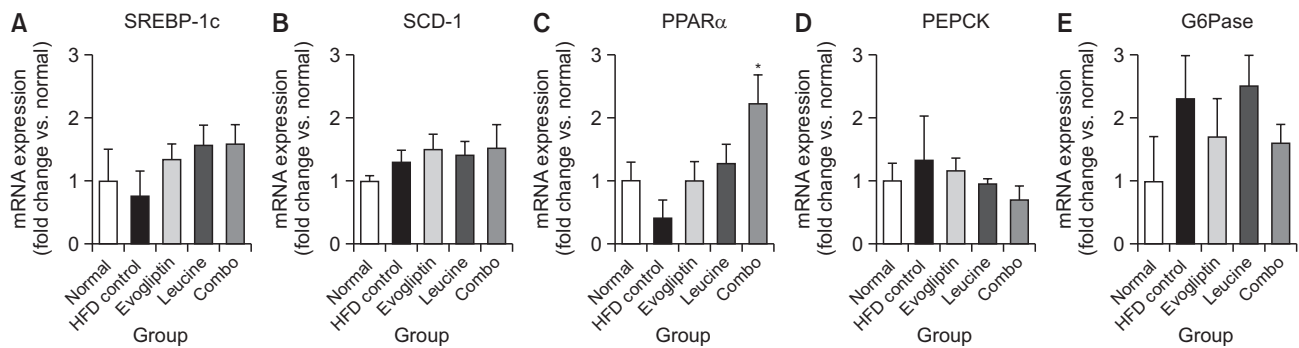
Fig. 5. Effects of evogliptin, leucine, and the combination of these two agents on gene expression in liver tissues. After eight weeks of the administration of evogliptin, leucine, or their combination, the gene expression levels of SREBP-1c (A), SCD-1 (B), PPARα (C), PEPCK (D), and G6Pase (E) in the liver tissues of HFD-fed mice were assessed by RT-qPCR (*n*=8/group). Data are expressed as the means ± SEM. *$p<0.05$ *vs.* HFD-fed control mice.

weight. Thus, we concluded that 1% leucine does not notably affect food consumption and body weight, although it increased GLP-1 secretion (Chen and Reimer, 2009). However, the combination of evogliptin and leucine decreased the HFD-induced weight gain by 45%, which indicates the synergistic effects of this combination on HFD-induced body weight gain.

Herein, the HFD-fed mice showed mild hyperglycemia and glucose and insulin intolerance, which is consistent with the results of a previous report (Kim *et al.*, 2017a). By reducing the blood glucose levels, evogliptin improved glucose tolerance in insulin-resistant mice. HFD-induced postprandial hyperglycemia and insulin resistance were not markedly affected by the addition of leucine, which is consistent with the results of a previous report (Fu *et al.*, 2015). However, the combination of leucine and evogliptin significantly decreased the postprandial glucose levels and the degree of insulin resistance in HFD-fed mice. Therefore, the synergistic effects of leucine and evogliptin on postprandial glycemic control and insulin sensitivity might be attributed to the evogliptin-induced inhibition of DPP4-mediated GLP-1 degradation (Kim *et al.*, 2012) and the leucine-induced promotion of GLP-1 secretion from the intestine (Chen and Reimer, 2009).

Hepatic steatosis, a hallmark of NAFLD, results from the accumulation of lipids, particularly triglycerides, in the liver (Qin and Tian, 2010). The pathogenesis of NAFLD has not yet been clearly defined; however, adipose tissue dysfunction characterized by insulin resistance is considered a central mechanism involved in the development of steatosis and transition to steatohepatitis in NAFLD (Wang *et al.*, 2010). The liver, which is the major organ responsible for storing excess carbohydrates by converting them to glycogen or lipids, disposes off 25-35% of the oral glucose load (Moore *et al.*, 2012). Recent studies have shown that BCAAs play an important role in the regulation of energy balance and metabolism (Zhang *et al.*, 2007; Macotela *et al.*, 2011). For example, Chen *et al.* (2018) reported that leucine supplementation reduces insulin resistance and hepatic steatosis in db/db mice, which suggests that dietary leucine supplementation is a potential nutritional intervention to attenuate hepatic steatosis and early diabetic neuropathy in type 2 diabetes. Although limited clinical data from non-randomized trials using small groups of patients with diabetes are available, several clinical studies have suggested that DPP4 inhibitors have beneficial effects on liver enzyme levels and histology in patients with NAFLD and type 2 diabetes (Iwasaki *et al.*, 2011; Yilmaz *et al.*, 2012; Mashitani *et al.*, 2016). As insulin resistance contributes to the development of hepatic steatosis, the use of an agent that decreases insulin resistance could be an ideal treatment for hepatic steatosis. Accordingly, we aimed to evaluate the combined effects of DPP4 inhibitors and leucine on the development of hepatic steatosis. The combination of evogliptin and leucine significantly suppressed the progression of fatty liver. The marked reduction in the hepatic TG content was associated with a decrease in the plasma ALT level, a marker of hepatic infiltration that is frequently elevated in hepatic steatosis. These data suggest that a decrease in insulin resistance results in a reduction of the hepatic lipid content and plasma ALT levels. Because caloric intake plays an important role in the pathogenesis of insulin resistance and hepatic steatosis, we measured the mean daily food intake. Our results showed that daily food intake did not differ markedly among the groups, which eliminated the possible effects of hyperphagia on insulin resistance.

Lipogenesis is a key process that underlies hepatic lipid accumulation in HFD-fed mice (Kim *et al.*, 2017a). DPP4 deficiency inhibits lipogenesis, thereby preventing hepatic steatosis in HFD-fed mice (Conarello *et al.*, 2003). Leucine supplementation has also been reported to reduce hepatic steatosis in db/db mice (Chen *et al.*, 2018). PPARα is highly expressed in the liver, and its activation stimulates the expression of genes related to peroxisomal β-oxidation (Rakhshandehroo *et al.*, 2009). Additionally, PPARα inhibits lipogenesis in the liver via both direct and indirect mechanisms (Kim *et al.*, 2017b). As the combination of evogliptin and leucine significantly prevented the formation of hepatic lipid droplets in the liver, we examined the expression of genes involved in hepatic lipogenesis and fatty acid oxidation to elucidate the mechanisms underlying the effects of this combination on hepatic steatosis. In this study, evogliptin did not affect the expression levels of SREBP-1c and SCD-1, which is consistent with the results of a previous study (Kim *et al.*, 2017a). However, evogliptin and leucine alone restored PPARα expression, although these effects were not statistically significant. Furthermore, the combination treatment elicited a significant increase in PPARα expression, compared with that in HFD-fed control mice. Therefore, we suggest that the combination treatment

reduces the degree of hepatic steatosis directly via the activation of PPARα expression or indirectly via the reduction of lipid accumulation. However, further studies are required to elucidate the mechanisms underlying these processes.

In summary, our results showed that the combination of evogliptin and leucine ameliorated hepatic steatosis by improving insulin resistance and upregulating PPARα expression. Therefore, this therapy might be beneficial in patients with type 2 diabetes and hepatic steatosis; however, more rigorous testing is needed to delineate the molecular mechanisms underlying the action of this combination.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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