Canadian Journal of Physiology and Pharmacology
Revue canadienne de physiologie et pharmacologie

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Journal:	<i>Canadian Journal of Physiology and Pharmacology</i>
Manuscript ID	cjpp-2016-0026.R1
Manuscript Type:	Article
Date Submitted by the Author:	19-May-2016
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Keyword:	DPP-4 inhibitor, metabolic syndrome, Western diet, adipose inflammation, non-alcoholic steatohepatitis

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Gemigliptin ameliorates Western diet-induced metabolic syndrome in mice

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Abstract

Dipeptidyl peptidase 4 (DPP-4) inhibitors are widely used anti-hyperglycemic agents for type 2 diabetes mellitus. Recently, increasing attention has been focused on the pleiotropic actions of DPP-4 inhibitors. The aim of the present study was to examine whether gemigliptin, a recently developed DPP-4 inhibitor, could ameliorate features of metabolic syndrome. Mice were fed a Western diet (WD) for 12 weeks and were subsequently divided into two groups: mice fed a WD diet alone or mice fed a WD diet supplemented with gemigliptin for an additional 4 weeks. Gemigliptin treatment attenuated WD-induced body weight gain, hypercholesterolemia, adipocyte hypertrophy, and macrophage infiltration into adipose tissue, which were accompanied by an increased expression of uncoupling protein 1 in subcutaneous fat. These events contributed to improved insulin sensitivity, as assessed by the homeostasis model assessment of insulin resistance and intraperitoneal insulin tolerance test. Furthermore, gemigliptin reduced WD-induced hepatic triglyceride accumulation via inhibition of de novo lipogenesis and activation of fatty acid oxidation, which was accompanied by AMP-dependent protein kinase activation. Gemigliptin ameliorated WD-induced hepatic inflammation and fibrosis through suppression of oxidative stress. These results suggest that DPP-4 inhibitors may represent promising therapeutic agents for metabolic syndrome beyond their current role as anti-hyperglycemic agents.

Keywords: DPP-4 inhibitor, metabolic syndrome, Western diet, adipocyte hypertrophy, adipose inflammation, non-alcoholic steatohepatitis, AMP-dependent protein kinase, oxidative stress

Introduction

Metabolic syndrome is a cluster of clinical features including central obesity, dyslipidemia, hypertension, and hyperglycemia (Eckel et al. 2005). The pathogenesis of metabolic syndrome is complex and poorly understood, but the main contributing factors are considered to be central obesity and insulin resistance. Marked accumulation of immune cells to hypertrophic adipose tissue during obesity has been demonstrated (Esser et al. 2014). These events eventually lead to systemic insulin resistance, metabolic syndrome, and cardiovascular disease. Non-alcoholic fatty liver disease (NAFLD) refers to a spectrum of conditions ranging from simple fat accumulation in the liver to non-alcoholic steatohepatitis (NASH) and cirrhosis. This condition is tightly associated with the individual components of metabolic syndrome and, thereby, can be considered as the hepatic manifestation of metabolic syndrome (Vanni et al. 2010).

Dipeptidyl peptidase 4 (DPP-4) inhibitors are widely used in the effective management of type 2 diabetes mellitus. DPP-4 is an enzyme that is responsible for the inactivation of incretin hormones such as glucagon-like peptide 1 (GLP-1) that enhance post-prandial insulin secretion from pancreatic β cells (Fadini et al. 2011). DPP-4 inhibition elevates the levels of active GLP-1 and reduces elevated blood glucose levels. The beneficial effects of DPP-4 inhibitors are believed to be primarily mediated by the physiological elevation of endogenous GLP-1 and its action on pancreas. However, the GLP-1 receptor is also widely expressed in extrapancreatic tissues (Holst 2007) and DPP-4 has several non-incretin substrates (Fadini et al. 2011). In recent years, extrapancreatic actions of DPP-4 inhibitors have been increasingly investigated. Accumulating evidence suggests that DPP-4 inhibitors may have therapeutic effects on insulin resistance, cardiovascular disease, and diabetic kidney disease in animal models (Aroor et al. 2015; Fadini et al. 2011; Fukuda-Tsuru et al. 2014; Kern et al. 2012; Kim et al. 2012; Tanaka et al. 2014). Several animal studies have reported on the therapeutic effect of DPP-4 inhibitors in inhibiting the accumulation of hepatic fat (Aroor et al. 2015; Kern et al. 2012; Ohyama et al. 2014; Shirakawa et al. 2011). In addition to hepatic steatosis, we recently showed that DPP-4 inhibition has a protective effect against methionine-choline-deficient (MCD) diet-induced hepatic inflammation and fibrosis (Jung et al. 2014).

The rising prevalence of metabolic syndrome parallels the increased consumption of a Western diet (WD) high in fat and fructose (Ahima 2011). Consumption of a Western dietary pattern promotes the development of metabolic syndrome (Lutsey et al. 2008). Feeding mice a WD results in obesity, dyslipidemia, hyperglycemia, insulin

1 resistance, and NASH, demonstrating this model to be clinically relevant to metabolic syndrome (Ishimoto et al.
2 2013; Vos et al. 2013).

3 Gemigliptin is a newly developed selective DPP-4 inhibitor that is currently used in Korea for the treatment of
4 type 2 diabetes mellitus (Yang et al. 2013). In the present study, we evaluated the therapeutic effects of DPP-4
5 inhibition with gemigliptin on the development of clinical manifestations associated with metabolic syndrome
6 including central obesity, adipose inflammation, and NASH in an animal model of metabolic syndrome.

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Materials and methods

Animals and diets

Eight-week-old C57BL/6J mice (Central Lab Animal Inc., Seoul, Republic of Korea) were fed a WD (D12079B; 41% energy as fat, 34.0% sucrose, 0.21% cholesterol; Research Diets Inc., New Brunswick, NJ, USA) for 12 weeks and were subsequently divided into two groups. The two groups of mice were treated with the following regimens: WD alone ($n=6$); and WD mixed with 300 mg/kg per day of gemigliptin (LG Life Sciences Ltd., Seoul, Republic of Korea) for an additional 4 weeks. The dose for gemigliptin was determined based on results from a previous study (Choi et al. 2015). As a control group, 8-week old C57BL/6J mice were maintained on a normal chow diet throughout the study period. Mice were housed at an ambient temperature (20–22°C) under a 12 h:12 h light-dark cycle with free access to water and food. At the end of the study and after overnight fasting, animals were sacrificed and blood and tissue (liver and adipose) samples were collected. All experimental procedures were performed in accordance with the guidelines for the care and use of laboratory animals from the National Institute of Health (USA) and were approved by the Kyungpook National University Institutional Animal Care and Use Committee.

Body composition analysis

Body composition analysis of mice was performed using the MiniSpec LF 50 (Bruker, Ettlingen, Germany). Body fat mass and lean mass were expressed as a percentage of body weight.

Plasma and tissue biochemical assays

Plasma levels of glucose, total cholesterol, and alanine transaminase (ALT) were measured using a Hitachi 7020 automatic analyzer (Hitachi, Tokyo, Japan). Plasma insulin levels were determined using an Ultrasensitive Insulin ELISA kit (ALPCO, Windham, NH, USA) according to the manufacturer's instructions. Plasma levels of active GLP-1 were measured using an ELISA kit (BioVendor, Brno, Czech Republic) in accordance with the manufacturer's instructions. Total hepatic triglyceride levels were assessed using a BioVision triglyceride assay kit (Mountain View, CA, USA) and hepatic 8-OHdG levels were measured using an ELISA kit (Abcam, San Francisco, CA, USA), both according to the manufacturer's instructions.

Homeostasis model assessment of insulin resistance

The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting plasma glucose and insulin levels using the following formula: $\text{HOMA-IR} = \text{fasting plasma glucose (mg/dL)} \times \text{fasting plasma insulin } (\mu\text{U/mL})/405$.

Intraperitoneal insulin tolerance test

For the intraperitoneal insulin tolerance test (IPITT), mice were fasted for 5 h and injected intraperitoneally with human insulin (1 U/kg body weight). Blood samples were collected from the tail vein at different time points. The area under the curve (AUC) was calculated using the trapezoidal rule.

Quantitative real-time RT-PCR

Total RNA was isolated from tissues using Trizol[®] reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed using the ViiA[™] 7 Real-Time PCR System (Life Technologies, Norwalk, CT, USA) and Power SYBR Green PCR Master Mix (Applied Biosystems Life Technologies, Foster City, CA, USA). Primer sequences were as follows: fatty acid translocase (FAT/CD36) forward primer 5'-TGAGCCTTCACTGTCTGTTGGA-3' and reverse primer 5'-CTGCTACAGCCAGATTCAGAACTG-3'; peroxisome proliferator-activated receptor alpha (PPAR α) forward primer 5'-GAACAAAGACGGGATGCTGATC-3' and reverse primer 5'-ACAGAACGGCTTCCTCAGGTT-3'; PPARgamma coactivator-1alpha (PGC-1 α) forward primer 5'-TGCGGGATGATGGAGACA-3' and reverse primer 5'-GCGAAAGCGTCACAGGTGTA-3'; carnitine palmitoyltransferase 1 (CPT1) forward primer 5'-TTATTAAGAACACAAATGTGCAAGCA-3' and reverse primer 5'-AGTTTGCGCCGATACATGATC-3'; long-chain acyl-CoA dehydrogenase (LCAD) forward primer 5'-GGCACAAAAGAACAGATCGAGAA-3' and reverse primer 5'-ATGGCTATGGACCGATACACT-3'; medium-chain acyl-CoA dehydrogenase (MCAD) forward primer 5'-TGACGGAGCAGCCAATGAT-3' and reverse primer 5'-TCTTAATGGCCGCCACATC-3'; microsomal triglyceride transfer protein (MTTP) forward primer 5'-TCTGGCTCTGGCATTCTGAG-3' and reverse primer 5'-CCGACAGCATTTTGGACATC-3'; F4/80 forward primer 5'-TGGCTGCCTCCCTGACTTT-3' and reverse

1 primer 5'-TCCTTTTGCAGTTGAAGTTTCCAT-3'; tumor necrosis factor-alpha (TNF α) forward primer 5'-
2 GACGTGGAAGTGGCAGAAGAG-3' and reverse primer 5'-CCGCCTGGAGTTCTGGAA-3'; interleukin-1beta
3 (IL-1 β) forward primer 5'-GAGCACCTTCTTTTCCTTCATCTT-3' and reverse primer 5'-
4 TCACACACCAGCAGGTTATCATC-3'; transforming growth factor-beta (TGF β) forward primer 5'-
5 TGCTAATGGTGGACCGCAA-3' and reverse primer 5'-CACTGCTTCCCGAATGTCTGA-3'; alpha-smooth
6 muscle actin (α SMA) forward primer 5'-TCCTGACGCTGAAGTATCCGAT-3' and reverse primer 5'-
7 GGCCACACGAAGCTCGTTATAG-3'; plasminogen activator inhibitor-1 (PAI-1) forward primer 5'-
8 CCACAAAGGTCTCATGGACCAT-3' and reverse primer 5'-TGAAAGTGTGTGCCCTCCAC-3'; type 1
9 collagen forward primer 5'-TGTGTTCCCTACTCAGCCGTCT-3' and reverse primer 5'-
10 CATCGGTCATGCTCTCTCCAA-3'; cytochrome P450 2E1 (CYP2E1) forward primer 5'-
11 GCATCCAAAGAGAGGCACACT-3' and reverse primer 5'-GGCTGGCCTTTGGTCTTTTT-3'; 18S rRNA
12 forward primer 5'-CTGGTTGATCCTGCCAGTAG-3' and reverse primer 5'-CGACCAAAGGAACCATAACT-3'.
13 As an internal standard, 18S rRNA was chosen.

14
15 **Western blot analysis**

16 Adipose and liver tissues were prepared using a lysis buffer (20 mM Tris-HCl (pH 7.4), 1% NP40, 5 mM EDTA, 2
17 mM Na₃VO₄, 100 mM NaF, 10 mM Na₄P₂O₇, 100 μ M PMSF, 7 μ g/mL aprotinin, and 7 μ g/mL leupeptin). Proteins
18 were resolved by SDS-PAGE and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). After
19 blocking, the membrane was incubated with primary antibodies. The primary antibodies used were as follows: anti-
20 uncoupling protein 1 (UCP1) antibody (Abcam); anti-sterol regulatory element-binding protein-1c (SREBP1c; BD
21 Biosciences, San Diego, CA, USA); anti-fatty acid synthase (FAS), anti-AMP-activated protein kinase (AMPK),
22 anti-phospho-AMPK (pAMPK), and anti-phospho-eukaryotic initiation factor 2 alpha (p-eIF2 α) were all purchased
23 from Cell Signaling Technology (Danvers, MA, USA); anti-activating transcription factor 4 (ATF4) and anti- β -
24 actin were purchased from Sigma-Aldrich (St. Louis, MO, USA); anti-CCAAT-enhancer-binding protein
25 homologous protein (CHOP; Santa Cruz Biotechnology, Dallas, TX, USA); and anti- β -tubulin (Applied Biological
26 Materials, Richmond, BC, Canada). The membrane was washed and incubated with a horseradish peroxidase-
27 conjugated secondary antibody and signals were detected using an ECL Western blotting detection system
28 (ImageQuant LAS4000; GE Healthcare Life Sciences, Pittsburgh, PA, USA).

Immunofluorescence analysis

Adipose tissues were rapidly removed from each mouse. The tissues were immediately fixed in PBS containing 4% paraformaldehyde and were embedded in paraffin. Paraffin-embedded tissues were sectioned and slides were prepared for immunofluorescence. The slides were incubated with anti-UCP1 antibody overnight at 4°C and were subsequently incubated with Alexa -568-labeled secondary antibody. Nuclei were stained with DAPI and confocal images were acquired using a confocal microscope (FluoView™ FV1000; Olympus, Tokyo, Japan).

Histological and immunohistochemical analysis of adipose tissue

Adipose tissue slides were prepared as described above and were stained with hematoxylin and eosin (H&E). Relative adipocyte size in H&E sections were estimated by counting the size of adipocytes in 10 random high-powered fields in each sample. The relative cell size was normalized to that of chow diet-fed mice. Immunohistochemical staining was performed using an anti-F4/80 primary antibody (Thermo Fisher Scientific) followed by staining with horseradish peroxidase-conjugated anti-rat IgG secondary antibody (Santa Cruz Biotechnology). Images were captured using an Olympus BX53 microscope. The size of the adipocytes was determined using Image J software (National Institutes of Health, Bethesda, MD, USA). The total number of cells and F4/80-positive crown-like structures (CLS), characterized by isolated dead adipocytes surrounded by macrophages, were counted in 10 different high-power fields from each section, and the ratio of CLS per 1000 cells was calculated.

Histological analysis of liver tissue

Liver tissues was fixed in PBS containing 4% paraformaldehyde, embedded in paraffin, and then stained with H&E and Masson's trichrome staining. Masson's trichrome staining was performed using HT15 Trichrome Staining (Masson) Kit (Sigma-Aldrich) according to the manufacturer's instructions. Images were captured using an Olympus BX53 microscope.

Statistical analysis

Data are expressed as the mean $\pm$ standard error of the mean (SEM). The differences between the groups were

assessed using Student’s *t*-test or one-way analysis of variance (ANOVA) with Bonferroni’s post hoc test. A *P* value less than 0.05 was considered statistically significant.

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Results

Effects of gemigliptin on body weight gain and hypercholesterolemia in WD-fed mice

WD-fed mice were treated with gemigliptin for 4 weeks to examine whether DPP-4 inhibition has a therapeutic potential in metabolic syndrome. Significant increases in body weight (Fig. 1A) and food intake (Fig. 1B) were observed in WD-fed mice compared with chow diet-fed mice. The body weight of gemigliptin-treated mice was significantly lower than that of WD-fed mice (Fig. 1A), while food intake was not affected by gemigliptin (Figure 1B). Plasma GLP-1 levels of gemigliptin-treated mice were significantly higher than those of chow diet-fed mice or WD-fed mice at the end of the study (Fig. 1C). WD feeding resulted in increased plasma levels of total cholesterol in mice and was significantly attenuated by gemigliptin treatment (Fig. 1D).

As gemigliptin treatment reduced body weight gain in WD-fed mice, the effects of gemigliptin on body composition in mice were investigated. Gemigliptin treatment significantly reduced body fat mass (Fig. 1E), while lean body mass remained unaffected (Fig. 1F).

Effects of gemigliptin on adipocyte hypertrophy and adipose inflammation in WD-fed mice

Hypertrophic adipocytes are more lipolytic and resistant to insulin action than small adipocytes, thereby adipocyte hypertrophy is considered as an important contributing factor to the development of metabolic diseases (Bays et al. 2008). Next, we examined the effect of gemigliptin on WD-induced adipocyte hypertrophy. A marked increase in adipocyte size in both epididymal white adipose tissue (WAT) and brown adipose tissue (BAT) was observed in WD-fed mice and these increases were attenuated by gemigliptin treatment (Figs. 2A-C).

Adipose inflammation is characterized by macrophage infiltration and plays a critical role in the development of insulin resistance and metabolic complications in obesity (Esser et al. 2014). Interrupting the accumulation of macrophages within hypertrophic adipose tissue suppresses adipose inflammation and ameliorates systemic insulin resistance. We performed immunohistochemical staining of F4/80, a macrophage-specific marker, in epididymal WAT of mice to evaluate the effect of WD and gemigliptin on adipose inflammation. A marked increase in the number of CLS in epididymal WAT (Figs. 2D and 2E) was found in WD-fed mice. Gemigliptin treatment significantly reduced the number of CLS in the epididymal WAT of WD-fed mice (Figs. 2D and 2E). In addition, mRNA expression levels of proinflammatory markers including F4/80, TNF α , and IL-1 β were upregulated by WD

feeding and were significantly attenuated by gemigliptin treatment (Fig. 2F).

Effect of gemigliptin on UCP1 expression in the subcutaneous fat of WD-fed mice

As gemigliptin treatment was found to result in reduced body weight gain and adiposity and did not affect food intake, we hypothesized that gemigliptin treatment may increase thermogenic gene expression to activate adaptive thermogenesis and energy expenditure in adipose tissues of WD-fed mice. Expression of UCP1 protein was significantly higher in the inguinal WAT of gemigliptin-treated mice than in that of untreated mice (Fig. 3A). Immunofluorescence analysis showed an increased expression of UCP1 in the inguinal WAT of gemigliptin-treated mice (Fig. 3B).

Effect of gemigliptin on systemic insulin resistance in WD-fed mice

Fasting glucose levels, fasting insulin levels, and HOMA-IR of WD-fed mice were significantly higher than those of chow diet-fed mice (Figs. 4A-C). After 4 weeks of treatment with gemigliptin, all of these parameters were significantly reduced in WD-fed mice (Figs. 4A-C). Furthermore, the IPITT results demonstrated that gemigliptin treatment improved the glucose-lowering effect of insulin (Fig. 4D), with a significantly lower AUC (Fig. 4E). These results suggest that the beneficial effects of gemigliptin against central obesity, adipocyte hypertrophy, and adipose inflammation that was accompanied by increased UCP1 expression in subcutaneous fat, contributes to improved systemic insulin sensitivity.

Effect of gemigliptin on hepatic steatosis in WD-fed mice

As hepatic steatosis is considered as the hepatic component of metabolic syndrome (Vanni et al. 2010), we next investigated the effect of gemigliptin on hepatic steatosis induced by WD. Gemigliptin treatment significantly reduced liver weight (Fig. 5A) and hepatic triglyceride levels (Fig. 5B) in WD-fed mice. H&E staining and Oil Red O staining also showed a significant reduction in the liver triglyceride content in gemigliptin-treated mice (Fig. 5C). The effect of gemigliptin on hepatic triglyceride metabolism was examined to obtain a mechanistic understanding of its anti-steatotic action. Expression of FAT/CD36, a key enzyme involved in fatty acid uptake, was significantly increased in WD-fed mice, but was not affected by gemigliptin treatment (Fig. 6A). Treatment with gemigliptin significantly increased the expression of genes involved in fatty acid oxidation including PPAR α , PGC-1 α , CPT1,

and MCAD in the livers of WD-fed mice (Fig. 6B). To examine the effect of gemigliptin on de novo lipogenesis, protein levels of SREBP-1c and FAS in the liver were assessed. Markedly higher levels of SREBP-1c and FAS were found in the livers of WD-fed mice than in chow diet-fed mice (Fig. 6C). Treatment with gemigliptin resulted in a significant reduction in their increased expression (Fig. 6C). In addition, mRNA expression of MTP, a lipid transfer protein required for the assembly and secretion of apolipoprotein B-containing lipoproteins, was also increased in WD-fed mice, but was not affected by gemigliptin treatment (Fig. 6D). We next examined the effect of gemigliptin on AMPK phosphorylation as AMPK plays an important role in suppressing de novo lipogenesis and increasing fatty acid oxidation (Viollet et al. 2009). Gemigliptin treatment significantly increased AMPK phosphorylation in the livers of WD-fed mice (Fig. 6E).

Effect of gemigliptin on hepatic inflammation and fibrosis in WD-fed mice

The effect of gemigliptin on WD-induced hepatic inflammation and fibrosis was investigated. H&E staining and Masson's trichrome staining revealed that WD feeding causes ballooning degeneration and fibrosis in livers and these changes were attenuated by gemigliptin treatment (Fig. 7A). Treatment with gemigliptin also significantly reduced plasma ALT levels in WD-fed mice (Fig. 7B). In addition, mRNA expression levels of inflammation-related (TNF α and IL-1 β ; Fig. 7C) and fibrosis-related (TGF β , α SMA, PAI-1, and type 1 collagen; Fig. 7D) genes in livers of WD-fed mice were significantly attenuated by gemigliptin treatment.

As oxidative stress play an important role in the pathogenesis of NASH (Jung et al. 2014), we examined the effect of gemigliptin on oxidative stress induced by WD feeding. Gemigliptin treatment significantly decreased the elevated mRNA expression of CYP2E1, a major pro-oxidant enzyme, in the livers of WD-fed mice (Fig. 7E). Moreover, the expression of 8-OHdG, a marker of oxidative DNA damage, was increased in the livers of WD-fed mice and this increase was significantly attenuated by gemigliptin treatment (Fig. 7F).

Discussion

In the present study, we investigated the therapeutic effects of gemigliptin, a newly developed DPP-4 inhibitor, on metabolic syndrome in a clinically relevant animal model. Major novel findings in the present study were as follows: gemigliptin treatment increased UCP1 expression in subcutaneous fat of WD-fed mice; gemigliptin attenuated hepatic steatosis via inhibition of de novo lipogenesis and activation of fatty acid oxidation, which was accompanied by AMPK activation; and gemigliptin ameliorated obesity-induced hepatic inflammation and fibrosis, which was accompanied by suppression of oxidative stress.

A Western dietary pattern is characterized by consumption of high levels of fat and fructose. Cross-sectional clinical studies show that a Western dietary pattern is closely associated with metabolic syndrome (Esmailzadeh et al. 2007; Paradis et al. 2009). Recently, Lutsey and colleagues evaluated the association between dietary pattern and the risk of developing metabolic syndrome over 9 years of follow-up in men and women enrolled in the Atherosclerotic Risk in Communities study (Lutsey et al. 2008). This large prospective study demonstrated that consumption of a Western dietary pattern promotes the incidence of metabolic syndrome. Furthermore, an increased risk of NAFLD in adults has been shown to be significantly associated with a Western dietary pattern (Oddy et al. 2013; Zelber-Sagi et al. 2007). Consistent with the results from previous clinical studies, feeding animals a WD results in a significant induction of obesity, dyslipidemia, hyperglycemia, insulin resistance, and NASH (Ishimoto et al. 2013; Vos et al. 2013). Thus, WD feeding may be considered as a clinically relevant animal model of metabolic syndrome. In the present study, gemigliptin treatment was demonstrated to ameliorate features of metabolic syndrome induced by WD feeding, suggesting that DPP-4 may be an important therapeutic target for the treatment of metabolic syndrome. These results parallel the findings of clinical studies that have suggested a positive relationship between DPP-4 expression and metabolic syndrome (Lamers et al. 2011; Sell et al. 2013). Lamers and co-workers reported that levels of DPP-4 released from adipose tissue positively correlated with an increasing risk score for metabolic syndrome in lean and obese subjects (Lamers et al. 2011). Sell et al. demonstrated a significantly higher DPP-4 release from adipose tissue in obese and insulin-resistant subjects compared with lean and insulin-sensitive controls (Sell et al. 2013).

Recently, extrapancreatic action of DPP-4 inhibitors has received much attention because their clinical benefits are not fully explained only by its glucose-lowering effects (Fadini et al. 2011). In the present study, DPP-4

inhibition with gemigliptin treatment was found to reduce body weight gain and fat mass in WD-fed mice. In agreement with our findings, several studies have shown that animals treated with other DPP-4 inhibitors exhibited a significant resistance to diet-induced obesity (Fukuda-Tsuru et al. 2014; Kim et al. 2012; Shimasaki et al. 2013). These findings suggest that the body weight-lowering effect is likely to be a class effect of DPP-4 inhibitors. Furthermore, for the first time, we demonstrated that gemigliptin treatment significantly increases the expression of UCP1 in subcutaneous fat, suggesting that DPP-4 inhibition induces browning of subcutaneous fat in mice. Browning is an important process in increasing energy expenditure and controlling body weight in mice and humans (Bartelt, et al. 2014). Given that food intake was not affected by gemigliptin treatment, the anti-obesity action of gemigliptin may be largely attributed to increased energy expenditure by its browning-inducing effect. Consistent with our findings, an indirect calorimetry study of mice lacking DPP-4 revealed an increase in energy expenditure (Conarello et al. 2003), and treatment with a DPP-4 inhibitor prevented diet-induced obesity accompanied with elevated energy expenditure in mice (Fukuda-Tsuru et al. 2014). Further studies are required to examine the molecular mechanism of the browning-inducing effect of DPP-4 inhibitors. However, because DPP-4 inhibitors show no effect on body weight in clinical practice, caution is necessary when extrapolating findings from animal studies to humans.

During progression of obesity, adipocyte size increases and hypertrophic cells are prone to macrophage infiltration, leading to the development of systemic insulin resistance (Esser et al. 2014). In the present study, WD feeding resulted in increased adipocyte size, macrophage infiltration into adipose tissue, and elevated expression of proinflammatory genes and treatment with gemigliptin significantly attenuated these events. Furthermore, gemigliptin treatment also improved WD-induced systemic insulin resistance. These results are consistent with previous studies showing that DPP-4 inhibitors have an anti-inflammatory effect in adipose tissue (Dobrian et al. 2011; Shirakawa et al. 2011). Interrupting the accumulation of macrophages into hypertrophic adipose tissue suppresses adipose inflammation and ameliorates systemic insulin resistance (Bays et al. 2008). Thus, the therapeutic effect of gemigliptin on systemic insulin resistance appears to be attributed, at least in part, to its anti-inflammatory action in adipose tissue. The mechanism underlying the anti-inflammatory effect of DPP-4 inhibitors in adipose tissue remains unknown, although the direct regulation of immune cell function by DPP-4 inhibition may be an important contributing factor (Iwata et al. 1999; Kim et al. 2010).

Accumulating evidence from animal studies demonstrates that DPP-4 inhibitors have a therapeutic effect against

hepatic steatosis (Aroor et al. 2015; Kern et al. 2012; Ohyama et al. 2014; Shirakawa et al. 2011). Hepatic steatosis is a manifestation of excessive triglyceride accumulation in the liver and may be due to the increased delivery of free fatty acids from the diet or adipose lipolysis, increased de novo lipogenesis, impaired fatty acid oxidation, and/or reduced hepatic export of triglycerides (Postic et al. 2008). In the present study, gemigliptin treatment was observed to decrease levels of protein expression of genes related to de novo lipogenesis and increased mRNA expression of genes related to fatty acid oxidation, while fatty acid uptake and hepatic export of triglycerides were not affected. Previous studies performed with other DPP-4 inhibitors demonstrate conflicting results regarding the mechanism underlying their anti-steatotic effect (Aroor et al. 2015; Ohyama et al. 2014; Shirakawa et al. 2011). These discrepancies may be explained by the differences in the properties of each DPP-4 inhibitor, genetic background and age of mice used, dietary composition, and feeding duration. In the present study, gemigliptin treatment induced the phosphorylation of AMPK in the liver of mice. AMPK plays an important role in suppressing de novo lipogenesis and increasing fatty acid oxidation, preventing the development and progression of NAFLD (Viollet et al. 2009). Our findings suggest that activation of AMPK in the liver by gemigliptin treatment may be responsible for its anti-steatotic effect.

Whereas many studies have focused on the anti-steatotic effect of DPP-4 inhibitors, their potential therapeutic effect on NASH have been rarely examined. Recently, we reported that sitagliptin protects against MCD diet-induced hepatic inflammation and fibrosis by regulating lipid accumulation, endoplasmic reticulum stress, and oxidative stress (Jung et al. 2014). While revealing the therapeutic effect of DPP-4 inhibition on NASH, our previous study was limited to the MCD diet, which may be regarded as a clinically irrelevant model of NASH. This model has several disadvantages such as significant weight loss, low serum leptin levels, and increased peripheral insulin sensitivity, and are quite different to human NASH (Larter et al. 2008). In the present study, we evaluated the effect of gemigliptin on NASH by using a clinically relevant animal model. DPP-4 inhibition with gemigliptin was demonstrated to have a therapeutic effect against WD-induced NASH by regulating lipid metabolism and oxidative stress. Although several clinical studies show the therapeutic effect of DPP-4 inhibitors against NAFLD (Iwasaki et al. 2011; Yilmaz et al. 2012), these studies were non-randomized trials and were performed in small groups of diabetic patients. Based on the results from the present and previous studies, large well-designed clinical trials are warranted to assess the therapeutic potential of DPP-4 inhibitors in human NASH.

In conclusion, the present study demonstrated that DPP-4 inhibition with gemigliptin treatment reduced WD-

1 induced body weight gain, hypercholesterolemia, adipocyte hypertrophy, and adipose inflammation, and was
2 accompanied by increased UCP1 expression in subcutaneous fat. The effects of gemigliptin treatment resulted in an
3 improvement in insulin sensitivity. Moreover, gemigliptin treatment ameliorated WD-induced NASH via inhibition
4 of de novo lipogenesis, activation of fatty acid oxidation, and suppression of oxidative stress. These results support
5 the idea that DPP-4 inhibitors may represent promising therapeutic agents for metabolic syndrome beyond their
6 current role as anti-hyperglycemic agents.

8 **Acknowledgments**

9 This work was supported by a National Research Foundation of Korea grant awarded by the Korean Ministry of
10 Education (2012R1A2A1A03670452) and a grant of the Korea Health Technology R&D project through the Korea
11 Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea
12 (HI16C1501).

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Figure legends

Fig. 1. Gemigliptin attenuated body weight gain and hypercholesterolemia in Western diet (WD)-fed mice. Eight-week old C57BL/6J mice were fed a WD for 12 weeks and were subsequently divided into two groups. One group was fed a WD alone and the other group was fed a WD mixed with 300 mg/kg per day of gemigliptin for an additional 4 weeks. As a control group, mice were maintained on a chow diet throughout the entire study. (A) Body weight at the end of the 16-week study. (B) Food intake. (C) Plasma levels of active glucagon-like peptide 1 (GLP-1). (D) Plasma levels of total cholesterol. Body fat mass (E) and lean mass (F) were expressed as a percentage of body weight. Body composition was measured by nuclear magnetic resonance. $n = 6$ per group. All data are expressed as mean $\pm$ SEM. $*P < 0.05$. NS, not significant.

Fig. 2. Gemigliptin reduced adipocyte cell size and macrophage infiltration into epididymal white adipose tissue in Western diet (WD)-fed mice. Epididymal white adipose tissue (WAT) and brown adipose tissue (BAT) were stained with hematoxylin and eosin (H&E). (A) Representative images of epididymal WAT and BAT from mice fed a chow diet, WD, or WD mixed with gemigliptin. Original magnification, $\times 100$. Adipocyte cell sizes in epididymal WAT (B) and BAT (C) were determined using Image J software and presented as a value relative to that for chow diet-fed mice. (D) Representative images of sections stained with anti-F4/80 antibody. Arrows indicate the crown-like structures (CLS). Original magnification, $\times 400$. (E) The total number of F4/80-positive CLS was counted in 10 different high-power fields from each section, and the ratio of CLS per 1000 cells was calculated. (F) Real-time RT-PCR analysis of F4/80, tumor necrosis factor- α (TNF α), and interleukin-1 β (IL-1 β) mRNA in epididymal WAT of mice. $n = 6$ per group. All data are expressed as mean $\pm$ SEM. $*P < 0.05$.

Fig. 3. Gemigliptin increased protein expression of uncoupling protein 1 in subcutaneous fat of Western diet (WD)-fed mice. (A) Western blot analysis of the expression of uncoupling protein 1 (UCP1) and β -tubulin in inguinal fat from mice fed a chow diet, WD, or WD mixed with gemigliptin. (B) Immunofluorescence analysis of UCP1 in subcutaneous fat. Red color indicates UCP1 staining and blue color indicates DAPI staining of cell nuclei. $n = 6$ per group. All data are expressed as mean $\pm$ SEM. $*P < 0.05$. NS, not significant.

Fig. 4. Gemigliptin ameliorated insulin resistance in Western diet (WD)-fed mice. Levels of fasting plasma glucose (A), levels of fasting plasma insulin (B), and homeostasis model assessment of insulin resistance (HOMA-IR) values (C) in mice fed a chow diet, WD, or WD mixed with gemigliptin. HOMA-IR was calculated using the fasting plasma glucose and insulin. (D) Intraperitoneal insulin tolerance test (IPITT). An IPITT was performed as described in the Materials and methods. The area under the curve (AUC) for the IPITT (E) was presented as a value relative to that for chow diet-fed mice. $n = 6$ per group. All data are expressed as mean $\pm$ SEM. $*P < 0.05$. $^{\#}P < 0.05$ compared with WD-fed mice.

Fig. 5. Gemigliptin attenuated hepatic triglyceride accumulation in Western diet (WD)-fed mice. Liver weight (A) and liver triglyceride content (B) of mice fed a chow diet, WD, or WD mixed with gemigliptin. (C) Representative images of hematoxylin and eosin (H&E) staining and Oil Red O staining in mice livers. Original magnification, $\times 100$. $n = 6$ per group. All data are expressed as mean $\pm$ SEM. $*P < 0.05$.

Fig. 6. Gemigliptin decreased hepatic triglyceride accumulation via inhibition of de novo lipogenesis and activation of fatty acid oxidation, which was accompanied by AMP-activated protein kinase activation. (A) Real-time RT-PCR analysis of fatty acid translocase (FAT/CD36) mRNA in the livers of mice fed a chow diet, Western diet (WD), or WD mixed with gemigliptin. (B) Western blot analysis of the expression of sterol regulatory element-binding protein-1c (SREBP1c), fatty acid synthase (FAS), and β -tubulin in mice livers. (C) Real-time RT-PCR analysis of peroxisome proliferator-activated receptor alpha (PPAR α), PPARgamma coactivator-1alpha (PGC-1 α), carnitine palmitoyltransferase 1 (CPT1), long-chain acyl-CoA dehydrogenase (LCAD), and medium-chain acyl-CoA dehydrogenase (MCAD) mRNA in mice livers. (D) Real-time RT-PCR analysis of Microsomal triglyceride transfer protein (MTTP) mRNA in mice livers. (E) Western blot analysis of the expression of phospho-AMP-activated protein kinase (pAMPK), AMPK, and β -actin in mice liver. $n = 6$ per group. All data are expressed as mean $\pm$ SEM. $*P < 0.05$. NS, not significant.

Fig. 7. Gemigliptin ameliorated hepatic inflammation and fibrosis, which was accompanied by suppression of oxidative stress in Western diet (WD)-fed mice. (A) Representative images of hematoxylin and eosin (H&E) staining (original magnification, $\times 200$) and Masson's trichrome staining (original magnification, $\times 100$) in the livers

1 of mice fed a chow diet, WD, or WD mixed with gemigliptin. Arrows indicate ballooning degeneration of
2 hepatocytes. (B) Plasma levels of alanine aminotransferase (ALT) in mice livers. (C) Real-time RT-PCR analysis of
3 tumor necrosis factor-alpha ($\text{TNF}\alpha$) and interleukin-1beta ($\text{IL-1}\beta$) mRNA in mice livers. (D) Real-time RT-PCR
4 analysis of transforming growth factor-beta ($\text{TGF } \beta$), alpha-smooth muscle actin (αSMA), plasminogen activator
5 inhibitor-1 (PAI-1), and type 1 collagen mRNA in mice livers. (E) Real-time RT-PCR analysis of cytochrome P450
6 2E1 (CYP2E1) mRNA in mice livers. (F) 8-OHdG levels in mice livers. $n = 6$ per group. All data are expressed as
7 mean $\pm$ SEM. $*P < 0.05$.

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Draft

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