

DPP-4 inhibitors improve cognition and brain mitochondrial function of insulin-resistant rats

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Abstract

Recent evidence has demonstrated that insulin resistance is related to the development of type 2 diabetes mellitus. Our previous study found that high-fat diet (HFD) consumption caused not only peripheral and brain insulin resistance but also brain mitochondrial dysfunction and cognitive impairment. Vildagliptin and sitagliptin, dipeptidyl-peptidase-4 inhibitors, are recently developed anti-diabetic drugs. However, the effects of both drugs on cognitive behaviors and brain mitochondrial function in HFD-induced insulin-resistant rats have not yet been investigated. Sixty male Wistar rats were divided into two groups to receive either normal diet or HFD for 12 weeks. Rats in each group were then further divided into three treatment groups to receive either vehicle, vildagliptin (3 mg/kg per day), or sitagliptin (30 mg/kg per day) for 21 days. The cognitive behaviors of the rats were tested using the Morris Water Maze test. Blood samples were collected to determine metabolic parameters and plasma oxidative stress levels. Upon completion of the study, the animals were killed and the brains were removed to investigate brain and hippocampal mitochondrial function as well as to determine oxidative stress levels. We demonstrated that both drugs significantly improved the metabolic parameters and decreased circulating and brain oxidative stress levels in HFD-induced insulin-resistant rats. In addition, both drugs completely prevented brain and hippocampal mitochondrial dysfunction and equally improved the learning behaviors impaired by the HFD. Our findings suggest that the inhibition of dipeptidyl-peptidase-4 enzymes with vildagliptin or sitagliptin in insulin-resistant rats not only increases peripheral insulin sensitivity but also decreases brain dysfunction.

Key Words

- ▶ insulin resistance
- ▶ high-fat diet
- ▶ DPP-4 inhibitors
- ▶ memory decline
- ▶ brain mitochondrial dysfunction

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Introduction

Insulin resistance is a pathological condition in which tissues do not respond to normal physiological insulin concentrations resulting in increased insulin concentration required to maintain glucose homeostasis (Kahn

& Flier 2000, Petersen & Shulman 2006). Insulin resistance plays a major role in the development of type 2 diabetes mellitus (T2DM). Our previous studies have shown that high-fat diet (HFD) consumption for a period of 12 weeks

not only results in peripheral and neuronal insulin resistance (Prachayasakul *et al.* 2011) but also leads to negative effects on brain mitochondrial function (Pipatpiboon *et al.* 2012). Several previous studies have demonstrated that insulin resistance is associated with learning and memory decline (Greenwood & Winocur 2005, Stranahan *et al.* 2008, Valladolid-Acebes *et al.* 2011). In addition, increasing amounts of evidence demonstrate an association between dementia and T2DM (Janson *et al.* 2004, Zhao & Townsend 2009, Kim *et al.* 2010). In this context, anti-diabetic agents such as rosiglitazone and glucagon-like peptide (GLP-1) have been reported to negate cognitive decline (During *et al.* 2003, Escribano *et al.* 2009, Wang *et al.* 2011). Recently developed oral anti-diabetic drugs, DDP-4 inhibitors including sitagliptin and vildagliptin, have been used to treat T2DM patients. Previous studies have shown that DDP-4 inhibitors decrease insulin resistance without causing severe hypoglycemia (Burkey *et al.* 2005, Richter *et al.* 2008), via prolonged levels of endogenous active GLP-1(7–36) amide (Rosenstock & Zinman 2007, Richter *et al.* 2008). GLP-1 is an incretin hormone secreted from intestinal L-cells following nutrient digestion, which causes decreased glucagon secretion and increased insulin sensitivity (Baggio & Drucker 2007).

Previous studies on diabetes-related Alzheimer's disease rat models have demonstrated that GLP-1 positively affects learning and memory (During *et al.* 2003, Chen *et al.* 2011b). In addition, a recent study has shown that sitagliptin can increase the levels of active GLP-1 in the brain and improve memory behaviors in Alzheimer's disease mice models (D'Amico *et al.* 2010). However, the effects of vildagliptin and sitagliptin on metabolic parameters, cognitive behaviors, and brain mitochondrial function in HFD-induced insulin-resistant rats have never been investigated. In this study, we tested the hypothesis that vildagliptin and sitagliptin can attenuate the impairment of cognitive behaviors and brain mitochondrial dysfunction in rats with insulin resistance induced by 12 weeks of HFD consumption.

Materials and methods

Animal models

Sixty male Wistar rats, weighing 180–200 g (~6–7 weeks old), from the National animal center, Salaya campus, Mahidol University, Bangkok, Thailand, were used for this study. All experiments were conducted in accordance with approved protocol from the Faculty of Medicine, Chiang

Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines. All animals were housed in environmentally controlled conditions (25±0.5 °C, 12 h light:12 h darkness cycle) and allowed to acclimate for 1 week. Rats were then divided into two groups ($n=30$ /group). Each group was fed either the normal diet (ND) or the HFD for 12 weeks. Animals in the ND group were fed with standard laboratory pelleted diet (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand), containing 19.7% total energy from fat, whereas animals in the HFD group were fed a diet containing 59.3% total energy from fat, as described in our previous study (Prachayasakul *et al.* 2011). The composition of ND was 51.9% carbohydrate, 19.7% fat, and 28.4% protein, but the composition of HFD was 14.3% carbohydrate, 59.3% fat, and 26.4% protein. The carbohydrate and fat composition of ND was rice starch (51.9% w/w) and soya oil (19.7%, w/w). The carbohydrate composition of HFD was rice starch (14.3% w/w) and the composition of fat was from lard (57.6%) and cholesterol (1.7%). All animals were given free access to drink water and their respective diets. At the end of the 12th week, rats in each group were divided into three subgroups ($n=10$ /subgroup) to receive vehicle (normal saline solution; 2 ml/kg per day), vildagliptin (Galvus, Novartis; 3 mg/kg per day), or sitagliptin (Januvia, MSD, Bangkok, Thailand; 30 mg/kg per day) (Chen *et al.* 2011a) via gavage feeding for 21 days. It has been shown that 3 mg/kg per day of vildagliptin and 30 mg/kg per day of sitagliptin reduces peripheral insulin resistance in insulin-resistant and diabetic rat models (Burkey *et al.* 2005).

Experimental protocols

The open-field activity level test was performed at the end of week 12 (before and after pharmacological treatment; $n=10$ per subgroup) to determine the locomotive function of each animal. All animals with positive open-field activities further went to the test for cognitive behaviors. Cognitive behaviors were determined via the Morris Water Maze (MWM) test at the end of week 12 and at the end of week 15 (after pharmacological treatment; $n=10$ per subgroup). The body weight of all animals was recorded weekly. Blood samples were collected from a tail vein at week 0 (before dietary feeding), week 12, and week 15 for further plasma analysis ($n=10$ per subgroup). At the end of treatment, ten animals in each subgroup were divided into two sets of experiments. The first set of animals ($n=6$ per subgroup) was deeply anesthetized with isoflurane and killed via decapitation. Each brain was rapidly removed

and used to determine brain mitochondrial function and oxidative stress levels. The second set of animals ($n=4$ per subgroup) was used to determine the glucose metabolism by measuring plasma glucose and plasma insulin levels during an oral glucose tolerance test (OGTT; Pipatpiboon *et al.* 2012). At the end of the experiment, animals were killed and the hippocampus was rapidly isolated for determining hippocampal mitochondrial function.

Plasma analysis

Plasma glucose and cholesterol levels were determined via the colorimetric assay (Biotech, Bangkok, Thailand). Plasma HDL and LDL/VLDL levels were determined via a commercial colorimetric assay kit (Biovision, Milpitas, CA, USA). Plasma insulin and GLP-1(7–36 amide) levels were determined via the Sandwich ELISA kit (LINCO Research, St Charles, MO, USA). Peripheral insulin resistance was assessed via the homeostasis model assessment (HOMA) as described in the previous studies (Haffner *et al.* 1997, Appleton *et al.* 2005). Plasma malondialdehyde (MDA) was determined using a HPLC-based assay (Grotto *et al.* 2007).

Open-field test

The open-field test was used to screen locomotive activity and was modified from the methods of Arakawa (2005). The open field consisted of a black box with a floor (75×75 cm) and 40 cm walls. The box floor was painted with 6 mm white lines to form 25 equal squares. During a 2-min observation period, the animal was placed in the middle square of the apparatus. The total number of lines crossed was measured manually from video tape recording.

MWM test

The MWM test was modified from the methods of Vorhees & Williams (2006) and used for the purpose of cognitive or learning and memory behaviors assessment. The test was performed in a 170 cm diameter water pool virtually divided into four quadrants. The pool was filled with water ($26\pm 1^\circ\text{C}$) and made opaque with cassava flour. A clear platform of 10 cm in diameter was submerged ~ 1 cm beneath the water surface and located in a designated target quadrant. The MWM test was performed at the end of week 12 and week 15 for each rat. Each test included two different assessments: the acquisition test (existent platform) and the probe test (non-existent platform). The acquisition test was performed on five consecutive days of training with four trials per day. Animals were given 120 s to locate the hidden platform. Any animals that could not

find the platform within the 120-s period were guided to the platform. After the platform was found, the animal was allowed to remain on the platform for 15 s before the next test began by placing the animal at a starting point within the other three remaining quadrants. The acquisition time began at the moment the animal entered the water and ended at the moment the animal reached the submerged platform. In the probe test, animals were tested on the 6th day of training with only one starting point. The probe time was the amount of time the animals spent in the target quadrant during the 90-s testing period. The data analysis of MWM was done manually from video tape recording with the investigator blinded to the groups of rats.

Preparation of brain and hippocampal mitochondria

Mitochondria were isolated as described in our previous study (Pipatpiboon *et al.* 2012). Shortly after decapitation, the brain (or hippocampus) was removed and placed in 5 ml ice-cold MSE solution, transferred to 10 ml ice-cold MSE-nagarse solution (0.05% nagarse in MSE solution), and homogenized at 4 g using a homogenizer. Then, brain homogenate was centrifuged at 2000 g for 4 min. The supernatant was collected and further centrifuged at 12 000 g for 11 min. Mitochondrial pellets were collected and resuspended in 10 ml ice-cold MSE-digitonin solution (0.02% digitonin in MSE solution). Finally, the mitochondrial pellets were resuspended in respiration buffer (150 mM KCl, 5 mM HEPES, 5 mM $\text{K}_2\text{HPO}_4\cdot 3\text{H}_2\text{O}$, 2 mM L-glutamate, and 5 mM pyruvate sodium salt). Mitochondrial protein concentration was measured via the BCA assay (Thummasorn *et al.* 2011).

Experimental protocol for brain and hippocampal mitochondrial function

Brain and hippocampal mitochondrial functions in each group were measured. We used 2 mM H_2O_2 applied onto mitochondria for 30 min to induce oxidative stress before the measurement of reactive oxygen species (ROS) production, mitochondrial membrane potential changes, and mitochondrial swelling. For mitochondria isolated from the hippocampus, only mitochondrial ROS production was measured as the amount of isolated mitochondria from the hippocampus was very small and was sufficient only for the determination of mitochondrial ROS production.

Brain and hippocampal mitochondrial ROS assay

Both brain and hippocampal mitochondrial ROS were measured via the use of dichloro-hydrofluoresceindiacetate

(DCFHDA). Brain mitochondria (0.4 mg/ml) were incubated with 2 μ M DCFHDA at 25 °C for 20 min. The fluorescence was determined using a fluorescent microplate reader at the excitation wavelength of 485 nm and emission wavelength of 530 nm (Thummasorn *et al.* 2011, Pipatpiboon *et al.* 2012).

Brain mitochondrial membrane potential ($\Delta\Psi_m$) assay

The change in mitochondrial membrane potential ($\Delta\Psi_m$) within isolated brain mitochondria was measured via the fluorescent dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolcarbocyanine iodide (JC-1). JC-1 monomer form (green) fluorescence was excited at a wavelength of 485 nm and detected at the emission wavelength of 590 nm. JC-1 aggregate form (red) fluorescence was excited at a wavelength of 485 nm and detected at the emission wavelength of 530 nm. Brain mitochondria (0.4 mg/ml) were incubated with JC-1 dye at 37 °C for 15 min. Mitochondrial membrane potential was determined by fluorescent intensity via a fluorescent microplate reader. The change in mitochondrial membrane potential was calculated as the ratio of red to green fluorescent intensity (Thummasorn *et al.* 2011, Pipatpiboon *et al.* 2012).

Brain mitochondria swelling assay

Brain mitochondrial swelling was determined by measuring the change in the absorbance of the brain mitochondrial suspension. Brain mitochondria (0.4 mg/ml) were incubated in 2 ml respiration buffer. The suspension was read at 540 nm via the use of a microplate reader. Mitochondrial swelling was indicated by a decrease in the absorbance (Thummasorn *et al.* 2011, Pipatpiboon *et al.* 2012).

Determination of plasma and brain MDA levels

The HPLC method was used to evaluate concentrations of plasma and brain MDA, which acts as an indicator of oxidative stress (Candan & Tuzmen 2008). To briefly summarize, the brain tissue was homogenized in a phosphate buffer (pH 2.8). The plasma or brain homogenate was mixed with 10% trichloroacetic acid (TCA) containing butylated hydroxytoluene (BHT), incubated at 90 °C for 30 min, and centrifuged at 3300 g for 10 min. The supernatant was mixed with H₃PO₄ and thiobarbituric acid solution (TBA) and incubated at 90 °C for 30 min to produce TBA reactive substances (TBARS). TBARS levels were measured via the absorbance, which was read at 532 nm by the HPLC system. Absorbance was determined directly from the standard curve and reported as the equivalent concentration of MDA.

Statistical analysis

Data were expressed as mean \pm s.e.m. Comparison between the two groups before the treatment was performed using an independent *t*-test. Comparison among groups after pharmacological treatment was performed using the two-way ANOVA test followed by the Fisher's test as the *post hoc* analysis. *P* < 0.05 was considered statistically significant.

Results

Metabolic parameters and plasma oxidative stress levels

At baseline levels, the body weight, food intake, plasma glucose, insulin, total cholesterol, and plasma MDA levels did not differ between ND-fed rats and HFD-fed rats (Table 1). After the end of 12 weeks, HFD-fed rats had significantly increased body weight, plasma insulin levels,

Table 1 The metabolic parameters and oxidative stress levels of ND- and HFD-fed rats. Data are presented as mean \pm s.e.m. with independent *t*-test for the statistical analysis (*n* = 30/group)

Metabolic parameters	Week 0 or baseline		Week 12	
	ND	HFD	ND	HFD
Body weight (g)	198 \pm 10	192 \pm 9	465 \pm 6*	526 \pm 12*,†
Food intake (g/day)	22 \pm 0.9	20 \pm 6	22 \pm 1	20 \pm 6
Plasma insulin (ng/ml)	2.0 \pm 0.35	2.1 \pm 0.78	2.11 \pm 0.32	3.57 \pm 0.44*,†
Plasma glucose (mg/dl)	136 \pm 8.09	138.11 \pm 7.52	141.33 \pm 8.42	144.16 \pm 3.23
HOMA index	17.32 \pm 1.32	17.43 \pm 1.56	17.66 \pm 2.55	26.80 \pm 3.54*,†
Plasma total cholesterol (mg/dl)	83.12 \pm 4.82	83.16 \pm 7.72	83.34 \pm 9.09	152.43 \pm 7.16*,†
Plasma MDA (μ mol/ml)	2.14 \pm 0.23	2.14 \pm 0.12	2.65 \pm 0.43	6.43 \pm 0.11*,†

ND, normal diet; HFD, high-fat diet. **P* < 0.05 in comparison with the baseline of the same diet, †*P* < 0.05 in comparison with the 12-week ND group.

HOMA index, plasma cholesterol levels, and plasma MDA levels in comparison with ND-fed rats (Table 1). These findings indicated that HFD-fed rats developed peripheral insulin resistance characterized by increased plasma insulin levels and HOMA index without an increase in plasma glucose levels.

After 21 days of either vildagliptin or sitagliptin treatments in ND-fed rats, the metabolic parameters, including body weight, food intake, visceral fat, plasma insulin levels, plasma glucose levels, plasma cholesterol levels, plasma MDA levels, HOMA index, and plasma HDL levels were not significantly different from those of the vehicle-treated rats (NDV) (Table 2). Vehicle-treated HFD-fed rats (HFDV) also showed the characteristics of peripheral insulin resistance including increased body weight, visceral fat, plasma cholesterol levels, plasma insulin levels, and HOMA index as well as decreased plasma HDL levels in comparison with NDV rats (Table 2). However, HFD-fed rats treated with vildagliptin (HFDVil) and with sitagliptin (HFDSi) displayed significantly decreased HOMA index, plasma insulin levels, and plasma cholesterol levels as well as increased plasma HDL levels in comparison with the HFDV group. However, neither vildagliptin nor sitagliptin altered the body weight, food intake, visceral fat, or plasma glucose levels in comparison with the HFDV group (Table 2). In the OGTT study, the total area under the glucose curve (AUCg) and the total area under the insulin curve (AUCi) in the HFDV group was significantly greater than the NDV, NDVil, and NDSi groups ($P < 0.05$, Table 2). Vildagliptin and sitagliptin

significantly reduced AUCg and AUCi only in the HFD-fed rats ($P < 0.05$, Table 2). These findings also suggest that both vildagliptin and sitagliptin improve the peripheral insulin sensitivity. In addition, the plasma levels of active GLP-1 were significantly reduced in the HFDV group compared with the NDV group ($P < 0.05$, Table 2). Both vildagliptin and sitagliptin significantly increased the plasma level of active GLP-1 in the HFD-fed rats ($P < 0.05$, Table 2).

We also found that both plasma MDA levels and brain MDA levels within the HFDV group were significantly increased in comparison with the NDV group (Table 2). However, plasma and brain oxidative stress levels within the HFDVil group and HFDSi group significantly decreased in comparison with the HFDV group (Table 2). These findings suggest that HFD consumption not only induces peripheral insulin resistance but also leads to oxidative stress in the plasma and brain. The administration of either vildagliptin or sitagliptin reduces peripheral insulin resistance, peripheral oxidative stress, and brain oxidative stress induced by HFD consumption.

Vildagliptin and sitagliptin improved cognitive behaviors in HFD-fed rats assessed by the MWM test

Locomotive activity was determined by an open-field test. We found that the number of lines that the rats crossed during the test at the end of week 12 was not significantly different between the ND group (41.67 ± 3.91) and the HFD group (42.33 ± 2.54) as shown in Fig. 1A.

Table 2 Effects of vildagliptin (3 mg/kg per day) and sitagliptin (30 mg/kg per day) for 21 days on metabolic parameters and oxidative stress levels in normal and HFD-fed rats treated with vehicle, vildagliptin, or sitagliptin. Data are presented as mean \pm s.e.m. Two-way ANOVA followed by the Fisher's test was used for the statistical analysis ($n = 10$ /group)

Metabolic parameters	NDV	NDVil	NDSi	HFDV	HFDVil	HFDSi
Body weight (g)	445 \pm 7	460 \pm 8	454 \pm 13	552 \pm 11*	566 \pm 9*	549 \pm 14*
Food intake (g)	28 \pm 3	23 \pm 1	24 \pm 2	26 \pm 1	24 \pm 1	24 \pm 2
Visceral fat (g)	24 \pm 3	23 \pm 2	21 \pm 19	56 \pm 3*	48 \pm 2*	50 \pm 6*
Plasma insulin (ng/ml)	2.6 \pm 0.3	2.2 \pm 0.3	2.2 \pm 0.4	3.6 \pm 0.5*	2.5 \pm 0.6 [†]	2.6 \pm 0.5 [†]
Plasma glucose (mg/dl)	143 \pm 5	147 \pm 8	151 \pm 7	148 \pm 10	145 \pm 9	146 \pm 8
HOMA index	17 \pm 4	14.4 \pm 4	14.8 \pm 6	23.7 \pm 6*	16.1 \pm 5 [†]	16.9 \pm 6 [†]
Plasma total cholesterol (mg/dl)	87 \pm 11	81 \pm 6	88 \pm 11	158 \pm 9*	109 \pm 5 [†]	105 \pm 6 [†]
HDL cholesterol (mg/dl)	0.85 \pm 0.08	1.13 \pm 0.04	1.36 \pm 0.07	0.55 \pm 0.05*	1.51 \pm 0.23 [†]	1.23 \pm 0.03 [†]
LDL/VLDL cholesterol (mg/dl)	0.87 \pm 0.24	0.78 \pm 0.04	0.76 \pm 0.8	0.98 \pm 0.2	0.53 \pm 0.1	0.64 \pm 0.38
Plasma MDA (μ mol/ml)	2.5 \pm 0.1	2.7 \pm 0.1	2.8 \pm 0.1	7.1 \pm 0.1*	6.5 \pm 0.2* [†]	6.4 \pm 0.1* [†]
Brain MDA (μ mol/mg protein)	1.46 \pm 0.23	1.40 \pm 0.21	1.22 \pm 0.21	2.30 \pm 0.32*	1.62 \pm 0.14 [†]	1.44 \pm 0.45 [†]
Plasma glucose AUC (AUCg) (mg/dl \times min $\times 10^4$)	4.32 \pm 0.49	3.57 \pm 0.08 [†]	3.45 \pm 0.13 [†]	5.71 \pm 0.48*	3.79 \pm 0.31 [†]	4.18 \pm 0.36 [†]
Plasma insulin AUC (AUCi) (ng/ml \times min $\times 10^2$)	3.74 \pm 1.15	4.65 \pm 1.24 [†]	5.03 \pm 0.50 [†]	8.86 \pm 0.39*	5.28 \pm 0.50 [†]	3.37 \pm 0.46 [†]
Plasma GLP-1 (pM)	8.50 \pm 0.08	7.68 \pm 0.31	7.60 \pm 0.02	6.59 \pm 0.05*	7.98 \pm 0.46 [†]	8.00 \pm 0.21 [†]

* $P < 0.05$ compared to NDV and [†] $P < 0.05$ compared to HFDV, NDV, normal diet-fed rats with vehicle treatment; NDVil, normal diet-fed rats with vildagliptin treatment; NDSi, normal diet-fed rats with sitagliptin; HFDV, high-fat diet-fed rats with vehicle treatment; HFDVil, high-fat diet-fed rats with vildagliptin treatment; HFDSi, high-fat diet-fed rats with sitagliptin treatment.

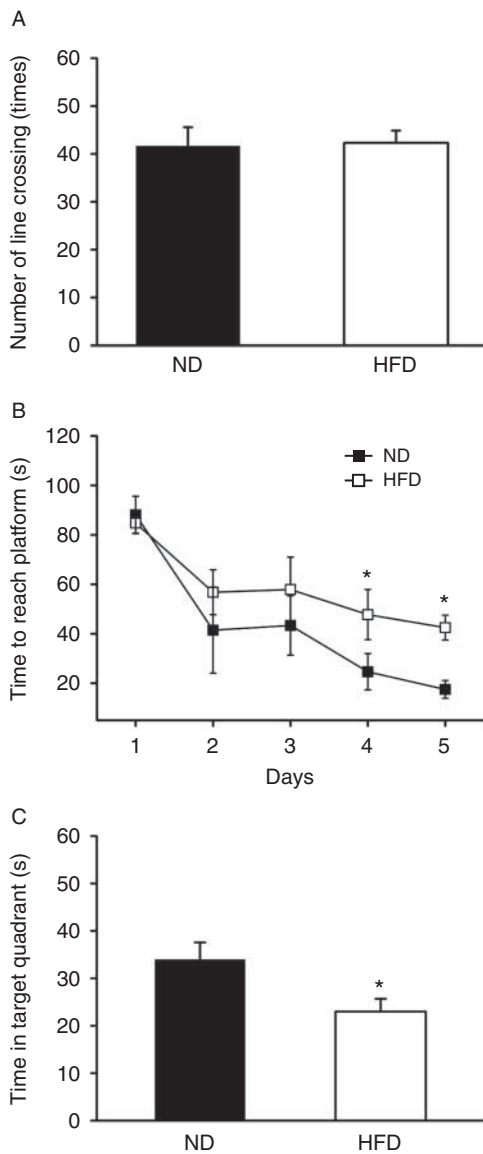


Figure 1

The impairment of learning and memory in HFD-fed rats measured by Morris Water Maze test. Open-field test was not different between ND and HFD groups (A). HFD consumption impaired learning and memory behaviors, indicated by increased time required to reach the platform in the acquisition test in comparison with the ND group (B) and decreased time spent in the target quadrant in the probe test, * $P < 0.05$ compared to ND (C). * $P < 0.05$ in comparison with the ND group, ND, normal diet-fed rats; HFD, high-fat diet-fed rats. Data are presented as mean \pm S.E.M. The independent t-test was used as the statistical analysis ($n = 30$ /group).

These findings indicate that the locomotive activity of all rats before pharmacological treatment did not differ between the two diet groups. The number of lines the rats crossed during the test at the end of vildagliptin and sitagliptin treatment in both dietary groups was not significantly different (Fig. 2A).

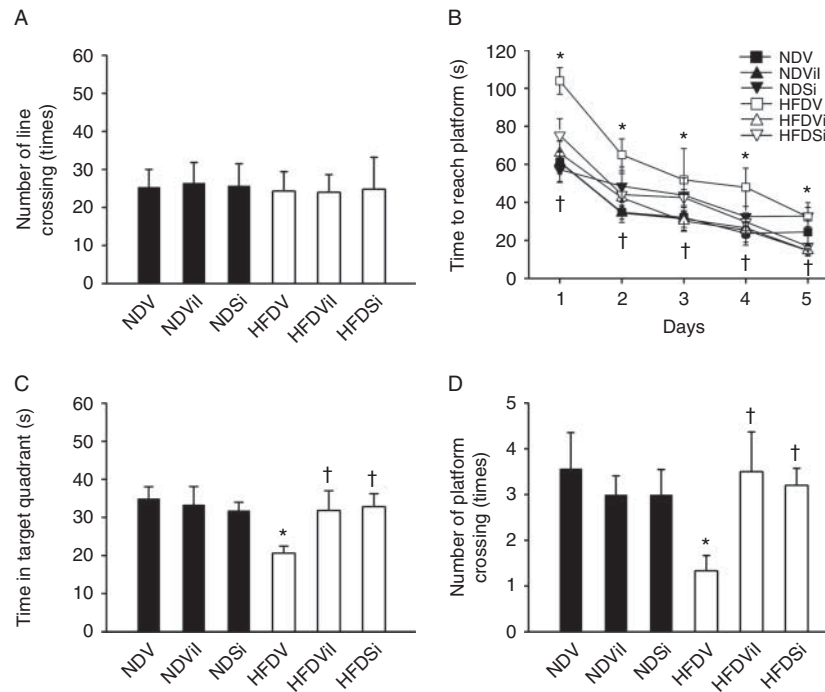
In the MWM test, before pharmacological treatment, we found that the time needed to reach the platform during the acquisition test, performed on days 4 and 5, in the HFD group was significantly increased in comparison with the NDV group (Fig. 1B). During the probe test, the time the rats spent in the target quadrant was significantly decreased within the HFD group in comparison with the ND group (Fig. 1C). These findings indicate that rats fed with the HFD for 12 weeks display significantly impaired learning and memory behaviors.

After 21 days of pharmacological treatment, we found that the HFDVil and HFDSi groups required significantly less time to reach the platform during the acquisition test in comparison with the HFDV group (Fig. 2B). In the probe test, the HFDVil and HFDSi groups spent significantly more time in the target quadrant in comparison with the HFDV group (Fig. 2C). In addition, the number of crossing the platform during the probe test in the HFDVil and HFDSi groups was significantly increased compared with the HFDV group (Fig. 2D). These findings suggest that vildagliptin and sitagliptin equally attenuate the impairment of learning and memory behaviors in HFD-induced insulin-resistant rats.

Vildagliptin and sitagliptin attenuated brain mitochondrial dysfunction and decreased brain oxidative stress levels in HFD-fed rats

To assess brain mitochondrial function, we measured the changes in brain mitochondria ROS production after H_2O_2 stimulation, $\Delta\Psi_m$ after H_2O_2 stimulation, and brain mitochondrial swelling. We found that 12 weeks of HFD consumption caused brain mitochondrial dysfunction as indicated by increased brain mitochondrial ROS production, brain mitochondrial membrane depolarization, and brain mitochondrial swelling in comparison with the ND group (Fig. 3). In HFD rats, treatment with either vildagliptin or sitagliptin significantly decreased brain mitochondrial ROS production (Fig. 3A), attenuated brain mitochondrial depolarization (Fig. 3B), and decreased brain mitochondrial swelling (Fig. 3C) in comparison with the HFDV group. In the mitochondria isolated from the hippocampus, we found that ROS production in the HFDV rats was also significantly higher than that in the NDV rats (Fig. 3D). Furthermore, both vildagliptin and sitagliptin could attenuate ROS production in the hippocampal mitochondria (Fig. 3D).

We also showed that vildagliptin and sitagliptin significantly reduced brain MDA levels in comparison with the HFDV group (Table 2). All these findings suggest

**Figure 2**

Effects of DPP-4 inhibitors on learning and memory. Open-field test was not different among all treatment groups (A). After 21 days of vildagliptin (3 mg/kg per day) and sitagliptin (30 mg/kg per day) administration, HFDVil- and HFDSi-fed rats showed significantly improved learning and memory behaviors, indicated by decreased time required to reach the platform in the acquisition test (B), increased time spent in the target quadrant in the probe test (C), and increased the number of platform crossing (D). * $P < 0.05$ in comparison with the NDV group, † $P < 0.05$ in

comparison with the HFDV group. NDV, normal diet-fed rats with vehicle treatment; NDVil, normal diet-fed rats with vildagliptin treatment; NDSi, normal diet-fed rats with sitagliptin treatment; HFDV, high-fat diet-fed rats with vehicle treatment; HFDVil, high-fat diet-fed rats with vildagliptin treatment; HFDSi, high-fat diet-fed rats with sitagliptin treatment. Data are presented as mean \pm s.e.m. Two-way ANOVA followed by the Fisher's test was used for the statistical analysis ($n = 10$ /group).

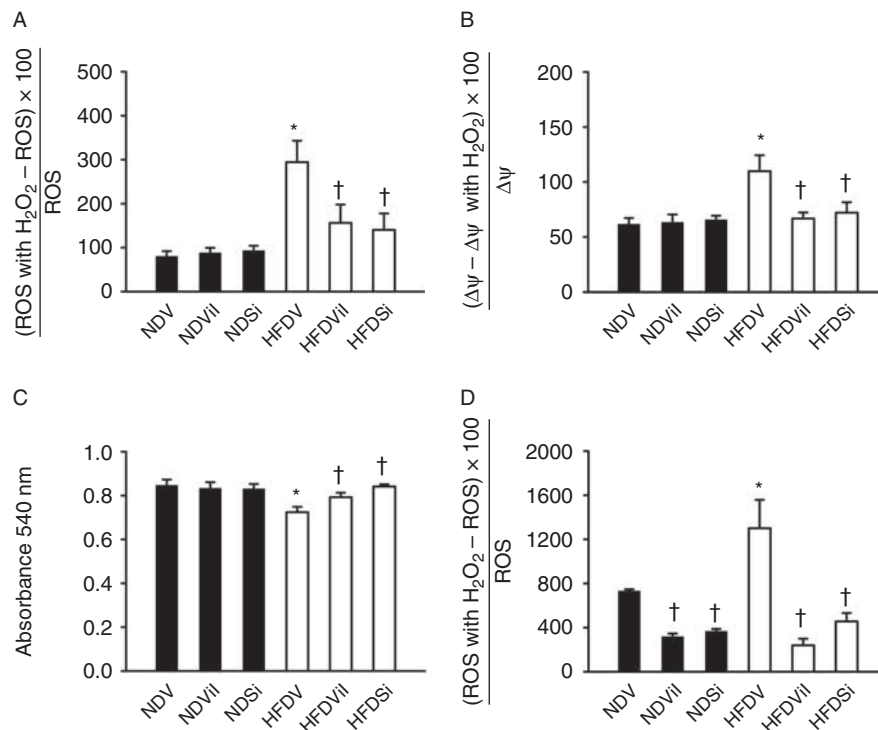
that vildagliptin and sitagliptin have equally beneficial effects resulting in decreased brain mitochondrial dysfunction and brain oxidative stress levels in HFD-induced insulin-resistant rats.

Discussion

Major findings from this study demonstrated that vildagliptin and sitagliptin equally i) improved metabolic parameters in HFD-induced insulin-resistant rats, ii) decreased plasma and brain oxidative stress levels in HFD-induced insulin-resistant rats, iii) restored brain mitochondrial function impaired by HFD by decreasing mitochondrial ROS production, preventing mitochondrial membrane depolarization, and preventing brain mitochondrial swelling, and iv) attenuated the impairment of learning and memory behaviors in HFD-induced insulin-resistant rats.

We demonstrated that 12 weeks of HFD consumption caused peripheral insulin resistance and brain

mitochondrial dysfunction. These findings are consistent with our previous studies (Pratchayasakul *et al.* 2011, Pipatpiboon *et al.* 2012). We also found that insulin resistance in rats induced by HFD consumption can lead to cognitive decline and an increase in oxidative stress within circulation as well as within the brain. These findings are consistent with previous studies (Greenwood & Winocur 2005, Stranahan *et al.* 2008). Changes in cognitive function following HFD consumption has been proposed to be due to the following: i) HFD consumption causes increased levels of triglyceride and cholesterol, which previous evidence has shown to be correlated with cognitive performance in patients with T2DM (Perlmutter *et al.* 1988); ii) 12 weeks of HFD consumption induced both hyperinsulinemia and increased brain oxidative stress, which can create a toxic environment for neurons as suggested by a previous study showing that hyperinsulinemia in a neuronal culture can sensitize neurons to stress-induced insults (Schafer & Erdo 1991); and iii) 12 weeks of HFD consumption caused an increase in

**Figure 3**

The effects of vildagliptin (3 mg/kg per day) and sitagliptin (30 mg/kg per day) treatment for 21 days on brain and hippocampal mitochondrial dysfunction in HFD-fed rats. Vildagliptin and sitagliptin administration completely attenuated an increase in brain ROS production following H_2O_2 application (A). Vildagliptin and sitagliptin significantly decreased brain mitochondrial membrane potential change following H_2O_2 application (B) and significantly increased absorbance values at 30 min, indicating the reduction of brain mitochondrial swelling (C). Vildagliptin and sitagliptin administration completely attenuated an increase in hippocampal ROS production following H_2O_2 application (D). * $P < 0.05$ in comparison with

the NDV group, † $P < 0.05$ in comparison with the HFDV group. NDV, normal diet-fed rats with vehicle treatment; NDVil, normal diet-fed rats with vildagliptin treatment; NDSi, normal diet-fed rats with sitagliptin; HFDV, high-fat diet-fed rats with vehicle treatment; HFDVil, high-fat diet-fed rats with vildagliptin treatment; HFDSi, high-fat diet-fed rats with sitagliptin treatment. Data are presented as mean \pm S.E.M. Two-way ANOVA followed by the Fisher's test was used for the statistical analysis. ($n = 10$ /group for brain mitochondrial function, $n = 4$ /group for hippocampal mitochondrial function).

circulating and brain glucocorticoid levels as shown in our previous study (Pratchayasakul *et al.* 2011). It has also been shown that an increase in brain glucocorticoid levels can perturb cognitive function (Green *et al.* 2006).

We also demonstrated that HFD rats had brain mitochondrial dysfunction as shown by increased ROS production, mitochondrial depolarization, and mitochondrial swelling. In the mitochondria, it has been shown that increased levels of ROS could cause the opening of the inner membrane anion channel (IMAC), thus leading to mitochondrial membrane depolarization (Aon *et al.* 2006, Zorov *et al.* 2006). The depolarization of mitochondria could also lead to the dysfunction of mitochondria to produce ATP synthesis (Aon *et al.* 2006). Furthermore, increased ROS levels could cause the opening of the mitochondrial permeability transitional pore (MPTP), thus allowing small metabolites and solute to diffuse into mitochondria, leading to mitochondrial

swelling, outer mitochondria membrane rupture, cytochrome *c* release, and finally cell death (Halestrap *et al.* 2004, Zorov *et al.* 2006). This could play a role in the cognitive decline observed in HFD rats. In this study, however, we did not investigate the interruption of the electron transport chain such as cytochrome *c* oxidase and UCP2 in relation to mitochondrial energy production. Future studies are needed to investigate the role of HFD on this issue.

In addition, we have shown previously that obesity and peripheral insulin resistance occurred after 8 weeks of HFD consumption, but neither brain insulin resistance nor the impairment of insulin-induced long-term depression (LTD) in hippocampus was observed (Pratchayasakul *et al.* 2011). However, brain insulin resistance with impaired insulin-induced LTD, together with brain mitochondrial dysfunction, was observed after 12 weeks of HFD consumption (Pipatpiboon *et al.* 2012). As brain insulin

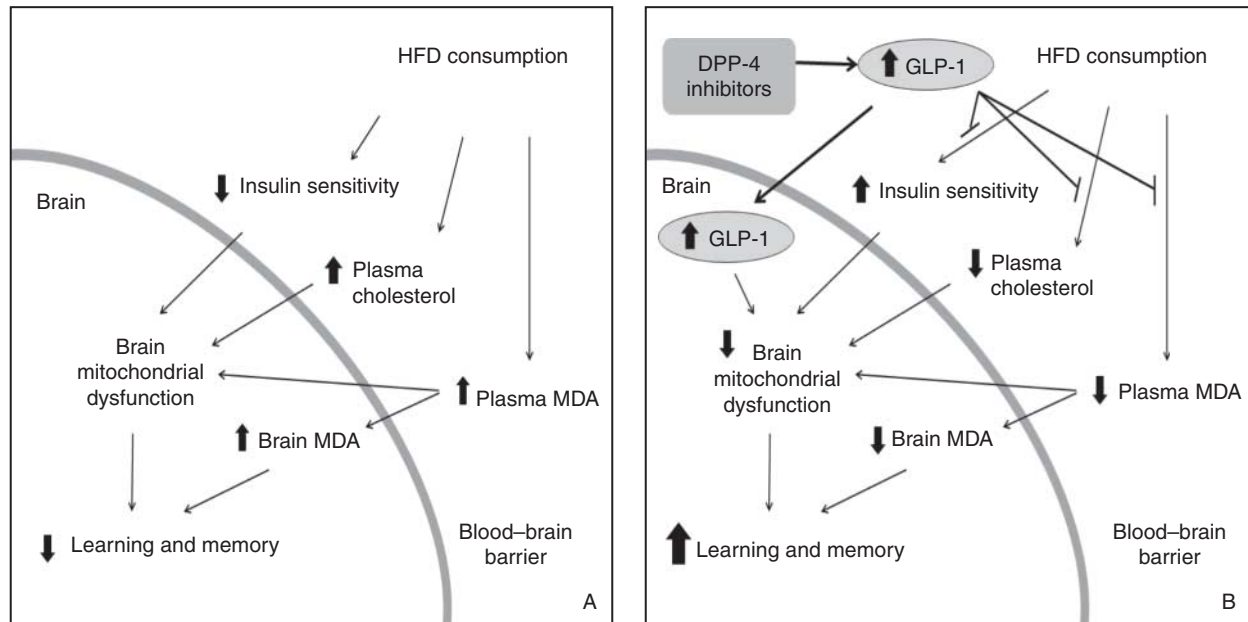
**Figure 4**

Diagram illustrates the proposed mechanism of HFD consumption-induced impairment of learning and memory (A) and the effects of DPP-4 inhibitors on attenuating this impairment (B). HFD consumption leads to peripheral insulin resistance (decreased insulin sensitivity) as well as increased oxidative stress and brain mitochondrial dysfunction, leading to impaired learning and memory. Vildagliptin and sitagliptin improve learning and

memory behaviors in insulin-resistant rats by increasing the GLP-1 levels, leading to the decrease in peripheral insulin resistance (i.e. increased insulin sensitivity), cholesterol levels, plasma and brain oxidative stress levels, and attenuating brain mitochondrial dysfunction. GLP-1, glucagon-like peptide-1; HFD, high-fat diet.

resistance was shown to be associated with cognitive decline (Stranahan *et al.* 2008), these findings indirectly suggest that observed changes in memory and brain mitochondrial function in HFD rats was secondary to alterations in obesity.

Vildagliptin and sitagliptin are new antidiabetic agents classified as DPP-4 inhibitors. It has been shown that DPP-4 inhibitors improve glycemic control in insulin resistance models and T2DM models (Richter *et al.* 2008). Consistent with this previous report, our study demonstrated that rats in the HFDVil or HFDSi groups displayed equally beneficial effects resulting in decreased peripheral insulin resistance observed as decreased plasma insulin levels, HOMA index, total plasma cholesterol levels, and increased plasma HDL levels. However, plasma glucose levels and LDL/VLDL levels within the HFDVil and HFDSi groups did not differ in comparison with the ND and HFDV groups. In addition, both HFDVil and HFDSi attenuated brain and circulation oxidative stress levels.

Moreover, we demonstrated that rats with high levels of plasma and brain oxidative stress induced by HFD consumption also developed brain mitochondrial dysfunction as well as impairment of cognitive function. These findings are consistent with our previous evidence

showing that 12 weeks of HFD consumption in rats caused brain mitochondrial dysfunction (Pipatpiboon *et al.* 2012). Previous studies as well as our findings suggest that the cognitive impairment caused by long-term HFD consumption could be related to brain mitochondrial dysfunction and oxidative stress (Winocur *et al.* 2005, Stranahan *et al.* 2008, White *et al.* 2009, Pintana *et al.* 2012). Interestingly, we found that both vildagliptin and sitagliptin completely restored brain mitochondrial function and improved learning and memory behaviors in rats with HFD-induced insulin resistance. Although stress during the MWM test could interfere with cognition, this influence should not play a significant role as rats in all groups were under the same MWM test condition and our results demonstrated that rats fed with the HFD for 12 weeks showed significant impaired learning and memory behaviors, but this was not the case in the ND rats. In addition, vildagliptin and sitagliptin equally attenuated the impairment of learning and memory behaviors in the HFD-induced insulin-resistant rats, but not in the vehicle-treated group. These finding strongly indicated the improved cognition by drug treatment.

The mechanism by which brain mitochondrial function is restored by treatment with vildagliptin and

sitagliptin in this study could be due to the inhibition of DPP-4 action leading to prolonged activity of GLP-1 action (Baggio & Drucker 2007, Richter *et al.* 2008). GLP-1 has been shown to protect against mitochondrial dysfunction in several cell types. For example, i) it has been shown that GLP-1 is involved in the mobilization of intracellular Ca^{2+} and the stimulation of mitochondrial ATP synthesis in cultured β -cells (Tsuboi *et al.* 2003), and ii) the study of isolated mouse hepatocytes found that GLP-1 entered cells and acted on hepatocyte mitochondria to modulate oxidative phosphorylation and to suppress oxidative stress and ROS production in ND-fed mice and diet-induced obese mice (Tomas *et al.* 2011). Furthermore, a recent study demonstrated that sitagliptin administration can increase active GLP-1 levels in the brain and improve memory behaviors in Alzheimer's disease mice models (D'Amico *et al.* 2010).

The proposed mechanism of DPP-4 inhibitors to ameliorate the effect of a HFD on insulin resistance could be due to their effects to increase plasma active GLP-1 levels. Increased plasma GLP-1 levels have been shown to increase insulin sensitivity, increase insulin secretion, but decrease plasma cholesterol and plasma MDA levels (D'Alessio *et al.* 1994, Pospisilik *et al.* 2002, Vaghiasya *et al.* 2010). All these beneficial effects could lead to the attenuation of insulin resistance caused by a HFD. In addition, the previous studies demonstrated that GLP-1 can cross the blood-brain barrier (Kastin *et al.* 2002, Baggio & Drucker 2007). Therefore, DPP-4 inhibitors could lead to increased brain active GLP-1 levels. It has been shown that brain GLP-1 acts as a neuroprotective agent (Baggio & Drucker 2007, Holst *et al.* 2011) and could lead to improved brain and hippocampal mitochondrial function and reduced brain MDA. Finally, these effects could lead to improved learning and memory. The summary of this proposed mechanism is illustrated in Fig. 4.

Conclusions

This study demonstrated that both vildagliptin and sitagliptin shared similar efficacy in attenuating peripheral insulin resistance, decreasing brain and circulation oxidative stress, and restoring brain and hippocampal mitochondrial function, thus leading to a prevention of learning and memory impairment in HFD-induced insulin-resistant rats.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

H P performed the experiments, analyzed the data, and wrote the manuscript. N A performed the experiments and analyzed the data. N C designed the study, analyzed the data, and wrote the manuscript. S C C designed the study, performed the experiments, analyzed the data, and wrote the manuscript.

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