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Glucose independent pleiotropic effects of a dipeptidyl peptidase-4 inhibitor on diabetic complications

DPP-4 억제제의 당뇨 합병증에서
다양한 혈당 비의존성 기전 연구

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By
Eunsoo Jung
정 은 수

Department of Veterinary Pathobiology and Preventive Medicine (Toxicology)
Graduate School of Seoul National University

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Supervised by Professor Myung-Haing Cho

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Supervisory Committee Approval of Thesis submitted by December 2015

Chairman: _____
Chan-Hee Chae, D.V.M., Ph.D.

Assistant chairman: _____
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Member: _____
Youngchan Park, D.V.M., Ph.D.

Member: _____
Choongman Hong, D.V.M., Ph.D.

Member: _____
Hwan-Goo Kang, D.V.M., Ph.D.

ABSTRACT

Dipeptidyl peptidase-4 (DPP-4) inhibitors are widely used as anti-diabetic agents in clinical practice. Gemigliptin, a new and selective DPP-4 inhibitor, has shown robust blood-glucose lowering effects in type 2 diabetic patients, but its effects on diabetic complications have not yet been reported.

This study evaluated the inhibitory effects of gemigliptin, a highly selective dipeptidyl peptidase-4 inhibitor, on the formation of advanced glycation end products (AGEs) and AGE cross-links with proteins in *in vitro* as well as in type 2 diabetic *db/db* mice. In *in vitro* assay, gemigliptin dose-dependently inhibited methylglyoxal-modified AGE-bovine serum albumin (BSA) formation (IC_{50} = 11.69 mM). AGE-collagen cross-linking assays showed that gemigliptin had a potent inhibitory effect (IC_{50} = 1.39 mM) on AGE-BSA cross-links to rat tail tendon collagen, and its activity was stronger than aminoguanidine (IC_{50} = 26.4 mM). In addition, gemigliptin directly trapped methylglyoxal in a concentration-dependent manner *in vitro*. To determine whether gemigliptin inhibits the *in vivo* glycation processes, gemigliptin (100 mg/kg/day) was orally administered into type 2 diabetic *db/db* mice for 12 weeks. Elevated serum levels of AGEs in *db/db* mice were suppressed by the administration of gemigliptin. These inhibitory effects of gemigliptin on the glycation process in both *in vitro* and *in vivo* suggest its therapeutic potential for ameliorating AGE-related diabetic complications.

Podocytes participate in the formation and regulation of the glomerular filtration barrier. Loss of podocytes occurs during the early stages of diabetic nephropathy and impairs glomerular filtration. Dipeptidyl peptidase-4 (DPP-4) inhibitors are widely used as

anti-diabetic agents in clinical practice. In this study, we showed that gemigliptin, a novel DPP-4 inhibitor, reduced podocyte apoptosis in type 2 diabetic *db/db* mice without reducing hyperglycemia. Gemigliptin (100 mg/kg/day) was administered orally for 12 weeks in db/db mice. Blood glucose levels and albuminuria were measured. The renal cortex was collected for histological examination, and molecular assays were used to detect 8-hydroxydeoxyguanosine, advanced oxidation protein products (AOPP), the receptor for advanced glycation end products (RAGE), and integrin-linked kinase (ILK). Type 2 diabetic *db/db* mice exhibited albuminuria, renal histopathological changes, and podocyte loss. Administration of gemigliptin to *db/db* mice suppressed albuminuria, enzyme activity and expression of DPP-4, and podocyte apoptosis. The effect of gemigliptin on diabetes-induced podocyte loss was associated with the suppression of oxidative damage, AOPP accumulation, RAGE expression, and ILK expression. These results indicate the possible benefits of using gemigliptin in diabetes patients to treat renal impairment without affecting glycemic control.

Retinal pericyte loss and neovascularization are characteristic features of diabetic retinopathy. Gemigliptin, a dipeptidyl peptidase-4 (DPP-4) inhibitor, has shown robust blood-glucose lowering effects in type 2 diabetic patients, but its effects on diabetic retinopathy have not yet been reported. We evaluated the efficacy of gemigliptin on retinal vascular leakage in *db/db* mice, which is an animal model for type 2 diabetes, and neovascularization in oxygen-induced retinopathy (OIR) mice, which is an animal model for ischemic proliferative retinopathy.

Gemigliptin (100mg/kg/day) was orally administered to the *db/db* mice for 12 weeks. C57BL/6 mice on postnatal day 7 (P7) were

exposed to 75% hyperoxia for 5 days, followed by exposure to room air from P12 to P17 to induce OIR. Gemigliptin (50 mg/kg/day) was intraperitoneally injected daily from P12 to P17. Retinal neovascularization was analyzed in flat-mounted retinas on P17. We determined the efficacy and possible mechanism of gemigliptin on high glucose-induced apoptosis of primary human retinal pericytes. The oral administration of gemigliptin for 4 months significantly ameliorated retinal pericyte apoptosis and vascular leakage in the *db/db* mice. Gemigliptin also ameliorated retinal neovascularization in the OIR mice. Gemigliptin attenuated the overexpression of plasminogen activator inhibitor-1 (PAI-1) in the retinas of diabetic and OIR mice. Gemigliptin and PAI-1 siRNA significantly inhibited pericyte apoptosis by inhibiting the overexpression of PAI-1, which is induced by high glucose. Our results suggest that gemigliptin has potent anti-angiogenic and anti-apoptotic activities via suppressing DPP-4 and PAI-1, and the results support the direct retinoprotective action of gemigliptin.

Keywords: Diabetic mellitus, Dipeptidyl peptidase-4 inhibitor, Diabetic complications, Diabetic nephropathy, Diabetic retinopathy
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General Introduction

1. Diabetes mellitus

Diabetes mellitus (DM) is a metabolic disorder characterized by a loss of glucose homeostasis with disturbances of carbohydrate, lipid and protein metabolism due to an impaired insulin secretion or decrease in the biological activity of insulin (Schneider et al., 2003). Diabetes is due to either the β cells of the pancreas not producing enough insulin (type 1), or the peripheral cells not responding properly to the insulin produced (type 2). The prevalence of DM for all age-groups worldwide is increasing with estimated projection of approximately 300 million patients by year 2025 (King et al., 1998).

2. Complications of diabetes mellitus

Uncontrolled hyperglycemia in both type 1 and type 2 diabetes lead to the development of complications (Weiss and Sumpio, 2006). Diabetic complications include cardiovascular diseases, hypertension, chronic kidney disease, retinal injury and nerve damage. Chronic hyperglycemia elicits an enhanced production of mitochondrial reactive oxygen species (ROS), which contribute to the development of diabetic complications in several organs (Weiss and Sumpio, 2006). The metabolic pathways activated by hyperglycemia include the formation of advanced glycation end product (AGE), polyol pathway, hexosamine pathway and the protein kinase C (PKC) pathway (Fig. 1).

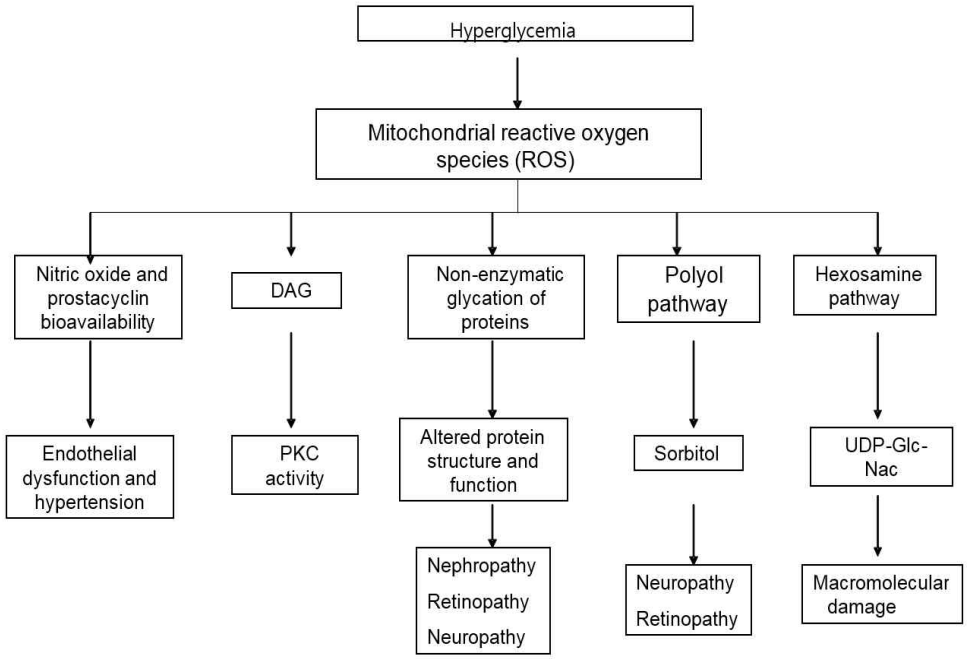

Figure 1. Metabolic pathways activated by chronically elevated blood glucose levels as well as the resultant long term complications of diabetes mellitus

2.1. Diabetic nephropathy

The hallmark of diabetic nephropathy is increased urinal excretion of proteins, mainly albumin. The progression of diabetic nephropathy is a descending path from normo-albuminuria to micro-albuminuria and overt proteinuria and eventually to end-stage renal disease (Fig. 2). Micro-albuminuria is defined as urinal albumin excretion rate (AER) 20-200 mg/min in overnight urine specimens (Mogensen et al., 1985). A urinal albumin levels above this value is called macro-albuminuria and considered a typical symptom of diabetic nephropathy.

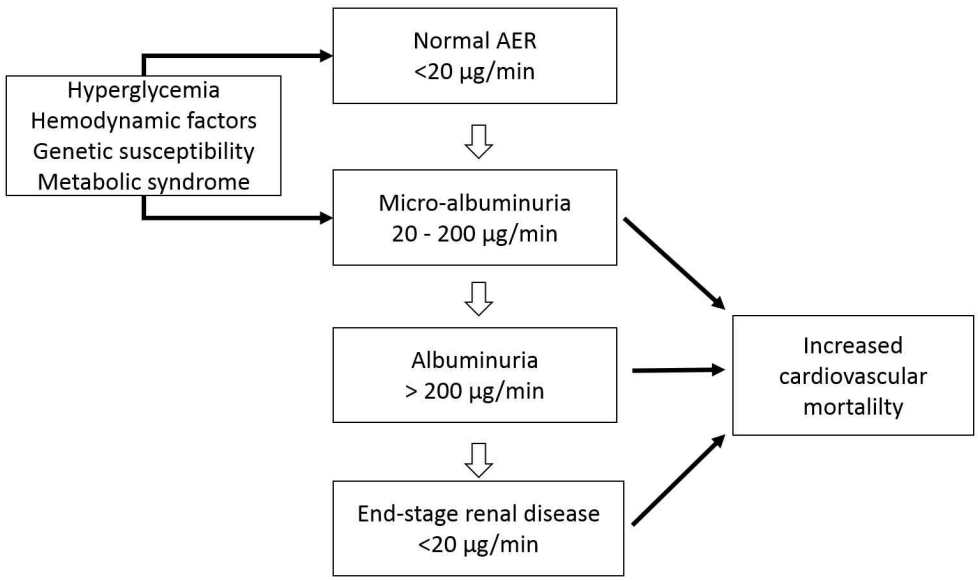

Figure 2. The progression of albumin excretion (AER), risk factors and prognosis

The incidence of diabetic nephropathy have increased rapidly after ten years of diabetes (Andersen et al., 1983). Approximately 35 ~ 45% of diabetic patients developed diabetic nephropathy during forty years of diabetes (Krolewski et al., 1985). About 50% of patients after duration of 20 years of diabetes had micro-albuminuria. (Krolewski et al., 1985). In patients with type 1 diabetes, micro-albuminuria is rare during the first years. However, micro-albuminuria is often observed in persons first diagnosed with type 2 diabetes (Uusitupa et al., 1987). This high incidence of micro-albuminuria in patients with type 2 diabetes can result from a difficulty in determining exact time at the onset of type 2 diabetes (Parving et al., 1992). Moreover, in patients with the metabolic syndrome, micro-albuminuria has been

also associated with a more generalized vascular damage (Nannipieri et al., 1997).

2.2. Diabetic retinopathy

Diabetes leads to formation of typical lesions in the retinal microvessels, such as vascular leakage, a formation of microaneurysms and hemorrhages. These vascular changes are defined as non-proliferative diabetic retinopathy (NPDR). NPDR do not threaten visual acuity unless they are located in the macular area, where they can cause macular edema. However, in advanced stage of diabetic retinopathy, capillary closure can lead to areas of impaired perfusion, which can result in retinal ischemia and neovascularization, defined as proliferative diabetic retinopathy (PDR) (Fig. 3). Retinal neovascularization can be responsible for intraocular hemorrhage and visual impairment. The formation of fibrous tissue in endovascular areas may eventually cause retinal detachment and blindness (Kohner, 1993).

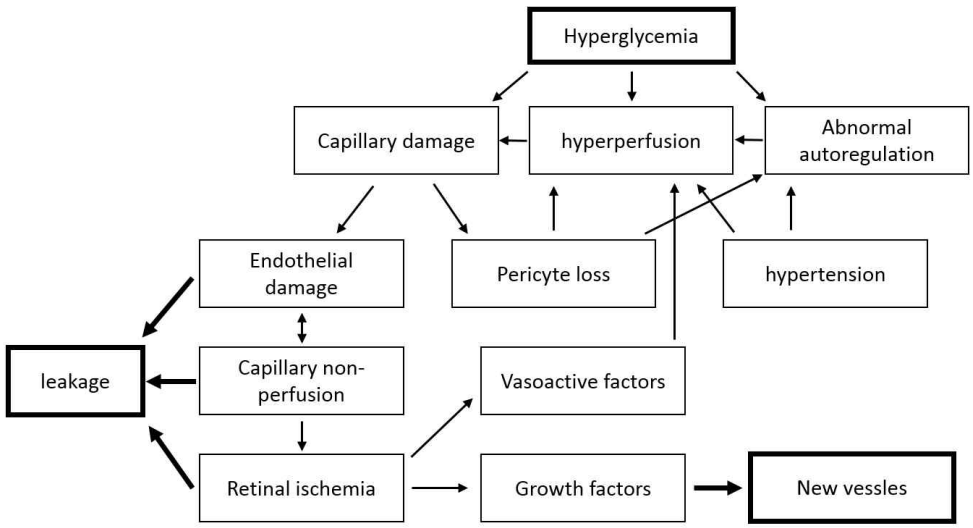

Figure 3. Role of hyperglycemic in the progression of diabetic retinopathy.

Age, duration of diabetes, blood glucose levels and treatment for glycemic control can contribute the prevalence of diabetic retinopathy (Agardh et al., 1989; Henricsson et al., 1996; Klein et al., 1984). The Wisconsin Epidemiological Study of Diabetic Retinopathy (WESDR) (Klein et al., 1984) reported that younger-onset group (diagnosed before 30 years of age) had no retinopathy during the first 5 years of disease, but more than 90% of the patients had some degree of retinopathy after 15 years of diabetes. About 20% of the older onset patients (diagnosed after 30 years of age) had some signs of retinopathy during the first 5 years and after a long duration still 20% of the patients did not have diabetic retinopathy. The incidence of PDR increased by 50% of the younger-onset patients and by 10 ~ 30 % in the older-onset patients after 25 years of diabetes. The United Kingdom Prospective Diabetes Study (UKPDS) showed that diabetic retinopathy was observed in about one third of first diagnosed type 2 diabetic patients (Kohner et al., 1998).

3. Dipeptidyl peptidase 4 (DPP-4) inhibitor

Medical nutrition therapy including increased physical activity and reduced food intake is the traditional first line of treatment for type 2 diabetes followed by the addition of oral anti-diabetes therapies and exogenous insulin as required (DeFronzo, 1999). A lot of attempts have been made to get near normal glycemic control and variety of drugs are now available (Takei and Kasatani, 2004). As a new therapeutic class of anti-diabetic drugs, the numerous DPP-4 inhibitors, often called as 'gliptins', have been developed in varying stages of clinical development with seven already approved in Korea.

Table 1. Approved DPP-4 inhibitors

Name	Approval (Company)
Sitagliptin (Januvia)	FDA approved in 2006 (Merck & Co)
Vildagliptin (Galvus)	EU approved in 2007 (Novartis)
Saxagliptin (Onglyza)	FDA approved in 2009 (BMS)
Linagliptin (Tradjenta)	FDA approved in 2011 (BI & Eli lily)
Gemigliptin (Zemiglo)	Korea approved in 2012 (LG Life Sciences)
Anagliptin (Suiny)	Japan approved in 2012 (Sanwa and Kowa)
Teneligliptin (Tenelia)	Japan approved in 2012 (Mitsubishi Tanabe)
Alogliptin (Nesina)	FDA approved in 2013 (Takeda)
Trelagliptin (Zafatek)	Japan approved in 2015 (Takeda)
Omarigliptin (Marizev)	Japan approved in 2015 (Merck & Co)
Evogliptin	Korea approved in 2015 (Dong-A ST)

3.1. Mode of actions of DPP-4 inhibitors

DPP-4 is an integral membrane serine protease that cleaves dipeptides from the N-terminus of proteins and substrate peptides that containing proline or alanine in the second amino terminal position (Huang et al., 2012; Silveira et al., 2013). DPP-4 is expressed on the surface of most cells and also exists as a soluble form in plasma. DPP-4 participates in numerous biological processes. Proteolytic activity of DPP-4 leads to inactivation or activation of some peptides, cytokines and growth factors (Cohen et al., 2004; Cunningham and O'Connor, 1997; Lambeir et al., 2003). DPP-4 also cleave many hormones, including two major incretin hormones glucagon-like peptide- 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which enhance the insulin secretion (Flatt et al., 2008). As shown in Fig. 4, the incretin hormones, such as GLP-1 and GIP, are synthesized in the small intestine and secreted in response to food (Velarde-Salcedo et al., 2013). Incretin hormones elicit glucose dependent insulin secretion in the pancreatic α-cells (Silveira et al., 2013), which leads to a lowering of the blood glucose levels (Flatt et al., 2008). It has been estimated that the incretin hormones, especially GLP-1 and GIP are responsible for over 50% of the total insulin secretion after a meal (Silveira et al., 2013; Velarde-Salcedo et al., 2013). However, DPP-4 cleaves and inactivates GLP-1 and GIP within a few minutes, thereby losing their insulinotropic activities (Li-Chan et al., 2012). The inactivation of GLP-1 and GIP by DPP-4 cannot stimulate insulin-release in the pancreas (Flatt et al., 2008). DPP-4 inhibitors have been developed to inhibit this rapid inactivation of GLP-1 and GIP, thus leading to prolonged incretin action and improving glucose tolerance and can thereby be used as a drug to treatment type 2 diabetes (Flatt et al.,

2008).

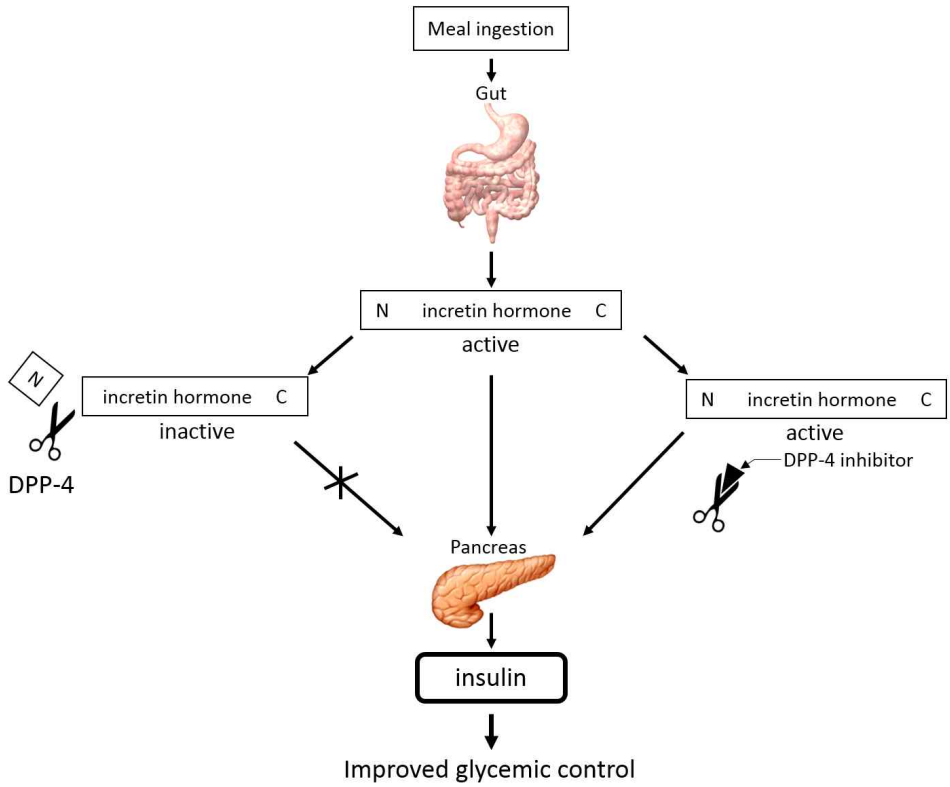

Figure 4. Overview of DPP-4 regulation in relation to the glucose level (Flatt et al., 2008).

3.2. Pleiotropic effect of DPP-4 inhibitors

Several biological molecules apart from the incretin hormones are potentially substrates for DPP-4 (Mentlein, 1999). The inhibition of DPP-4 enzyme activity may exert pleiotropic effects through activation or inactivation of biological molecules that are substrates for DPP-4. Stromal-derived factor (SDF)-1α is a small peptide chemokine that is a substrate for DPP-4 since its active form is

rapidly degraded to an inactive form by DPP-4 (Proost et al., 1998). A recent study demonstrated that SDF-1α is also expressed in pancreatic islets, and its expression is enhanced by cellular injury. SDF-1α increases the expression of prohormone convertase-1 in α-cells, which increases the production of GLP-1 in islet (Liu et al., 2011). In pancreatic damage by oxidative stress or glucolipotoxicity, SDF-1α expression is increased with increased production of GLP-1. Therefore, DPP-4 inhibition facilitates further the intra islet GLP-1 production when inactivation of SDF-1α by DPP-4 is inhibited.

Recently, Krijnenet al. observed a marked decrease of microvascular endothelial DPP-4 expression in recently infarcted human hearts (Krijnen et al., 2012). DPP-4 can prevent fibrin polymerization and clot formation via cleaving N-terminal Gly-Pro from the fibrin α-chain, (Mentlein and Heymann, 1982). The treatment of diprotin A, a DPP-4 inhibitor, in human umbilical cord vein endothelial cells (HUVECs) increased the expression of endothelial tissue factor and consequently induced adherence of platelets to the ECs, although platelet aggregation was not increased (Krijnen et al., 2012). Sitagliptin has been shown to inhibit platelet aggregation through its inhibitory effects on thrombin-induced intracellular free calcium accumulation and on thrombin-induced tyrosine phosphorylation of multiple proteins in human platelets (Gupta et al., 2012). This finding suggests that DPP-4 has an antithrombotic effect.

Sitagliptin promotes reverse cholesterol transport through reduced intestinal cholesterol absorption in a mouse model (Briand et al., 2012). Vildagliptin was also shown to decrease the level of hepatic mRNA transcript for farnesyl diphosphate transferase in dual incretin receptor knockout mice (Flock et al., 2007). Farnesyl diphosphate is a substrate for the synthesis of dolichol, coenzyme Q and cholesterol.

Although the postprandial lipid lowering effect of DPP-4 inhibitor is dependent on GLP-1 (Hsieh et al., 2010), DPP-4 inhibition may have a direct effect on cholesterol metabolism by affecting the isoprenoid pathway (Choe et al., 2014; Monami et al., 2012).

Several previous studies have shown that many DPP-4 inhibitors improve endothelial function in diabetic patients (van Poppel et al., 2011) in GLP-1 dependent as well as independent manners (Yoon and Lee, 2011). Alogliptin induced a dose-dependent vasorelaxation through Src-Akt-endothelial nitric oxide synthase pathway in a mouse model (Shah et al., 2011). In a recent study, linagliptin treatment led to a decrease in lipid and protein oxidation in a rat model of renovascular hypertension (Chaykovska et al., 2013). DPP-4 can bind mannose-6-phosphate/insulin-like growth factor II receptor (M6P/IGF-IIR) which plays an important roles in two distinct biological processes; protein trafficking and transmembrane signal transduction (Hawkes and Kar, 2004). The interaction between DPP-4 and M6P/IGF-IIR is responsible for T cell activation (Busek et al., 2004). DPP-4 can directly contribute to stimulate ROS production and receptor for advanced glycation end products (RAGE) gene expression via the interaction with M6P/IGF-IIR in HUVECs (Ishibashi et al., 2013). In addition, linagliptin inhibited AGEs-induced soluble DPP-4 production, ROS generation and gene expressions of RAGE, intercellular adhesion molecule-1 and plasminogen activator inhibitor-1 in HUVECs (Ishibashi et al., 2013). Soluble DPP-4 proteins stimulated the proliferation of vascular smooth muscle cells (VSMCs), but DPP-4 inhibitor suppressed the proliferationof VSMCs by inhibiting ERK phosphorylation (Ervinna et al., 2013). Sitagliptin treatment suppressed proliferation of VSMCs, promoted apoptosis of VSMCs and reduced inflammation and MMP-2 and MMP-9 expressions in

the injured artery (Lim et al., 2012). Short-term treatment with vildagliptin prevented left ventricular hypertrophy induced by continuous infusion of isoproterenol through the amelioration of perivascular fibrosis and expression of genes associated with glucose uptake (GLUT4) and inflammation (TNF-α and IL-6) (Miyoshi et al., 2014).

In renal tissues of diabetic mice, the DPP-4 protein levels were enhanced as compared with normal kidneys. Diabetic glomerulosclerosis and tubulointerstitial fibrosis are associated with increased both DPP-4 protein levels and enzyme activity and increased transforming growth factor-β2 signaling pathway (Kanasaki et al., 2014). Linagliptin treatment ameliorated these pathophysiologic changes in the diabetic kidney (Kanasaki et al., 2014).

Many studies have provided the strong evidence to support the anti-atherosclerotic and cardiovascular and renal protective effects of DPP-4 inhibitors (Shah et al., 2011). Recent meta-analysis of clinical trial data have shown more favorable CV outcomes with DPP-4 inhibitors than with other classes of anti-diabetic agents (Cameron-Vendrig et al., 2014; Monami et al., 2013; Wu et al., 2014).

4. Aim of the study

Gemigliptin is a selective and long-lasting DPP-4 inhibitor, and it is approved by the Korea Ministry of Food and Drug Safety for clinical use in patients with type 2 diabetes (Lim et al., 2009). Most research on gemigliptin has focused on the normalization of blood glucose in patients with type 2 diabetes (Kim et al., 2013). However, this is the first study demonstrating that gemigliptin has protective effects in the diabetic nephropathy and retinopathy by a mechanism independent of lowered blood glucose. In addition, we investigate the possible mechanism of action whereby gemigliptin exert its blood glucose pleiotropic effects. The study had the following objectives.

1) To evaluate the inhibitory effects of gemigliptin on the formation of AGEs and the cross-linking between preformed AGE-BSA and proteins *in vitro*. In addition, we also investigated whether the inhibitory activity of gemigliptin on the glycation process is due to its reactivity with reactive dicarbonyl compounds. Moreover, because various classes of structurally different DPP-4 inhibitors are currently available, we also compared the effectiveness of gemigliptin with that of the other DPP-4 inhibitors, vildagliptin and saxagliptin. Subsequently, we compared AGE formation and cross-links in type 2 diabetic *db/db* mice with and without administration of gemigliptin.

2) To determine whether gemigliptin reduced diabetic nephropathy independently of its glucose-lowering property, we have studied the effect of gemigliptin diabetes-induced podocyte injury in type 2 diabetic *db/db* mice.

3) To the best of our knowledge, there have been no reports on the effects of DPP-4 inhibitors on retinal pericyte injury and neovascularization. Therefore, we studied whether gemigliptin inhibits retinal pericyte injury through the modulation of apoptotic cascades in type 2 diabetic *db/db* mice. Additionally, we evaluated whether gemigliptin effectively prevents retinal neovascularization in oxygen-induced retinopathy (OIR) mice.

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Part I.

Gemigliptin, a novel dipeptidyl peptidase-4 inhibitor, exhibits potent anti-glycation properties *in vitro* and *in vivo*

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Abstract

This study evaluated the inhibitory effects of gemigliptin, a highly selective dipeptidyl peptidase-4 inhibitor, on the formation of advanced glycation end products (AGEs) and AGE cross-links with proteins in *in vitro* as well as in type 2 diabetic *db/db* mice. In *in vitro* assay, gemigliptin dose-dependently inhibited methylglyoxal-modified AGE-bovine serum albumin (BSA) formation (IC_{50} = 11.69 mM). AGE-collagen cross-linking assays showed that gemigliptin had a potent inhibitory effect (IC_{50} = 1.39 mM) on AGE-BSA cross-links to rat tail tendon collagen, and its activity was stronger than aminoguanidine (IC_{50} = 26.4 mM). In addition, gemigliptin directly trapped methylglyoxal in a concentration-dependent manner *in vitro*. To determine whether gemigliptin inhibits the *in vivo* glycation processes, gemigliptin (100 mg/kg/day) was orally administered into type 2 diabetic *db/db* mice for 12 weeks. Elevated serum levels of AGEs in *db/db* mice were suppressed by the administration of gemigliptin. These inhibitory effects of gemigliptin on the glycation process in both *in vitro* and *in vivo* suggest its therapeutic potential for ameliorating AGE-related diabetic complications.

1. Introduction

The glycation process is a spontaneous non-enzymatic chemical reaction (called glycosylation) of sugar molecules with proteins, DNA and lipids to form Amadori product in a biological environment. The Amadori product irreversibly undergoes a variety of dehydration and rearrangement reactions, resulting in the formation of complex group known as advanced glycation end products (AGEs) (Brownlee et al., 1988). AGEs accumulate in all tissues and on plasma lipoproteins and bind to a specific receptor for AGE (RAGE) (Moritoh et al., 2009). Enhanced binding of AGEs to RAGE have been shown in diabetic patients, and the AGE/RAGE interaction has an important role to develop diabetes related complications (Sato et al., 2006) Moreover, AGEs can covalently cross-link with proteins, which changes the biochemical structures and functions of those proteins. Intracellular AGEs are also generated from sugar-derived dicarbonyl precursors. Methylglyoxal, a reactive dicarbonyl metabolite, is physiologically produced as an intermediate in glycolysis. The levels of methylglyoxal are increased in the plasma and tissue of patients and animals with diabetes (Haik et al., 1994). Methylglyoxal is more chemically reactive than blood sugars. Thus, methylglyoxal easily cross-links with free amino acid residues in proteins, which leads to the generation of stable end products (Bourajjaj et al., 2003). Evidence for methylglyoxal-derived modifications in human and animal tissues has been reported (Horan et al., 2007 Miyata et al., 1997 Shamsi et al., 1998).

It has been suggested that suppression of the glycation reaction may prevent the progress of diabetic complications. Aminoguanidine, a nucleophilic hydrazine compound, could inhibit the glycation reaction

in vitro as well as *in vivo* (Hammes et al., 1991 Kumari et al., 1991). The mechanism of action of aminoguanidine may involve trapping dicarbonyl metabolites, such as methylglyoxal (Lo et al., 1994). The roles of potential glycation reaction inhibitors have not yet been investigated thoroughly. Gemigliptin (previously identified as LC15-0444) is a novel and selective dipeptidyl peptidase-4 (DPP-4) inhibitor used for the treatment of type 2 diabetes (Pennock et al., 2013). Although the conformational structure of gemigliptin differs from that of aminoguanidine, a very recent report has shown that another chemically similar DDP-4 inhibitor, sitagliptin, reduces serum levels of glycated albumin in type 2 diabetic subjects (Shima et al., 2014) and reduces the AGE content in lenses in streptozotocin-induced diabetic rats (Pandit et al., 2013). However, it remains unclear whether DPP-4 inhibitors have inhibitory effects on the glycation processes and cross-linking with proteins.

The purpose of this study was to evaluate the inhibitory effects of gemigliptin on the formation of AGEs and the cross-linking between preformed AGE-BSA and proteins *in vitro*. In addition, we also investigated whether the inhibitory activity of gemigliptin on the glycation process is due to its reactivity with reactive dicarbonyl compounds. Moreover, because various classes of structurally different DPP-4 inhibitors are currently available, we also compared the effectiveness of gemigliptin with that of the other DPP-4 inhibitors, vildagliptin and saxagliptin. Subsequently, we compared AGE formation and cross-links in type 2 diabetic *db/db* mice with and without administration of gemigliptin.

2. Materials and Methods

2.1 *In vitro* tests

2.1.1 Inhibitory activity on AGEs formation

Bovine serum albumin (Sigma Chemicals, MO, USA) was incubated at 4 °C for 7 days with methylglyoxal (5mM) in sodium phosphate buffer (0.1 M, pH 7.4). All of the reagent and samples were sterilized by filtration through 0.2 mm membrane filters. The reaction mixture was then mixed with gemigliptin (LG Life Sciences, Seoul, Korea, 99.2% pure by HPLC analysis), vildagliptin (Beijing HuiKang BoYuan Chemical Tech, Beijing, China, 99.4% pure by HPLC) or saxagliptin (Beijing HuiKang BoYuan Chemical Tech, Beijing, China, 99.0% pure by HPLC). Aminoguanidine (Sigma Chemicals, MO, USA) was used as a positive inhibitor. The levels of AGE were determined by measuring AGE-specific fluorescence using a spectrofluorometer (excitation at 370 nm and emission at 440 nm, Synergy HT, BIO-TEK, VT, USA). We calculated the 50% inhibition concentration (IC_{50}) of AGE formation.

2.1.2 AGE cross-linking assay

The ability of DPP-4 inhibitors to inhibit cross-linking of preformed AGE-BSA with collagen was examined. Preformed AGE-BSA (TransGenic Inc, Kobe, Japan) was mixed with DPP-4 inhibitors or aminoguanidine. This mixture was incubated in a rat tail tendon collagen-coated 96-well plate (Sigma, MO, USA) for 4 h at 37 °C. The cross-linked complexes of preformed AGE-BSA-collagen were detected using a mouse anti-AGE antibody (TransGenic, Kobe, Japan) and horseradish peroxidase-conjugated secondary antibody (Santa

Cruz, CA, USA). Peroxidase activity was quantified using tetramethylbenzidine. Inhibition of preformed AGE-BSA and collagen cross-linking was expressed as the percentage of optical density.

2.1.3 Scavenging of carbonyl intermediates of AGE formation

We evaluated the ability of DPP-4 inhibitors to interact with methylglyoxal *in vitro* according to our previously reported method (Kim et al., 2011). Aminoguanidine was used as a positive control to determine the relative concentration of the remaining methylglyoxal.

2.2 *In vivo* tests

2.2.1 Animals

All mice were handled according to the approved procedure (LGMD13-083) by Institutional Animal Care and Use Committee of LG Life Sciences. Seven week-old male C57BL/KsJ-*db/db* mice (*db/db,* SLC, Shizuoka, Japan) and their lean littermates (*db/+*, normal) were randomly assigned to three (n = 10) groups. One group of *db/db* mice was orally administered gemigliptin (100 mg/kg body weight) and another group was administered the same amount of vehicle via oral gavage for 12 weeks. Non-diabetic littermates received the same vehicle treatment. Blood glucose level and body weight were measured weekly.

2.2.2 Quantification of serum AGEs levels

At necropsy, serum samples were collected and serum AGE levels were analyzed using an AGE ELISA kit (MyBioSource Inc, CA,

USA) according to the manufacturer's instruction.

2.2.3 RBC-IgG assay

Immunoglobulin G (IgG) is cross-linked to membrane protein of red blood cells (RBCs). RBC-IgG are formed before other AGE cross-links *in vivo*. The amount of RBC-IgG can be used to estimate protein cross-linking levels (Vasan et al., 1996). To test the inhibitory effect of gemigliptin on AGE cross-links, RBCs from heparinized whole blood were collected and RBC-IgG levels were determined using an anti-IgG ELISA.

2.3 Statistical analysis

All results are expressed as the mean ± standard error of the mean (S.E.M.). The IC_{50} values were determined by interpolation from the concentration-inhibition curve. Differences between groups were assessed by Student's t-test for single comparisons or by one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. Differences were considered significant at $P<0.01$. The statistical differences and IC_{50} values were determined using Prism 4.0 program (Graphpad, CA, USA).

3. Results

3.1 Inhibitory effect of DPP-4 inhibitors on AGEs formation *in vitro*

Three DPP-4 inhibitors were examined to evaluate inhibitory effects on AGE-BSA formation. As shown in Fig. 1, gemigliptin dose-dependently inhibited the formation of AGE-BSA (IC_{50} = 11.69 ± 0.13 mM). The inhibitory activity of gemigliptin was less than aminoguanidine (IC_{50} = 2.69 ± 0.06 mM) and other two DPP-4 inhibitors did not show anti-AGE formation activity.

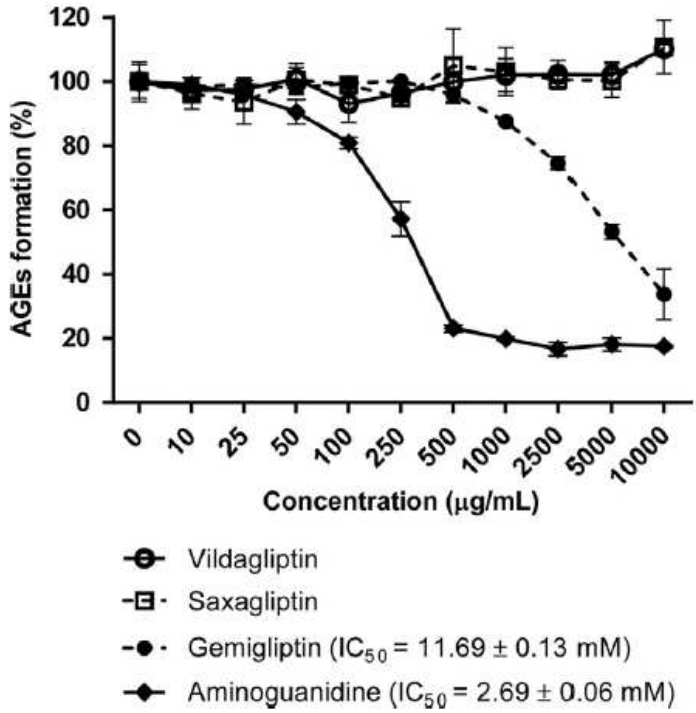

Figure 1. Inhibitory effect of DPP-4 inhibitors and aminoguanidine on AGEs formation. Data are expressed as the mean ± S.E.M., n=4. The IC_{50} values were determined from the plotted graph of AGE inhibition activity.

3.2 Inhibitory effect of DPP-4 inhibitors on cross-linking of preformed AGE-BSA with rat tail tendon collagen *in vitro*

The inhibition of cross-links between preformed AGE-BSA and collagen under various concentrations of DPP-4 inhibitors was examined (Fig. 2). Gemigliptin dose-dependently suppressed the cross-linking of preformed AGE-BSA with rat tail tendon collagen (IC_{50} = 1.39 ± 0.10 mM), and its inhibitory activity was greater than that of aminoguanidine (IC_{50} = 26.4 ± 1.20 mM). Additionally, other two DPP-4 inhibitors did not show inhibitory effects on AGE cross-linking with collagen.

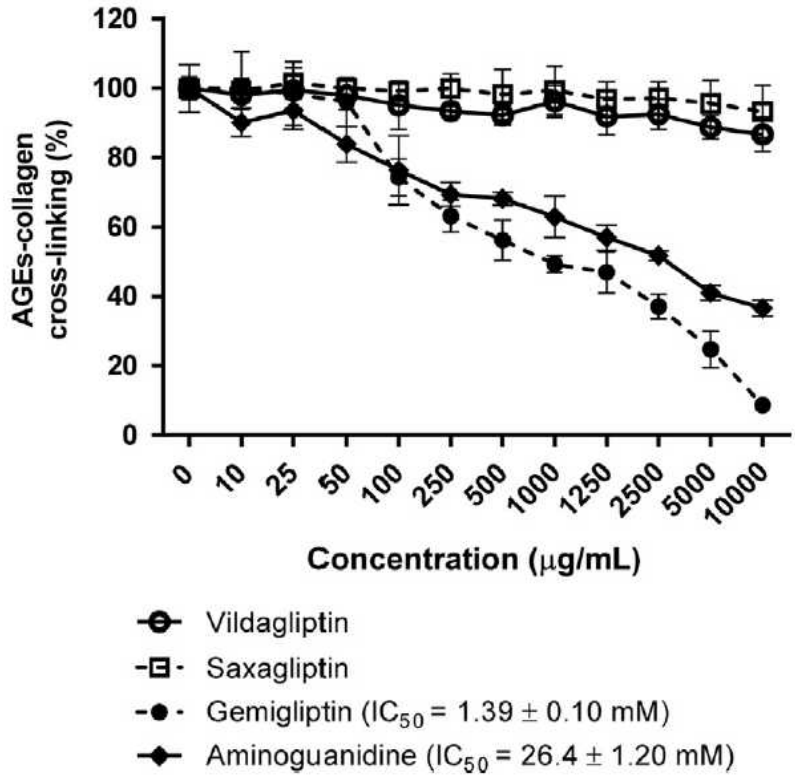

Figure 2. Inhibitory effect of DPP-4 inhibitors on the cross-links of preformed AGE-BSA with rat tail tendon collagen *in vitro* All results are expressed the mean ± S.E.M., n = 4. The IC_{50} values were determined from the plotted graph of cross-link inhibition activity.

3.3 Methylglyoxal scavenging effect of DPP-4 inhibitors

Many AGE inhibitors can interact with reactive dicarbonyl metabolites during the glycation process (Edelstein and Brownlee, 1992). Therefore, we tested whether three DPP-4 inhibitors can also interact with methylglyoxal *in vitro* (Fig. 3). Gemigliptin dose-dependently scavenged methylglyoxal (IC_{50} = 1.15 ± 0.02 mM), and its activity was greater than aminoguanidine (IC_{50} = 7.02 ± 0.16 mM).

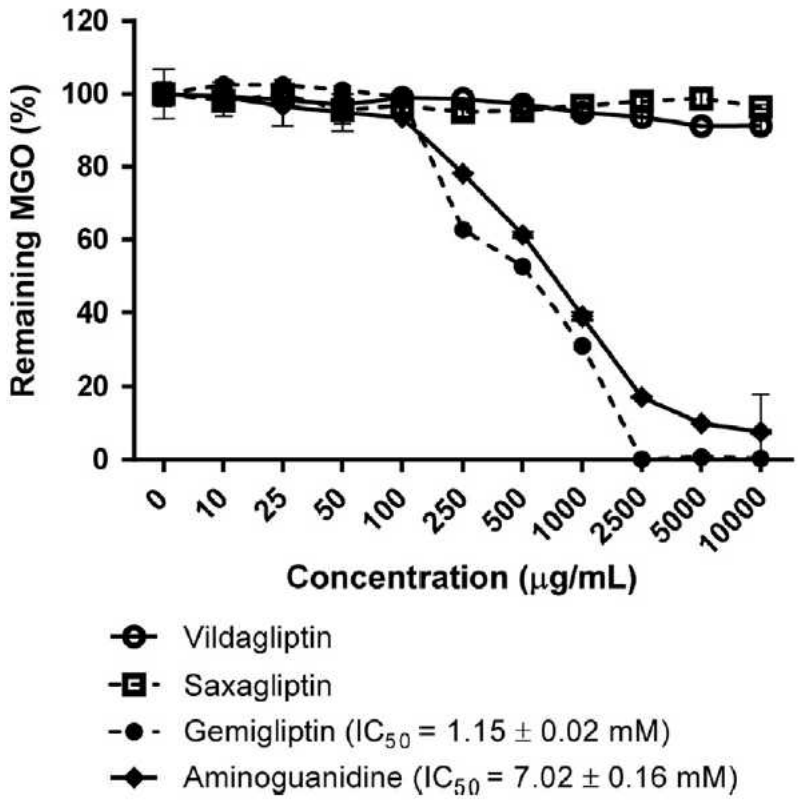

Figure 3. Carbonyl trapping activity of DPP-4 inhibitors and aminoguanidine All results are expressed as the mean ± S.E.M., n = 4. The IC_{50} values were determined from the plotted graph of scavenging activity.

3.4 Gemigliptin inhibits AGEs formation and AGE cross-links *in vivo*

The *in vivo* inhibitory effect of gemigliptin on AGEs formation in diabetic animal models was examined. After a 12 week-treatment, the body weight of *db/db* mice was greater than normal *db/+* mice. Severe hyperglycemia was observed in *db/db* mice ($P<0.01$ vs. normal *db/+* mice). Gemigliptin did not reduce blood glucose levels below that of *db/db* mice (Table 1). At the end of the study, *db/db* mice showed significantly increased serum AGE levels compared to normal *db/+* mice, but gemigliptin significantly reduced circulating AGE levels by 44.5 % in serum compared to vehicle-treated *db/db* mice (Fig. 4A). We next conducted the RBC-IgG assay to evaluate AGE cross-links. As shown in Fig. 4B, the RBC-IgG content of normal *db/+* mice was 0.21±0.01 and the content of vehicle-treated *db/db* mice was 0.61±0.04 ($P<0.01$). However, treatment with gemigliptin significantly reduced RBC-IgG content by 38.6% (0.41±0.06; $P<0.01$) compared with vehicle-treated *db/db* mice.

Table 1. Metabolic and physical parameters

		NOR	DM	Gemigliptin
Body weight (g)	Initial	25.1±0.8	34.61.9[a]	34.6±3.7
	Final	29.0±1.2	35.4±1.9[a]	33.2±8.0
Blood glucose (mg/dl)	Initial	133.4±17.0	459.4±46.2[a]	413.8±93.9
	Final	139.8±27.7	531.6±42.9[a]	510.81±115.4

NOR, normal *db/+* mice; DM, *db/db* mice; Gemigliptin, *db/db* mice treated with gemigliptin (100 mg/kg/day). All data are expressed as the mean ± S.E.M. [a]$P<0.01$ vs. NOR group.

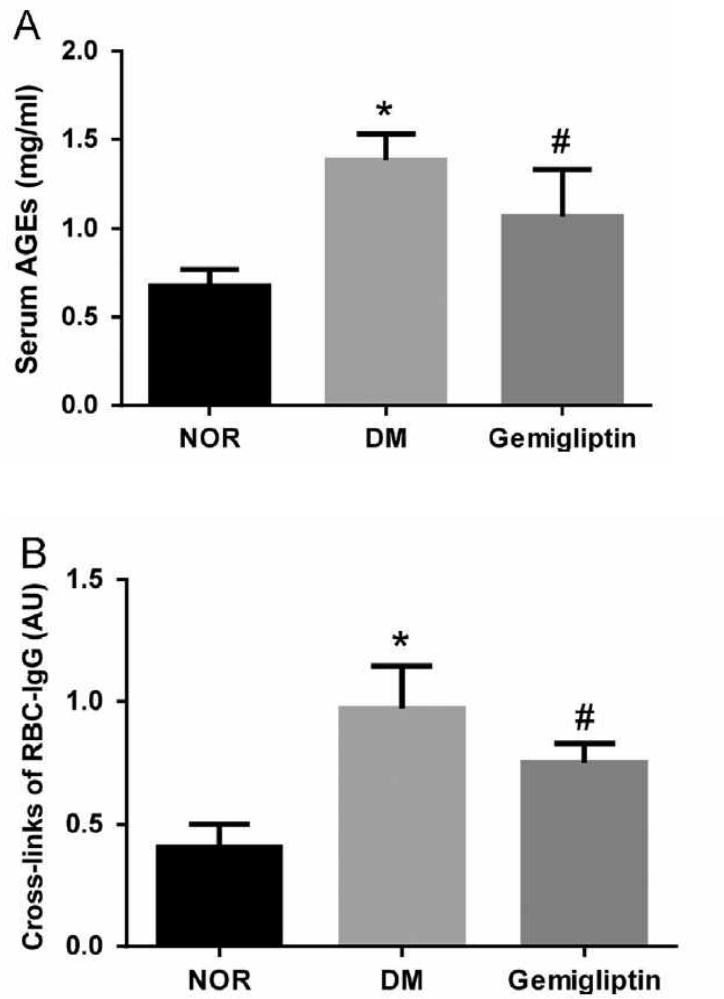

Figure 4. Effect of gemigliptin on AGE formation (A) and IgG cross-linked to the RBC surface (B) in blood of *db/db* mice. The values in the bar graph represent mean ± S.E.M., n = 10. *P<0.01 vs. normal *db/+* mice, # P<0.01 vs. vehicle-treated *db/db* mice.

4. Discussion

Many previous studies have reported that the formation of AGEs and their accumulation in tissues and cells play a crucial role in the pathogenic processes of diabetic complications (Sato et al., 2006). The cross-linked complex of AGEs with target proteins, including collagen, is permanent and irreversible. In the present study, we showed that a novel DPP-4 inhibitor, gemigliptin, possessed the potent activities of an AGE inhibitor and a carbonyl scavenger. Although a relatively high concentration (1 to 10 mM) of gemigliptin was required to block the glycation process *in vitro*, gemigliptin may be more potent than aminoguanidine as an AGE cross-linking inhibitor and methylglyoxal scavenger.

The cytotoxic effects of AGEs under diabetic conditions have been shown in many previous studies (Brownlee, 1995). Human body does not have any enzymes to break AGE structures. Thus, the importance of inhibiting AGE formation has been shown using a number of AGE inhibitors. Aminoguanidine prevented AGE formation resulting in inhibition of diabetic nephropathy, retinopathy and neuropathy in numerous diabetic animal models (Thornalley, 2003). However, because of safety concerns about its pro-oxidant activities (Suji and Sivakami, 2006) and inhibition of NO synthase (Tilton et al., 1993), aminoguanidine has currently not been used to treat diabetic complications (Turgut and Bolton, 2010). New antiglycating agents with higher levels of efficacy and safety in human are continually pursued.

Gemigliptin is an oral anti-diabetic agent that is approved by the Korean Food and Drug Administration and has an excellent safety record (Pennock et al., 2013). The detailed mechanism of action of gemigliptin as an AGE-inhibitor is still unclear. AGE inhibitors,

including aminoguanidine, pyridoxamine and 2-Isopropylidenehydrazono -4-oxo-thiazolidin-5-ylacetanilide (OPB-9195), suppress AGE formation through interaction with the reactive dicarbonyl species and acting as dicarbonyl scavenger. Aminoguanidine reacts with the α -dicarbonyls of reactive dicarbonyl species to form triazine derivatives (Lo et al., 1994 Thornalley et al., 2000). Pyridoxamine interacts with glyoxal, glyoxalaldehyde and methylglyoxal, resulting in the formation of adducts (Nagaraj et al., 2002). Our *in vitro* study showed that gemigliptin directly trapped methylglyoxal, and its activity was more potent than aminoguanidine. It is suggested that the scavenging activity of gemigliptin could participate in the inhibition of AGE formation and may prevent or delay the development of AGE-related diabetic complications.

Interestingly, among the three DPP-4 inhibitors used in this study, gemigliptin is the most potent anti-AGE inhibitor and methylglyoxal scavenger. Vildagliptin and saxagliptin had no inhibitory effects on the glycation processes. Consistent with our result, vildagliptin did not suppress circulating serum AGE levels in type 2 diabetic OLETF rats (Matsui et al., 2011). DPP-4 inhibitors include diverse structural types. Structurally, DPP-4 inhibitors are divided into two main classes, those that interact covalently with DPP-4 and those that do not (Horan et al., 2007). Vildagliptin and saxagliptin are covalent DPP-4 inhibitors and cyanopyrrolidine derivatives. Gemigliptin is a non-covalent DPP-4 inhibitor and piperazine derivative (Safavi et al., 2013). Vardhan et al. reported that piperazine analogues are potential AGE inhibitors (Vardhan et al., 2013). A cognition-enhancing drug tenilsetam, (+)-3-(2-thienyl)-2-piperazine, acts as an inhibitor of AGE formation and also methylglyoxal scavenger. Although the mechanisms of action of tenilsetam have yet to be explained,

tenilsetam inhibits the protein cross-linking by acting with sugars and glycated proteins (Rahbar and Figarola, 2003). In addition, recent *in vivo* studies showed that sitagliptin, a piperazine derivate, reduced the serum levels of glycated albumin in type 2 diabetic subjects (Shima et al., 2014) and reduced the AGE contents in lenses in streptozotocin-induced diabetic rats (Pandit et al., 2013). These results suggest that a piperazine derivative, such as gemigliptin, is among numerous DPP-4 inhibitors that can act as both AGE inhibitor and methylglyoxal scavenger.

In our *in vivo* study, gemigliptin induced only a minor decrease in blood glucose levels. Consistent with our observation, sitagliptin and vildagliptin also failed to improve hyperglycemia in similarly designed *db/db* mice studies (Moritoh et al., 2009). In addition, DPP-4 inhibitor, valine-pyrrolidide improved glucose intolerance in 6-week-old *db/db* mice in early stage of diabetes but not in 23-week-old *db/db* mice (Nagakura et al., 2003), which suggests that the glucose-lowering effect of DPP-4 inhibitors is more effective in the presence of healthy pancreatic β-cells. In our present study, gemigliptin has an inhibitory effect on the glycation processes despite continued hyperglycemia in *db/db* mice. These results suggest that even in hyperglycemia, it is possible to attenuate AGE-related tissue injury by gemigliptin.

In conclusion, our study showed that gemigliptin is a potent inhibitor of AGE formation and its cross-linking reaction with proteins. These activities may be explained in large part by its scavenging effect on methylglyoxal. Our results indicate the potential utility of gemigliptin as an AGE inhibitor and provide a possibility for treating diabetic complications associated with AGEs. Further clinical studies are needed to assess the value and possible application of gemigliptin as

an anti-glycation agent in diabetic patients.

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Part II.

Gemigliptin improves renal function and attenuates podocyte injury in mice with diabetic nephropathy

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Abstract

Podocytes participate in the formation and regulation of the glomerular filtration barrier. Loss of podocytes occurs during the early stages of diabetic nephropathy and impairs glomerular filtration. Dipeptidyl peptidase-4 (DPP-4) inhibitors are widely used as anti-diabetic agents in clinical practice. In this study, we showed that gemigliptin, a novel DPP-4 inhibitor, reduced podocyte apoptosis in type 2 diabetic *db/db* mice without reducing hyperglycemia. Gemigliptin (100 mg/kg/day) was administered orally for 12 weeks in *db/db* mice. Blood glucose levels and albuminuria were measured. The renal cortex was collected for histological examination, and molecular assays were used to detect 8-hydroxydeoxyguanosine, advanced oxidation protein products (AOPP), the receptor for advanced glycation end products (RAGE), and integrin-linked kinase (ILK). Type 2 diabetic *db/db* mice exhibited albuminuria, renal histopathological changes, and podocyte loss. Administration of gemigliptin to *db/db* mice suppressed albuminuria, enzyme activity and expression of DPP-4, and podocyte apoptosis. The effect of gemigliptin on diabetes-induced podocyte loss was associated with the suppression of oxidative damage, AOPP accumulation, RAGE expression, and ILK expression. These results indicate the possible benefits of using gemigliptin in diabetes patients to treat renal impairment without affecting glycemic control.

1. Introduction

Diabetic nephropathy represents one of the most frequent and serious microvascular complications that develop in patients with diabetes (White and Bilous, 2000). It is characterized by albuminuria and a decreased glomerular filtration rate (Ruggenenti et al., 2004). The association between the onset of albuminuria and progressive loss of renal function is well described, but the underlying pathophysiological mechanisms are still unclear. Albuminuria could be the result of impaired function of the glomerular filtration barrier or tubular reabsorption (Gorriz and Martinez-Castelao, 2012). The glomerular filtration barrier consists of a highly specialized structure with three layers: the endothelium, basement membrane, and podocytes (Siddiqi and Advani, 2013). Damage or loss of podocytes is a primary cause of the decreased ability of the barrier to filter out albumin. The density of podocytes per glomerulus is markedly reduced in patients with diabetes (Drummond and Mauer, 2002 Pagtalunan et al., 1997). In animal models of diabetes, podocyte depletion represents one of the earliest cellular injuries affecting the kidney (Susztak et al., 2006). Podocytes are highly differentiated cells with limited potential for proliferation (Wiggins, 2007). Thus, podocytes that are lost from the glomerulus are slowly replenished leading to podocytopenia (Spurney and Coffman, 2008). This has focused attention on podocytes as the primary target for developing therapies against diabetic nephropathy (Mathieson, 2012).

Currently, there are no commercially available drugs that specifically target podocytes. However, many agents originally developed for other clinical applications are used to treat glomerular disease by preserving the structural integrity of podocytes (Meliambro et al.,

2013). Glucocorticoids (Ransom et al., 2005), abatacept (Yu et al., 2013), thiazolidinedione (Kanjanabuch et al., 2007), angiotensin-converting -enzyme inhibitors (Mifsud et al., 2001), and rituximab (Fornoni et al., 2011) have been successfully repurposed to treat renal podocyte injury. Dipeptidyl peptidase-4 (DPP-4) inhibitors are a major new class of anti-diabetic drugs that increase the level of a gastrointestinal incretin hormone, glucagon-like peptide-1 (GLP-1), by inhibiting its cleavage. DPP-4 inhibitors show glucose-dependent activity by increasing insulin secretion, inhibiting glucagon secretion, and increasing β-cell mass, thereby resulting in lower blood glucose levels (Baetta and Corsini, 2011). Several recent studies have suggested that DPP-4 inhibitors owe their protective effects against diabetic nephropathy to a therapeutic off target action. Vildagliptin reduced diabetic renal injury by reducing the production of transforming growth factor-β1 in the kidney of streptozotocin (STZ)-induced diabetic rats (Liu et al., 2012). Linagliptin reduced renal oxidative stress and also reduced albuminuria in STZ-induced diabetic mice (Nakashima et al., 2014). Sitagliptin prevented diabetes-induced renal inflammation and apoptosis in Zucker diabetic fatty rats (Marques et al., 2014). It is noteworthy that vildagliptin and linagliptin both reduced diabetic nephropathy independently of their glucose-lowering properties. However, the precise mechanisms, beyond the effect of DPP-4 inhibitors on blood glucose, have not yet been elucidated. To the best of our knowledge, there are no reports on the effects of DPP-4 inhibitors on podocytes under hyperglycemic conditions. To address this, we have studied the effect of gemigliptin, a novel DPP-4 inhibitor, on diabetes-induced podocyte injury in type 2 diabetic *db/db* mice.

2. Materials and Methods

2.1 Animals and experimental design

Five-week-old male C57BL/KsJ-Lepr$^{db/db}$ diabetic mice (*db/db*) and nondiabetic heterozygous littermates (*db/+*) were purchased from Jung-Ang Lab Animal Inc. (Seoul, Korea). After 2 weeks of acclimatization, the *db/+* and *db/db* mice were divided into the following three groups: *db/+* group (NOR, n = 10), *db/db* group (DM, n = 10), and *db/db* + gemigliptin group (Gemigliptin, n = 10). Gemigliptin (100 mg/kg body weight) or an equal amount of vehicle (0.5% methylcellulose solution) was administered to diabetic *db/db* mice by oral gavage once a day for 12 weeks. Non-diabetic littermates received the same amount of methylcellulose solution. Body weight and blood glucose levels were recorded every week. Approval for animal studies was obtained from the Animal Welfare Review Board of LG Life Sciences (LGMD13-083; approval date 01/07/2014).

2.2 Measurement of albuminuria and histological analysis

After 12 weeks of treatment, urine samples were collected using metabolic cages for a 24 h period (Techniplast, Buguggiate, Italy). Urinary albumin level was measured using a mouse albumin enzyme-linked immunosorbent assay (ELISA) kit (Abnova, Taipei, Taiwan). After killing the mice, both kidneys were collected, and the kidney cortex was fixed in a 10% formalin solution and embedded in paraffin. Cortical tissue slices (4 μm thickness) were dewaxed and stained with periodic acid-Schiff.

2.3 Detection of 8-hydroxydeoxyguanosine and nephrin in urine samples

Urinary samples were collected over 24h and stored until analysis at -80°C under nitrogen to prevent the formation of artificial adducts. Urinary 8-hydroxydeoxyguanosine (8-OHdG) and nephrin levels were determined using an 8-OHdG Check ELISA kit (Japan Institute for the Control of Aging, Fukuroi, Japan) and a mouse nephrin ELISA kit (Exocell, Philadelphia, PA, USA), respectively. The concentrations of 8-OHdG and nephrin were calculated as ng per mg of total protein.

2.4 Renal DPP-4 enzymatic activity assay

Frozen renal cortex samples were weighed and homogenized in assay buffer (25 mM HEPES, 140 mM NaCl, 80 mM $MgCl_2$, pH 7.8) with Triton X-100 (1%, v/v). DPP-4 enzymatic activity was assessed using a DPP-4 Activity Assay Kit (Biovision, Milpitas, CA, USA) according to the manufacturer's instructions. DPP-4 activity was calculated using the cleavage rate of 7-amino-4-methylcoumarin per tissue weight (μM/min per g tissue).

2.5 Renal advanced oxidation protein products assay

Frozen renal cortex tissue (200 mg) was homogenized using a Teflon homogenizer in 2 ml of 100 mM KH_2PO_4 - K_2HPO_4buffer (pH7.4) containing digitonin (0.1%, v/v). Advanced oxidation protein products (AOPP) were measured using an OxiSelect™ AOPP Assay Kit (Cell Biolabs, San Diego, CA, USA) according to the

manufacturer's instructions. The levels of AOPP were expressed as the amount of chloramine per mg of protein (μM/mg total protein).

2.6 Immunostaining

Immunostaining was conducted according to a previously described protocol. (Park et al., 2013) For immunohistochemical analysis, the antibodies used were rabbit anti-8-OHdG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-receptor for advanced glycation end product (anti-RAGE, Abcam, MA, USA), and mouse anti-integrin-linked kinase (anti-ILK, Santa Cruz Biotechnology). Their expression was detected using an EnVision Plus detection system (DAKO, Carpinteria, CA, USA) and developed using 3,3′-diaminobenzidine tetrahydrochloride chromogen solution. The antibody used for immunofluorescence analysis was rabbit anti-synaptopodin (Santa Cruz Biotechnology). To detect synaptopodin, the slides were probed with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit immunoglobulin G (Santa Cruz Biotechnology). Negative control slides were incubated with non-immune normal serum as the primary antibody. Immunostaining intensity was evaluated quantitatively in 40 glomeruli per animal using cellSens software (Olympus, Tokyo, Japan).

2.7 Double immunofluorescence staining

To directly observe the apoptosis of renal podocytes, tissue sections were double stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and Wilms tumor antigen-1 (WT-1) according to a previously published procedure. (Park et al., 2013) Briefly, the sections were first labeled using the DeadEnd TUNEL system (Promega, Madison, WI, USA) and FITC-conjugated

streptavidin (Santa Cruz Biotechnology). The second sequence of staining using rabbit anti-WT-1 antibody (Santa Cruz Biotechnology) was performed on the same section with rhodamine-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology). To achieve dual-labeling for synaptopodin and integrin-linked kinase (ILK), a sequence of two single immunofluorescent staining procedures was performed. To prevent any unwanted reactions between the two staining sequences, sections were blocked with normal serum from the same species as the labeled antibody following the first staining. The number of cells positive for WT-1 was quantified from 40 glomeruli per animal using cellSens software (Olympus, Tokyo, Japan).

2.8 Western blotting analysis

Protein lysate (20 μg) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Biorad, Hercules, CA, USA). The membrane was labeled with mouse anti-DPP-4 (Santa Cruz Biotechnology), mouse anti-RAGE antibody (Abcam, Cambridge, MA, USA), and mouse anti-ILK antibody (Santa Cruz Biotechnology). The immunoreactive bands were detected using an enhanced chemiluminescence kit (Amersham, Piscataway, NJ, USA) and the density of the bands-of-interests was further measured using a LAS-3000 (Fujifilm, Tokyo, Japan).

2.9 Statistical analysis

Data are expressed as the mean ± standard error of the mean (S.E.M.). Differences between groups were assessed by one-way analysis of variance (ANOVA) with post hoc analysis using Prism

4.0 program (GraphPad, La Jolla, CA, USA). Differences were considered statistically significant with $P < 0.01$.

3. Results

3.1 Body weight and blood glucose

Compared to non-diabetic controls, *db/db* mice had an increased body weight, and this change was unaffected by gemigliptin administration (Table 1). Blood glucose and glycated hemoglobin (HbA1c) levels were significantly higher in *db/db* mice ($P < 0.01$ vs. normal *db/+*mice), but no significant reduction of these values was observed after gemigliptin treatment (Table 1).

Table 1. Body weight, blood glucose, and HbA1c levels in experimental animals

	NOR	DM	Gemigliptin
Body weight (g)	29.0 ± 1.2	35.4 ± 1.9[a]	33.2 ± 8.0
Blood glucose (mg/dl)	139.8 ± 27.7	531.6 ± 42.9[a]	510.81 ± 115.4
HbA1c (%)	4.80 ± 1.82	12.45 ± 1.99[a]	12.99 ±2.89

NOR, normal *db/+* mice; DM, *db/db* mice; Gemigliptin, *db/db* mice treated with gemigliptin (100 mg/kg/day); HbA1c, glycated hemoglobin. Data are expressed as the mean ± S.E.M.

[a] $P < 0.01$ vs. NOR group.

3.2 Gemigliptin inhibits diabetes-induced podocyte injury

Our first aim was to determine whether gemigliptin could prevent diabetes-induced renal impairment. Fig. 1A and 1B show diffuse mesangial expansion with sclerotic lesions (arrow) ad albuminuria in *db/db* mice. Compared to the vehicle-treated *db/db* mice, gemigliptin-treated mice showed reduction of diabetes-induced histopathological changes and albuminuria ($P<0.01$).

Next, to determine podocyte loss from renal tissue, urine specimens were tested for the urinary podocyte-specific protein, nephrin. At the end of the treatment period, urinary levels of nephrin had increased significantly in *db/db* mice (Fig. 2A, $P<0.01$ vs. *db/db* mice). To more accurately determine the extent of podocyte loss, podocytes were probed with anti-WT-1 and anti-synaptopodin antibodies (Park et al., 2013), and the mean number of positive-stained cells in a particular glomerulus were determined. In *db/db* mice, the number of WT-1-positive or synaptopodin-positive cells (red and green, respectively) significantly decreased compared to that in *db/+* mice (Fig. 2B to 2D). Interestingly, TUNEL and WT-1 double-positive cells (arrow, Fig.2B) were rare or absent in *db/+* mice. In contrast, the number of apoptotic podocytes increased in *db/db* mice with higher nephrinuria. However, gemigliptin ameliorated this apoptotic podocyte loss with low-grade nephrinuria (Fig.2B). Taken together, these results suggest that gemigliptin prevents diabetes-induced podocyte apoptosis and reduces albuminuria.

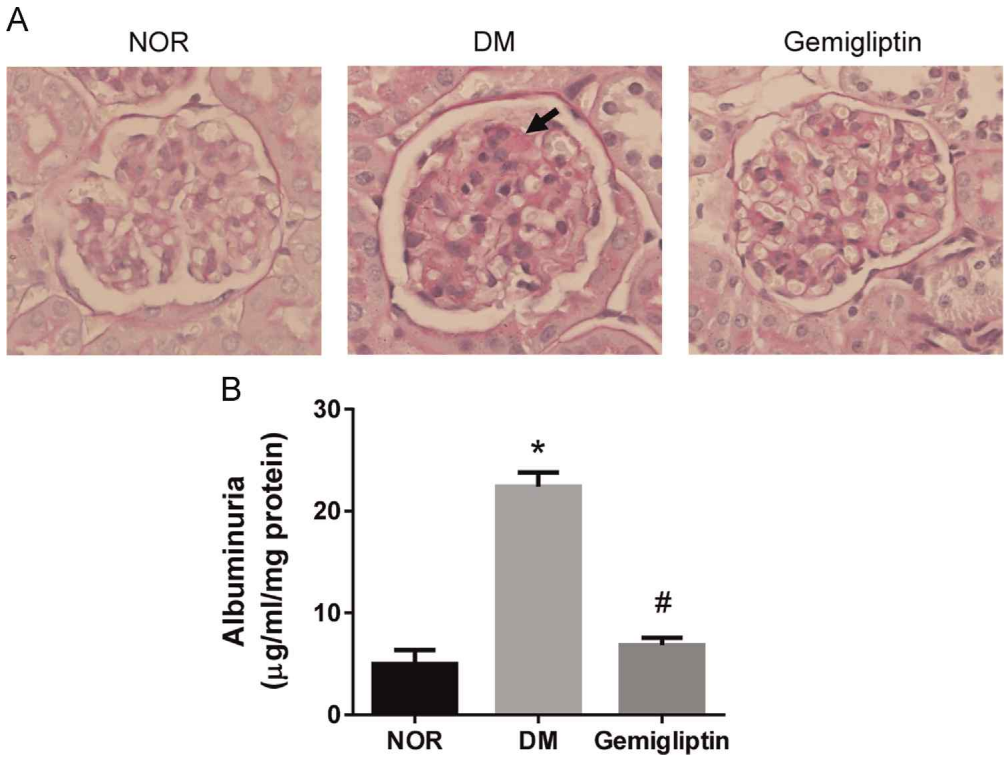

Figure 1. Renal histopathology and function. (A) Periodic acid-Schiff staining of glomeruli. x400 magnification. Arrows indicate sclerotic lesions. (B) Albuminuria. NOR, normal *db/+* mice; DM, *db/db* mice; Gemigliptin, *db/db* mice treated with gemigliptin (100 mg/kg/day). Data are expressed as mean ± S.E.M. (n = 10). *P < 0.01 vs. NOR group, #P < 0.01 vs. DM group.

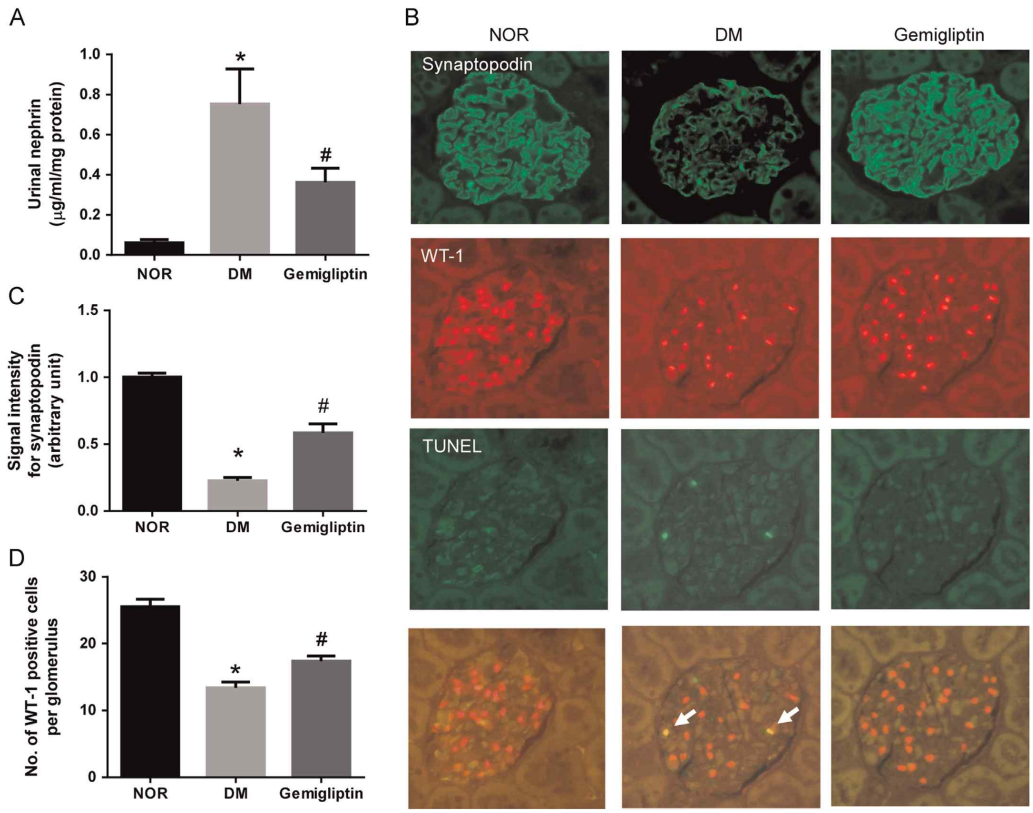

Figure 2. Inhibitory effects of gemigliptin on diabetes-induced podocyte loss. (A) ELISA analysis of urinary nephrin. (B) Immunofluorescence staining for synaptopodin (green) and a dual labeling for WT-1 (red) and TUNEL (green). x400 magnification. The renal cortex was stained using specific antibodies for synaptopodin and WT-1 (specific markers of podocytes). Double positive cell (arrow) indicates co-localization of the podocyte marker and apoptosis. Quantitative analyses of (C) signal intensity of synaptopodin, (D) and numbers of WT-1-positive cells. Data are expressed as mean ± S.E.M. (n = 10). *P < 0.01 vs. NOR group, #P < 0.01 vs. DM group.

3.3 Gemigliptin inhibits renal DPP-4 activity and protein expression

DPP-4 enzyme activity was significantly enhanced in the renal tissue of *db/db* mice compared to that in *db/+* mice ($P<0.01$, Fig.3A). Treatment with gemigliptin normalized enzyme activity in *db/db* mice. Similarly, diabetes led to increased DPP-4 protein levels compared to those in *db/+* mice, while gemigliptin prevented the increase in renal DPP-4 protein levels in *db/db* mice (Fig. 3B).

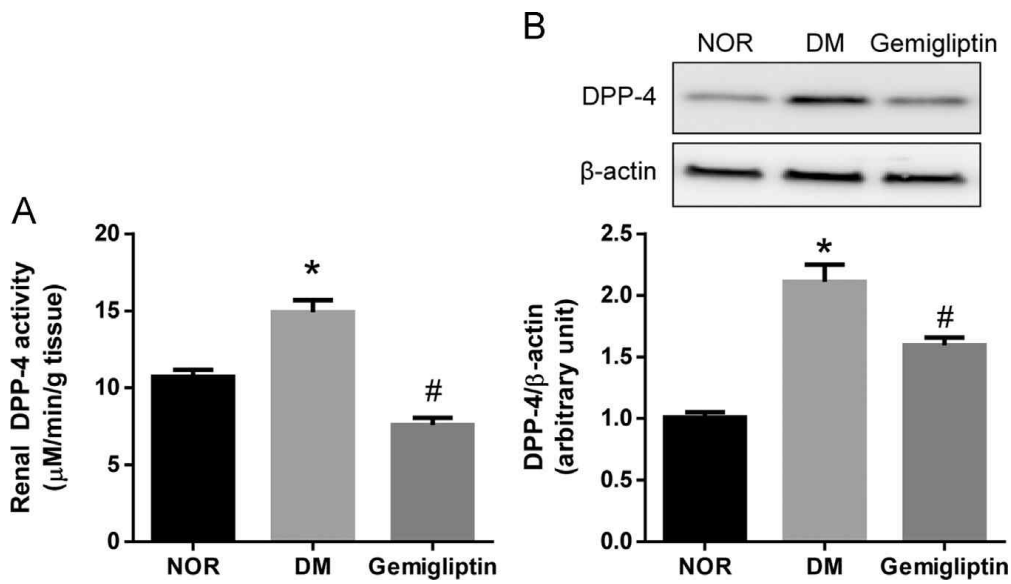

Figure 3. Renal DPP-4 activity and protein expression. (A) DPP-4 enzyme activity was measured by the cleavage of a fluorescent substrate. (B) The protein levels of DPP-4 were assessed by western blot analysis. The bands were detected and quantified using an image analyzer (LAS-3000, Fujifilm), and DPP-4 protein levels were normalized to β-actin levels. Data are expressed as mean ± S.E.M. (n = 10). *P < 0.01 vs. NOR group, #P < 0.01 vs. DM group.

3.4 Gemigliptin prevents the accumulation of AOPP and expression of its receptor in renal tissues

AOPP has been identified as a novel family of protein adducts that are cross-linked by dityrosine. They are known to occur more frequently in diseases associated with increased oxidative stress, including chronic renal disease and diabetes (Witko-Sarsat et al., 1998). AOPP induces apoptosis of podocyte (Zhou et al., 2009) and causes inflammatory response in endothelial cells via binding to RAGE (Guo et al., 2008). Thus, we performed immunostaining and a urinary ELISA for 8-OHdG to examine oxidative damage and measure renal AOPP and RAGE protein expression in *db/db* mice in the absence or presence of gemigliptin. Urinary excretion of 8-OHdG was significantly greater in the DM group than in the NOR group (Fig. 4A). However, this observed increase in urinary 8-OHdG was markedly reduced by treatment with gemigliptin (db/db mice, 11.64 ± 3.31 ng/ml/mg protein; gemigliptin-treated *db/db* mice, 7.94 ± 1.78 ng/ml/mg protein). A similar pattern was observed in urinary levels of 8-OHdG and albumin. As shown in Fig. 4B, the number of cells stained positively for 8-OHdG (arrow) was significantly higher in the DM group than in the NOR group. However, significantly fewer cells tested positive for 8-OHdG in *db/db* mice treated with gemigliptin. In addition, AOPP clearly accumulated in the renal tissue of *db/db* mice; it was significantly higher in the DM group than in the NOR group (Fig. 4C). However, AOPP accumulation was reduced in renal tissue of gemigliptin-treated *db/db* mice.

Next, we conducted immunostaining and immunoblotting assays for RAGE. Immunostaining demonstrated a significant increase in RAGE protein (arrow) in the glomeruli of *db/db* mice compared to that in

non-diabetic controls, but RAGE expression was down-regulated by gemigliptin (Fig. 5A). In the western blot analysis, increased levels of RAGE proteins were noted in the renal cortex of *db/db* mice. Gemigliptin significantly lowered the expression of RAGE proteins (Fig. 5B).

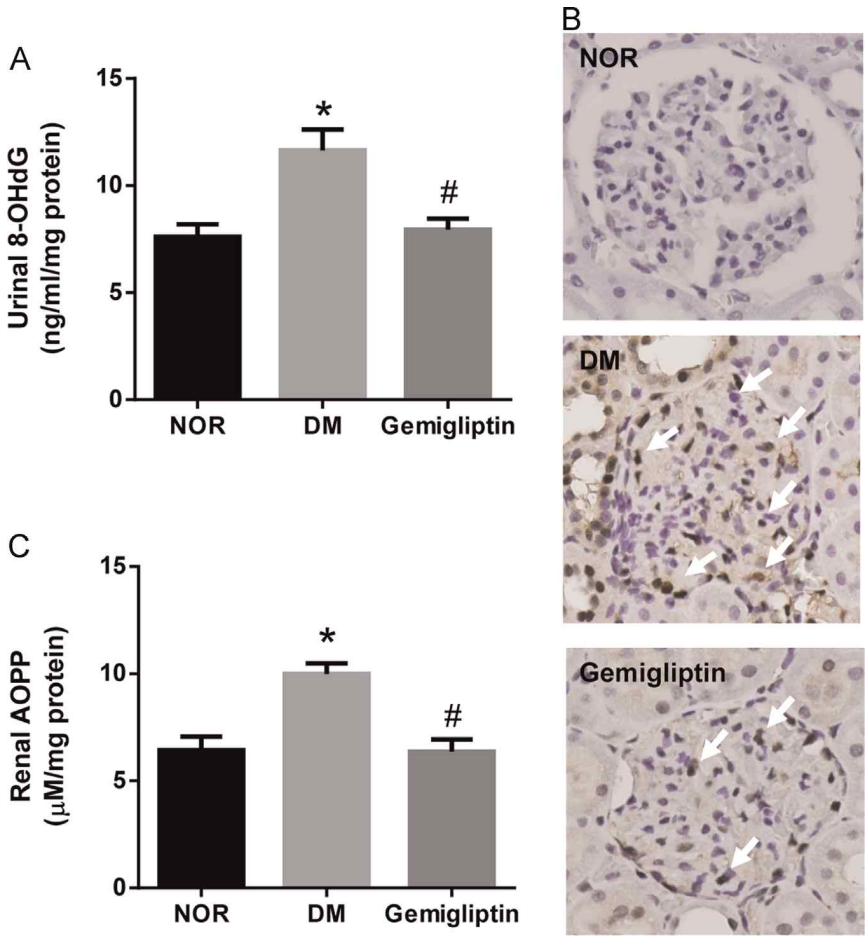

Figure 4. Effect of gemigliptin on 8-OHdG formation and AOPP accumulation in renal tissues. (A) Urinary 8-OHdG excretion levels. (B) Immunohistochemical staining for 8-OHdG. Arrows indicate 8-OHdG - positive nuclei in the glomerulus. (C) Renal AOPP levels. Data are expressed as mean ± S.E.M. (n = 10). *P < 0.01 vs. NOR group, #P < 0.01 vs. DM group.

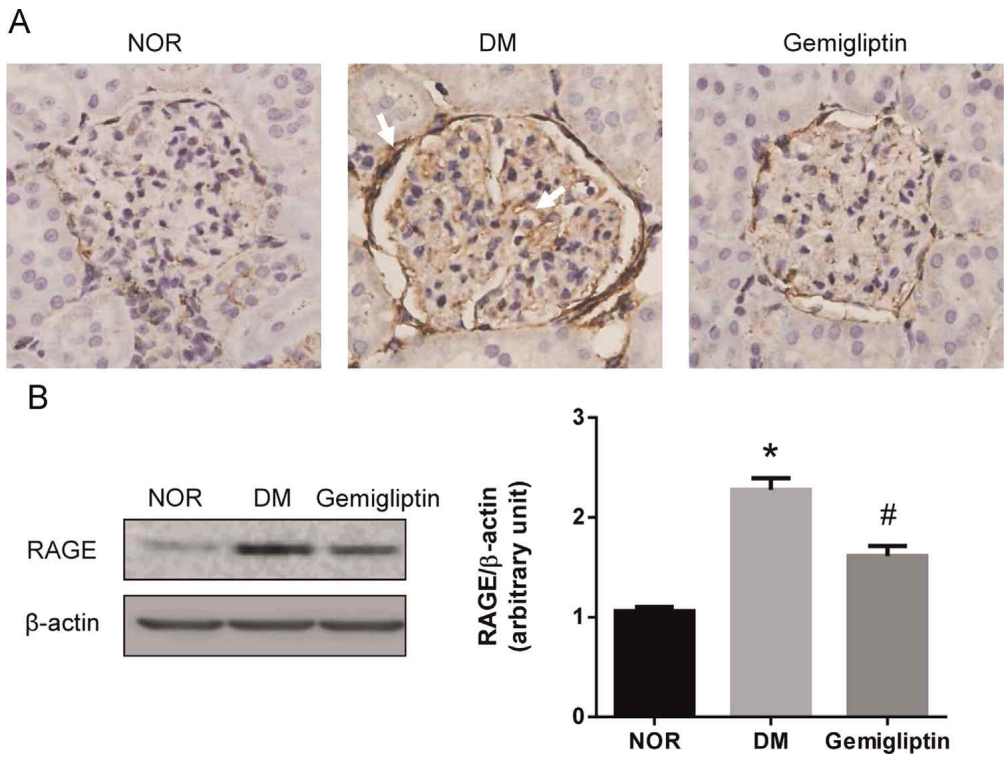

Figure 5. Effect of gemigliptin on RAGE protein expression in renal tissues. (A) Immunohistochemical staining for RAGE. Arrows indicate the area of RAGE expression. (B) Representative immunoblots of RAGE in protein extracts. Quantitative analysis of protein expression. RAGE protein levels were normalized to β-actin levels. Data are expressed as mean ± S.E.M. (n = 10). *P < 0.01 vs. NOR group, #P < 0.01 vs. DM group.

3.5 Gemigliptin inhibits ILK expression

ILK, a 59-kDa Ser/Thr kinase, is important for the control of podocyte-matrix adhesion and podocyte survival. Advanced glycation end products (AGEs) have been shown to reduce podocyte adhesion by up-regulating ILK expression (Cheng et al., 2013). Moreover, AGEs are structurally similar to AOPP and elicit similar biological responses (Kalousova et al., 2002). These findings suggest that there may be a link between AOPP accumulation, ILK regulation, and podocyte loss. Thus, we evaluated the effect of gemigliptin on ILK expression in *db/db* mice. As shown in Fig.6A, immunohistochemical staining showed little ILK protein present in the glomeruli of *db/+* mice. In *db/db* mice, ILK expression was up-regulated in the glomeruli (arrow). Double immunostaining for synaptopodin (green) and ILK (red) clearly confirmed ILK expression in podocytes. Although the number of podocytes reduced, co-localization (arrowhead) of synaptopodin and ILK was clearly observed in *db/db* mice (Fig. 6B). However, gemigliptin decreased ILK protein expression in the glomeruli of *db/db* mice. Similarly, we observed changes in the quantity of ILK protein using western blot analysis. The expression of ILK was consistent with their immunohistochemical response (Fig. 6B).

Compared to ILK expression in *db/+* mice, ILK expression was significantly higher in *db/db* mice, and gemigliptin treatment reduced ILK expression significantly. These observations suggested that the protective effect of gemigliptin on podocytes might be caused by down-regulation of ILK protein expression.

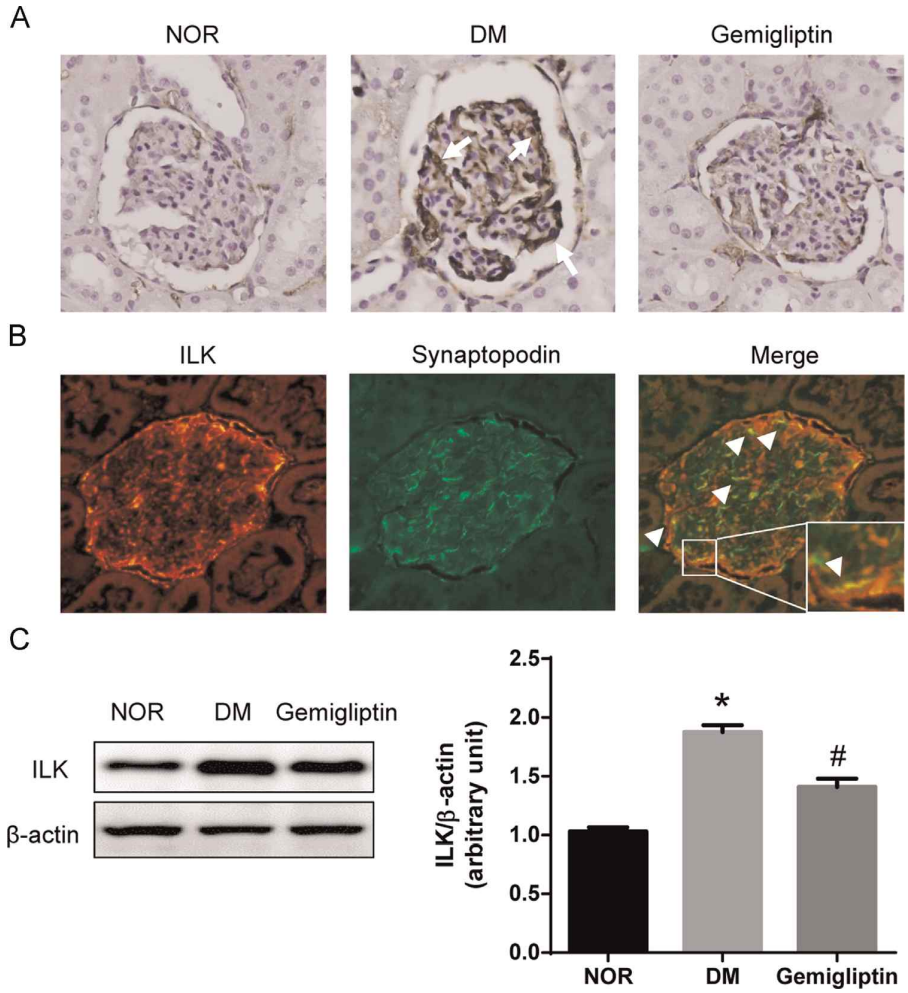

Figure 6. Gemigliptin inhibited the protein expression of ILK in renal tissue. (A) Immunohistochemical staining for ILK. Arrows indicate the area of ILK expression (B) Double immunofluorescence staining for ILK (red) and synaptopodin (green) to confirm the expression of ILK in podocytes. The specific co-staining (arrowhead) for ILK and synaptopodin is shown in the merged picture as yellow. (C) The protein expression of ILK was also examined by western blot. ILK protein levels were normalized to β-actin levels. Data are

expressed as mean ± S.E.M. (n = 10). *P < 0.01 vs. NOR group, #P < 0.01 vs. DM group.

4. Discussion

Gemigliptin is a selective and long-lasting DPP-4 inhibitor, and it is approved by the Korea Ministry of Food and Drug Safety for clinical use in patients with type 2 diabetes (Lim et al., 2009). Most research on gemigliptin has focused on the normalization of blood glucose in patients with type 2 diabetes (Kim et al., 2013). However, this is the first study demonstrating that gemigliptin has protective effects in the renal podocytes of diabetic animals by a mechanism independent of lowered blood glucose.

Podocytes, highly specialized epithelial cells found in the glomerulus, contribute to the formation of the glomerular filtration barrier. Increased renal podocyte loss is a well-established consequence of diabetes, and is one of the first detectable signs of diabetic nephropathy(Pagtalunan et al., 1997). Importantly, the reduction of podocyte number is observed even in patients with a relatively recent onset of diabetes, and podocyte injury could even occur before albuminuria (Steffes et al., 2001). Moreover, podocyte apoptosis and depletion correspond with the development of glomerulosclerosis in a time-dependent manner (Kriz and Lemley, 1999). Therefore, improving the health of podocytes is a reasonable therapeutic approach to prevent renal function impairment in the event of diabetic nephropathy (Leeuwis et al., 2010; Mathieson, 2012). Interestingly, gemigliptin successfully prevented podocyte apoptosis through the inhibition of AOPP accumulation and ILK up-regulation in *db/db* mice, despite continued hyperglycemia. These results indicate that gemigliptin has direct effects on podocytes that are independent of its anti-hyperglycemic effect.

It was previously reported that DPP-4 inhibitors were unable to

improve hyperglycemia in a late-stage animal disease model (Nagakura et al., 2003). Kawashima et al. reported that alogliptin did not show beneficial effects on blood glucose and β-cells in *db/db* mice aged between 8 and 16 weeks. Their research suggests that the ineffectiveness of DPP-4 inhibitors might be owing to the reduction of GLP-1 receptor expression in *db/db* mice (Kawashima et al., 2011). Consistent with previous reports, gemigliptin failed to reduce blood glucose levels in *db/db* mice. Therefore, this animal model is suitable for evaluating the effects of gemigliptin in the diabetic kidney independent of its glucose-lowering effects.

An oral dose of 50 mg/day gemigliptin is recommended for glycemic control in patients with diabetes. However, our recent study showed that oral administration of 100 mg/kg/day gemigliptin reduced plasma AGE levels in *db/db* mice (Jung et al., 2014). Min et al. also showed that oral consumption of 150 mg/kg/day gemigliptin in a mouse model of ureteral obstruction prevented renal interstitial fibrosis (Min et al., 2014). On the basis of these results, we selected a dose of 100 mg/kg gemigliptin for daily oral administration in *db/db* mice. Our finding regarding the effective dose of gemigliptin on kidney tissues in *db/db* mice is consistent with that of the Min et al. (Min et al., 2014). In a previous report, the average peak plasma concentration of gemigliptin was 62.7 ng/ml in humans after oral administration of a single 50 mg dose, and the area under the plasma concentration time curve (AUC) was 743.1 ng/ml • h^{-1}. The elimination half-life of gemigliptin was 17.1 h. Inhibition of DPP-4 activity was maintained for 24 h (Lim et al., 2008). However, in *db/db* mice, the peak plasma concentration of gemigliptin after oral administration of 100 mg/kg gemigliptin was 2614 ng/ml, but the elimination half-life of gemigliptin was 1.5 h. Thus, the AUC value of gemigliptin was 4510

ng/ml • h^{-1} in *db/db* mice (unpublished data). Although, this AUC value of gemigliptin after oral administration in human was 6-folds lower than that observed in mice, the elimination half-life of gemigliptin in human were 11-folds longer than in mice. Moreover, due to its short half-life in *db/db* mice, the DPP-4 inhibitory activity of gemigliptin for 24 h was equivalent to that seen in *db/db* mice after oral administration of 100 mg/kg gemigliptin. For this reason, gemigliptin may have renoprotective effect at a relatively lower dose in diabetic patients.

DPP-4 is a serine exopeptidase that cleaves Xaa-Ala or Xaa-Pro dipeptides at the N-terminus (Lambeir et al., 2003). Several studies have demonstrated the renoprotective effects of DPP-4 inhibitors. Linagliptin reduced renal fibrosis in STZ-induced diabetic mice (Kanasaki et al., 2014). Alogliptin ameliorated albuminuria in type 2 diabetes patients with early stage of nephropathy (Fujita et al., 2014), and vildagliptin attenuated renal sclerosis in Zucker diabetic fatty rats (Vavrinec et al., 2014). An important aspect of these studies is that DPP-4 inhibitors demonstrate renoprotective effects independent of glycemic control. Although the mechanism of action of DPP-4 inhibitors in diabetes-induced renal injury is associated with GLP-1, the enzymatic or non-enzymatic functions of DPP-4 may also regulate the progression of diabetic renal disease (Hocher et al., 2012). DPP-4 is widely expressed in the entire body (Barnett, 2006), and expressed at the highest level per organ weight in renal tissue. (Mentlein, 1999) It is also found in podocytes (Machado et al., 2012). In the present study, gemigliptin normalized renal DPP-4 activity. Similarly, vildagliptin normalized renal DPP-4 activity in STZ-induced diabetic rats, and significantly prevented albuminuria, oxidative DNA damage, and renal cell apoptosis (Vavrinec et al., 2014). The

amelioration of renal DPP-4 activity by gemigliptin may contribute to the significant renal protection observed. Moreover, gemigliptin suppressed DPP-4 activity as well as protein expression in renal tissue. This result agrees with that of other research showing that sitagliptin and linagliptin also inhibited renal DPP-4 protein expression in diabetic animals (Kanasaki et al., 2014; Marques et al., 2014). In our present study, gemigliptin inhibited renal DPP-4 activity than that of *db/+* mice, although DPP-4 protein level in gemigliptin-treated db/db mice was higher than normal *db/+* mice. Similarly, linagliptin also highly inhibited renal DPP-4 activity in STZ-induced diabetic mice than normal control mice. Linagliptin inhibited DPP-4 protein expression, but its protein level was higher than normal control mice (Kanasaki et al., 2014). This discrepancy between the protein amount and its enzyme activity could be explained by a mechanism of action of gemigliptin. Gemigliptin is a highly selective DPP-4 inhibitor and directly bind to DPP-4 in a reversible manner, which reduces its enzyme activity (Kim et al., 2013). Moreover, it was reported that DPP-4 protein expression was regulated by microRNA 29s in STZ-induced diabetic mice, not by DPP-4 inhibitor. Linagliptin restored microRNA 29s, leading to the suppression of DPP-4 protein levels (Kanasaki et al., 2014). Further studies are necessary to evaluate whether gemigliptin increases microRNA 29s in the kidney of diabetic animal models.

DPP-4 directly increased reactive oxygen species generation in endothelial cells (Ishibashi et al., 2013). Its deficiency inhibited H_2O_2-induced intracellular reactive oxygen species production in cardiomyocytes (Ku et al., 2013). Additionally, it has been demonstrated that reactive oxygen species overproduction induced by high-glucose leads to podocyte apoptosis in vitro and to progressive

podocytopenia in experimental animals, leading to diabetic nephropathy (Susztak et al., 2006). Although podocyte-specific reactive oxygen species generation by DPP-4 and podocyte-specific inhibition of reactive oxygen species production by gemigliptin was not shown in this study, we demonstrated that gemigliptin led to the reduction of renal DPP-4 protein expression and prevented renal AOPP accumulation, enhanced 8-OHdG formation and enhanced reactive oxygen species-mediated podocyte apoptosis in *db/db* mice. These findings suggest that DPP-4 inhibition by gemigliptin could reduce oxidative stress-related podocyte injury and reduce the risk of cell death.

There is increasing evidence to suggest that ILK has an important role in the onset and progression of diabetic nephropathy (Chen et al., 2014). Changes to the cytoskeletal architecture lead to weakening of the adhesion between podocytes and the glomerular basement membrane. ILKs are reported to be involved in these processes (Wu and Dedhar, 2001). ILK proteins are highly expressed in mesangial cells and glomerular epithelial cells of diabetes patients (Guo et al., 2001) and diabetic rats (Dai et al., 2012). ILK activation leads to the reduction of integrin α3β1 expression and impaired cell-cell contact of podocytes under high-glucose conditions (Chen et al., 2000). Moreover, it was reported that AGEs reduced podocyte adhesion via up-regulation of ILK expression (Cheng et al., 2013). The effects caused by AGEs are because of their interaction with the receptor, RAGE; AOPP is structurally similar to AGE and elicits similar biological responses via interaction with the same receptor (Guo et al., 2008). Blocking RAGE dramatically attenuated the effect of AOPP (Iwao et al., 2008). Moreover, it was recently reported that DPP-4 increases RAGE gene expression and the DPP-4 inhibitor, linagliptin,

prevented up-regulation of RAGE mRNA levels in endothelial cells (Ishibashi et al., 2013). Our results clearly showed that gemigliptin treatment leads to decreased RAGE and ILK protein expression in the kidney of diabetic mice. Although we did not show AOPP-specific up-regulation of ILK in podocytes, our observations suggest that gemigliptin therapy may be useful in regulating AOPP/RAGE interaction and ILK expression in the kidney of individuals with diabetes.

Our study demonstrated that gemigliptin has an anti-apoptotic effect in podocytes that is attributable to inhibition of AOPP accumulation, RAGE expression, and ILK up-regulation in *db/db* mice. These novel findings provide information regarding the renoprotective effects of gemigliptin that are independent of glycemic control.

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Part III.

Gemigliptin, a dipeptidyl peptidase-4 inhibitor, inhibits retinal pericyte injury in db/db mice and retinal neovascularization in mice with ischemic retinopathy

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Abstract

Retinal pericyte loss and neovascularization are characteristic features of diabetic retinopathy. Gemigliptin, a dipeptidyl peptidase-4 (DPP-4) inhibitor, has shown robust blood-glucose lowering effects in type 2 diabetic patients, but its effects on diabetic retinopathy have not yet been reported. We evaluated the efficacy of gemigliptin on retinal vascular leakage in *db/db* mice, which is an animal model for type 2 diabetes, and neovascularization in oxygen-induced retinopathy (OIR) mice, which is an animal model for ischemic proliferative retinopathy. Gemigliptin (100 mg/kg/day) was orally administered to the *db/db* mice for 12 weeks. C57BL/6 mice on postnatal day 7(P7) were exposed to 75% hyperoxia for 5 days, followed by exposure to room air from P12 to P17 to induce OIR. Gemigliptin (50 mg/kg/day) was intraperitoneally injected daily from P12 to P17. Retinal neovascularization was analyzed in flat-mounted retinas on P17. We determined the efficacy and possible mechanism of gemigliptin on high glucose-induced apoptosis of primary human retinal pericytes. The oral administration of gemigliptin for 4 months significantly ameliorated retinal pericyte apoptosis and vascular leakage in the *db/db* mice. Gemigliptin also ameliorated retinal neovascularization in the OIR mice. Gemigliptin attenuated the overexpression of plasminogen activator inhibitor-1 (PAI-1) in the retinas of diabetic and OIR mice. Gemigliptin and PAI-1 siRNA significantly inhibited pericyte apoptosis by inhibiting the overexpression of PAI-1, which is induced by high glucose. These results suggest that gemigliptin has potent anti-angiogenic and anti-apoptotic activities via its ability to suppress DPP-4 and PAI-1, and the results support the direct retinoprotective action of gemigliptin.

1. Introduction

Diabetic retinopathy is a common and serious microvascular complication of diabetes (Fong et al., 2002). The normal function of retinal vessels requires interactions between the inner endothelial cells and surrounding pericytes (Hammes et al., 2002). Pericyte loss is one of the earliest detectable lesions in the diabetic retina (Hammes, 2005) and may be responsible for the retinal vascular leakage and sight-threatening neovascularization (Arboleda-Velasquez et al., 2015). Pericytes express several angiogenic growth factors, which are critical for determining whether vessels are stable or will undergo angiogenesis (Tomasek et al., 2006). The current management strategy for diabetic retinopathy is focused on early detection and tight glycemic control to slow the progression of the disease. However, the persistence of hyperglycemic stress despite glucose normalization has been observed in the diabetic retina (Engerman and Kern, 1987; Ihnat et al., 2007). Previous studies have shown that vascular endothelial cells and retinal pigment epithelial cells previously exposed to high glucose continued to display a reactive oxygen species (ROS)-mediated cellular stress despite subsequent normalization of glucose concentration in the media (Ihnat et al., 2007). Indeed, although many classes of glucose-lowering oral agents have been available for clinical use, the prevalence of diabetic retinopathy is increasing (Girach et al., 2006).

Dipeptidyl peptidase-4 (DPP-4) inhibitors are a major new class of anti-diabetic drugs that increase the levels of a gastrointestinal incretin hormone, glucagon-like peptide-1 (GLP-1), by inhibiting its cleavage. DPP-4 inhibitors show glucose-dependent activity by increasing insulin secretion, inhibiting glucagon secretion, and increasing β-cell mass, thereby producing lower blood glucose levels

(Baetta and Corsini, 2011). Recently, Blaslov et al. reported that circulating DPP-4 activity is associated with the progression and severity of diabetic retinopathy (Blaslov et al., 2015). Several DPP-4 inhibitors were shown to have protective effects against diabetic retinopathy. Sitagliptin inhibited retinal vascular leakage, inflammation and neuronal apoptosis in both type 1 and type 2 diabetic rats (Goncalves et al., 2012; Goncalves et al., 2014). Vildagliptin down-regulated the gene expression levels of angiogenic growth factors, such as vascular endothelial growth factor (VEGF), intercellular adhesion molecule-1 (ICAM-1), plasminogen activator inhibitor-1 (PAI-1) and pigment epithelium-derived factor (PEDF) in the retinas of obese type 2 diabetic rats (Maeda et al., 2013). Saxagliptin improved retinal capillary flow in diabetic patients without the clinical signs of microvascular alteration (Ott et al., 2014). However, the precise mechanisms of DPP-4 inhibitors against diabetic retinopathy beyond its glucose-lowering role are still not well understood.

Gemigliptin is a highly selective DPP-4 inhibitor developed in Korea and has been clinically used as an oral agent for type 2 diabetes (Kim et al., 2013). Previous *in vitro* and *in vivo* data suggest that gemigliptin also exerts a number of additional activities, including anti-glycation, anti-inflammation and renoprotective effects (Hwang et al., 2015; Hwang et al., 2014; Jung et al., 2014; Min et al., 2014). To the best of our knowledge, there have been no reports on the effects of DPP-4 inhibitors on retinal pericyte injury and neovascularization. Therefore, we studied whether gemigliptin inhibits retinal pericyte injury through the modulation of apoptotic cascades in type 2 diabetic *db/db* mice. Additionally, we evaluated whether gemigliptin effectively prevents retinal neovascularization in oxygen-induced retinopathy

(OIR) mice.

2. Materials and Methods

2.1. Animals

Six-week-old male C57BL/KsJ-Leprdb/db diabetic mice (*db/db*) and nondiabetic heterozygous littermates (*db/+*) were purchased from Japan SLC (Shizuoka, Japan). C57BL/6 mice were purchased from Orient Bio (Seoul, Korea). The care and use of the animals were approved by the Animal Welfare Review Board of LG Life Sciences (LGMD13-083; approval date 01/07/2014).

2.2. Type 2 diabetic *db/db* mice

After 2 weeks of acclimatization, the *db/+* and *db/db* mice were divided into the following three groups: the *db/+* group (n = 10), the *db/db* group (n = 10) and the gemigliptin group (n = 10). Gemigliptin (100 mg/kg body weight; LG Life Sciences, Seoul, Korea) or an equal amount of vehicle (0.5% methylcellulose solution) was administered to diabetic db/db mice by oral gavage daily for 12 weeks. Non-diabetic littermates received the same amount of methylcellulose solution. The blood glucose levels were recorded every week.

2.3. OIR mice and analysis of retinal neovascularization

OIR was induced in C57BL/6 mice. On postnatal day 12 (P12), after the mice were exposed to 75 ± 2% oxygen for 5 days (P7-P12), they were returned to room air and randomly assigned to two groups: vehicle-treated OIR mice and gemigliptin-treated (50 mg/kg/day) OIR mice. The normal control mice were maintained under room conditions from birth until postnatal day 17 (P17). Gemigliptin was diluted with 5% dimethyl sulfoxide (Sigma, St Louis, MO, USA) in

saline. The mice were injected intraperitoneally with 100 μL of this solution daily for 5 days. The vehicle solution was injected in the normal control group. On P17, the mice were anesthetized and sacrificed. Fluorescein retinal angiography and Bandeiraea simplicifolia isolectin B4 staining for the quantification of preretinal neovascular tufts were performed according to a method described previously (Connor et al., 2009) Briefly, preretinal neovascularization areas were outlined and quantified in each quadrant of the retina as a percentage of total area of retina analyzed.

2.4. Trypsin digest preparation to isolate retinal vascular beds

Each retina was carefully isolated under a microscope. After fixation in 4% paraformaldehyde for 24 h, the retinas were incubated in 3% trypsin (Sigma, St Louis, MO, USA) in sodium phosphate buffer containing 100 mmol/L NaF for 1 h to inhibit the DNase activity. The retinal vascular beds were separated from the retinal tissue by gentle rinsing in phosphate-buffered saline (PBS) and transferred to microscope slides.

2.5. Determination of the endothelial cells/pericytes (E/P) ratio

The retinas (n=10) from each group were isolated, and the trypsin-digested retinal vessels were stained with periodic acid Schiff base reagent. Pericytes and endothelial cells were identified based on the morphology and relative location to the capillaries. The E/P ratio was calculated using a previously established method (Midena et al., 1989). To determine the E/P ratio, the total numbers of pericytes and endothelial cells were counted in 10 randomly selected areas

(magnification 400x) in the middle one-third of the retinal capillary area. Samples were examined by 3 ophthalmic pathologists in a blinded fashion.

2.6. TUNEL assay and immunofluorescence staining

The retinal digests were incubated with a mouse anti-NG2 antibody (Chemicon, Temecula, CA, USA), a mouse anti-PAI-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and a TUNEL fluorescein kit (Promega, Madison, WI, USA). Nuclei were counterstained with diamidinophenylindole (DAPI, Sigma). For the quantification of pericyte density, 4 fields of view (mm^2) were randomly collected in the retinal digests from each mouse. The number of NG-2-positive was counted, and the number of pericytes was standardized by the number of DAPI-positive cells (number of NG2-positive cells per 100 DAPI-positive cells). TUNEL-positive cells were determined by counting per mm^2 of capillary area.

2.7. Retinal fluorescein isothiocyanate-dextran leakage

Mice were deeply anesthetized with zolazepam (Virbac, Carros, France). Then, a solution of 50 mg/kg of fluorescein isothiocyanate (FITC)-dextran (a molecular weight of 4.4 kDa, Sigma) in PBS (pH 7.4) was injected into the left ventricle. After circulation for 10 min, the retinas were isolated and transferred onto a microscope slide. Pictures were taken using a fluorescence microscope (Olympus, Tokyo, Japan). To quantify retinal vascular permeability, the mice were perfused with PBS (500 ml/kg body weight) for 6 min. Plasma was also collected before perfusion. The retina of each eye was isolated, weighed and homogenized in 200 μL of distilled water. The homogenate was centrifuged at 15,000 rpm for 20 min, and the

supernatant was collected. The fluorescence in each 100 μL sample was measured using a spectrofluorophotometer (Bio-Tek, Winooski, VT, USA). The amount of FITC-dextran leakage into the retinal tissues was calculated using the following equation: retinal FITC-dextran leakage = [retinal FITC-dextran (μg)/retinal weight (g)]/[plasma FITC-dextran (μg/μL) x circulation time (min)]

2.8. Immunofluorescence staining for occludin in retinal whole mounts

The whole retinas from each group were fixed with 4% paraformaldehyde for 24 h. The retinas were blocked and permeabilized in 10% normal donkey serum with 0.3% Triton in PBS for 1 h. The retinas were incubated with mouse anti-occludin antibody (Invitrogen, Carlsbad, CA, USA) in block solution for 48 h at 4°C. The retinas were washed for 30 min at room temperature and then incubated for 2 h at 4°C with rhodamine-conjugated donkey anti-mouse immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, CA, USA). All retinas were flat mounted and viewed with a fluorescence microscope (Olympus, Tokyo, Japan).

2.9. Protein array

On P17, the mice were anesthetized and sacrificed. Each retina was carefully isolated under a microscope. The retinas were homogenized in PBS using protease inhibitors and centrifuged at 10000 x g for 5 minutes, and the total protein concentrations were quantified. To determine the expression levels of angiogenesis-related factors, protein arrays were performed using a kit (Proteome Profiler™ Mouse Angiogenesis Antibody Array Kit, R&D Systems, Abingdon, UK). Optical density measurements were performed using ImageJ software

(NIH, Bethesda, MD, USA). A list of the 55 factors can be found on the manufacturer's webpage.

2.10. DPP-4 enzymatic activity assay

Plasma DPP-4 enzymatic activity was assessed using a DPP-4 Activity Assay Kit (Biovision, Milpitas, CA, USA) according to the manufacturer's instructions. DPP-4 activity was calculated using the cleavage rate of 7-amino-4-methylcoumarin per min per mL.

2.11. Primary human retinal pericyte culture

Primary human retinal pericytes (Cell Systems, Kirkland, WA, USA) were maintained in the specified Pericyte Media (PromoCell, Heidelberg, Germany). Pericytes were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were plated in 6-well plates and were used in experiments when the cells were approximately 80% confluent.

2.12. Apoptosis assay using flow cytometry

Pericytes were treated with normal glucose (5 mmol/L) and high glucose (30 mmol/L) in the presence or absence of gemigliptin at various concentrations (0, 1, 10 and 100 mmol/L) for 1 week. Mannitol (30 mmol/L) was used as an osmotic control. To determine the effect of DPP-4 and PAI-1 suppression, pericytes were transfected with 40 nmol/L DPP-4 siRNA (Santa Cruz Biotechnology), PAI-1 siRNA (Santa Cruz Biotechnology), or a corresponding scrambled siRNA using Lipofectamine™ 2000 (Invitrogen, Frederick, MD, USA) 24 h before treatment with high glucose. Pericytes were then collected and stained with an Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit (Invitrogen)

according to the manufacturer's instructions. Apoptosis was examined using a flow cytometer (Becton Dickinson, San Jose, CA, USA). The cells that stained positively with annexin V but not with PI were considered apoptotic cells.

2.13. Western blot analysis

Protein lysates were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were labeled with mouse anti-DPP-4 (Santa Cruz Biotechnology) and mouse anti-PAI-1 antibody (Santa Cruz Biotechnology). The immunoreactive bands were detected using chemiluminescence detection reagents (Pierce, Rockford, IL, USA), and the density of the bands-of-interest was further measured using a LAS-3000 machine (Fujifilm, Tokyo, Japan).

2.14. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Differences between groups were assessed using one-way ANOVA and Tukey's multiple comparison test in the Prism 6.0 program (GraphPad, La Jolla, CA, USA), and $P<0.05$ was considered statistically significant.

3. Results

3.1. Gemigliptin inhibits diabetes-induced retinal pericyte injury

The mice with a blood glucose concentration exceeding 300 mg/dL were considered diabetic. In the normal *db/+* mice, blood glucose levels were <150 mg/dL. The blood glucose levels in the *db/db* mice were >300 mg/dL at 8 weeks of age and gradually increased over the treatment period. Interestingly, gemigliptin treatment did not affect the blood glucose levels throughout the treatment period (Fig. 1).

We used retinal digest preparations to determine the presence of pericyte loss. The E/P ratio was calculated to determine pericyte density in the retinal vasculature. The E/P ratio was significantly increased in the *db/db* mice compared to that of normal *db/+* mice, whereas the E/P ratio for the gemigliptin-treated mice was significantly reduced (Fig. 2A and B). To confirm pericyte loss, we performed immunofluorescence analysis for NG2, which is a pericyte marker. As shown in Fig. 2C, the number of NG2-positive cells significantly decreased in the vehicle-treated *db/db* mice compared with normal *db/+* mice. However, gemigliptin significantly inhibited the reduction of NG2-positive cells in *db/db* mice. We performed TUNEL staining to further confirm that the reduction in pericyte density in *db/db* mice was due to apoptosis. The retinal vessels of the *db/db* mice showed many TUNEL-positive pericytes and endothelial cells, whereas normal *db/+* mice and gemigliptin-treated *db/db* mice exhibited few positive cells (Fig. 2D). These findings suggest that the loss of retinal pericytes occurred in *db/db* mice and that gemigliptin might inhibit diabetes-induced pericyte apoptosis.

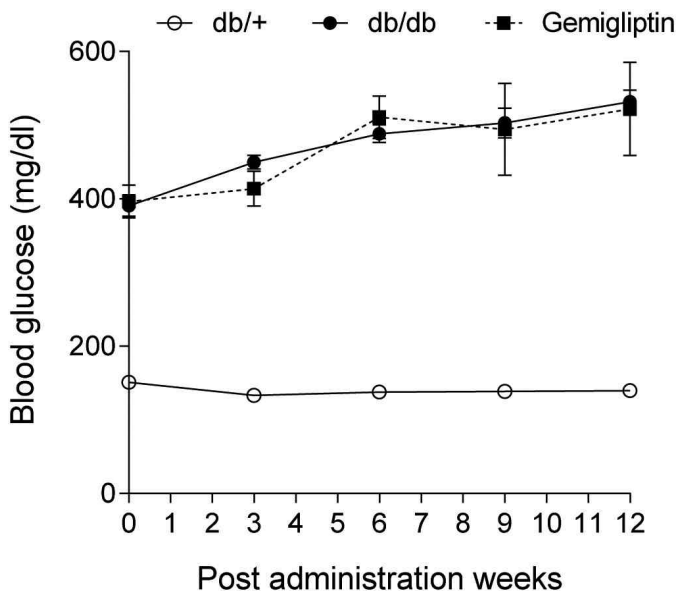
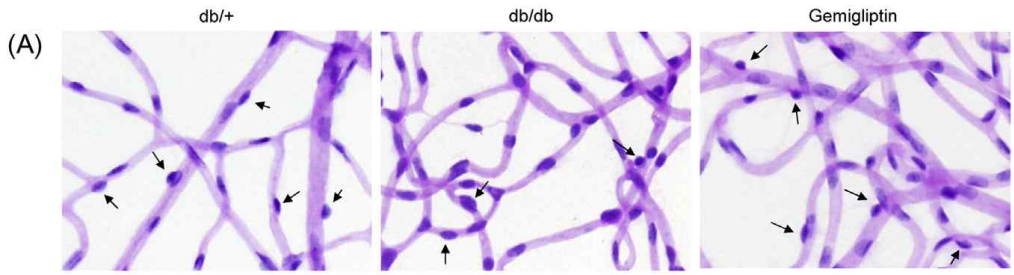
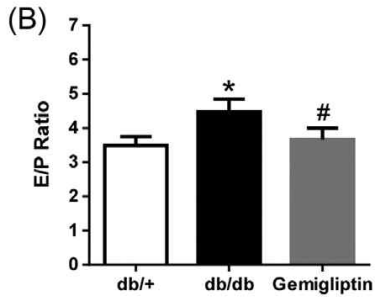
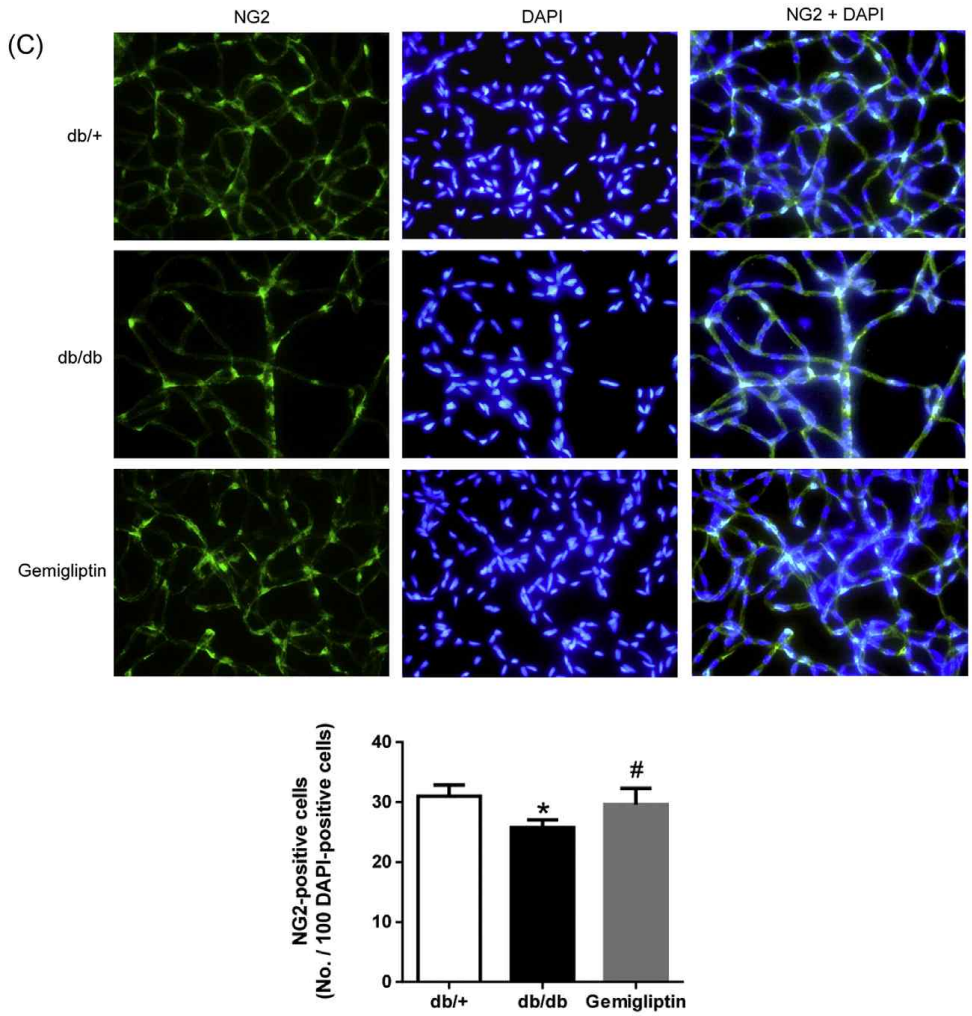


Fig. 1 Blood glucose levels. Values are expressed as the mean ± SEM, n = 10.


(A)
db/+
db/db
Gemigliptin


(B)
E/P Ratio
0
1
2
3
4
5
6
7
*
#
db/+
db/db
Gemigliptin


(C)
NG2
DAPI
NG2 + DAPI
db/+
db/db
Gemigliptin
NG2-positive cells
(No. / 100 DAPI-positive cells)
40
30
20
10
0
*
#
db/+
db/db
Gemigliptin

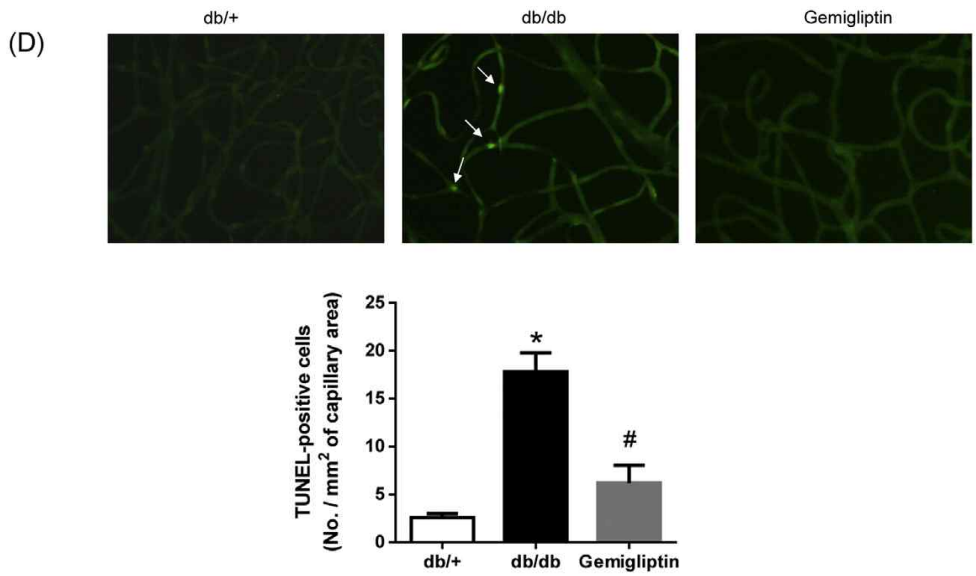


Fig. 2 Inhibitory effects of gemigliptin on diabetes-induced pericyte loss (A) Periodic acid Schiff and hematoxylin-stained retinal digested microvessels. Pericytes were identified in retinal digest preparations based on morphologic criteria (shape, staining intensity, and relative position in the capillary) and quantified. The arrows indicate representative pericytes. (B) The E/P ratio was calculated. (C) Immunofluorescence staining for NG2 (green) in retinal vessels. Vessels were counterstained with DAPI (blue). The number of pericytes was determined by counting the number of NG2 positive cells per mm^2 of capillary area. (D) TUNEL staining. TUNEL-positive nuclei were counted per mm^2 capillary area. Data are expressed as the mean ± SEM, n = 10. *P < 0.05 vs. *db/+* mice, #P < 0.05 vs. *db/db* mice.

3.2 Gemigliptin decreases diabetes-induced BRB disruption

We evaluated retinal vessel dysfunction using FITC-dextran microscopy. Oral gemigliptin treatment significantly reduced retinal vascular leakage in *db/db* mice compared with the vehicle-treated *db/db* mice, to a level that was similar to normal *db/+* mice (Fig. 3A and B). Next, the retinal vessels were immunostained for occludin to examine gemigliptin effects on tight junction protein loss in the retinal microvasculature. Retinal protein extracts were analyzed using Western blot analysis. Unlike the retinal vasculature in normal *db/+* mice, multiple alterations in the subcellular distribution of occludin proteins were observed in the retinal vessels of vehicle-treated *db/db* mice, but gemigliptin significantly restored the decrease in occludin protein levels in *db/db* mice compared with vehicle-treated *db/db* mice to levels that were comparable to the retinas of normal *db/+* mice (Fig. 3C and D).

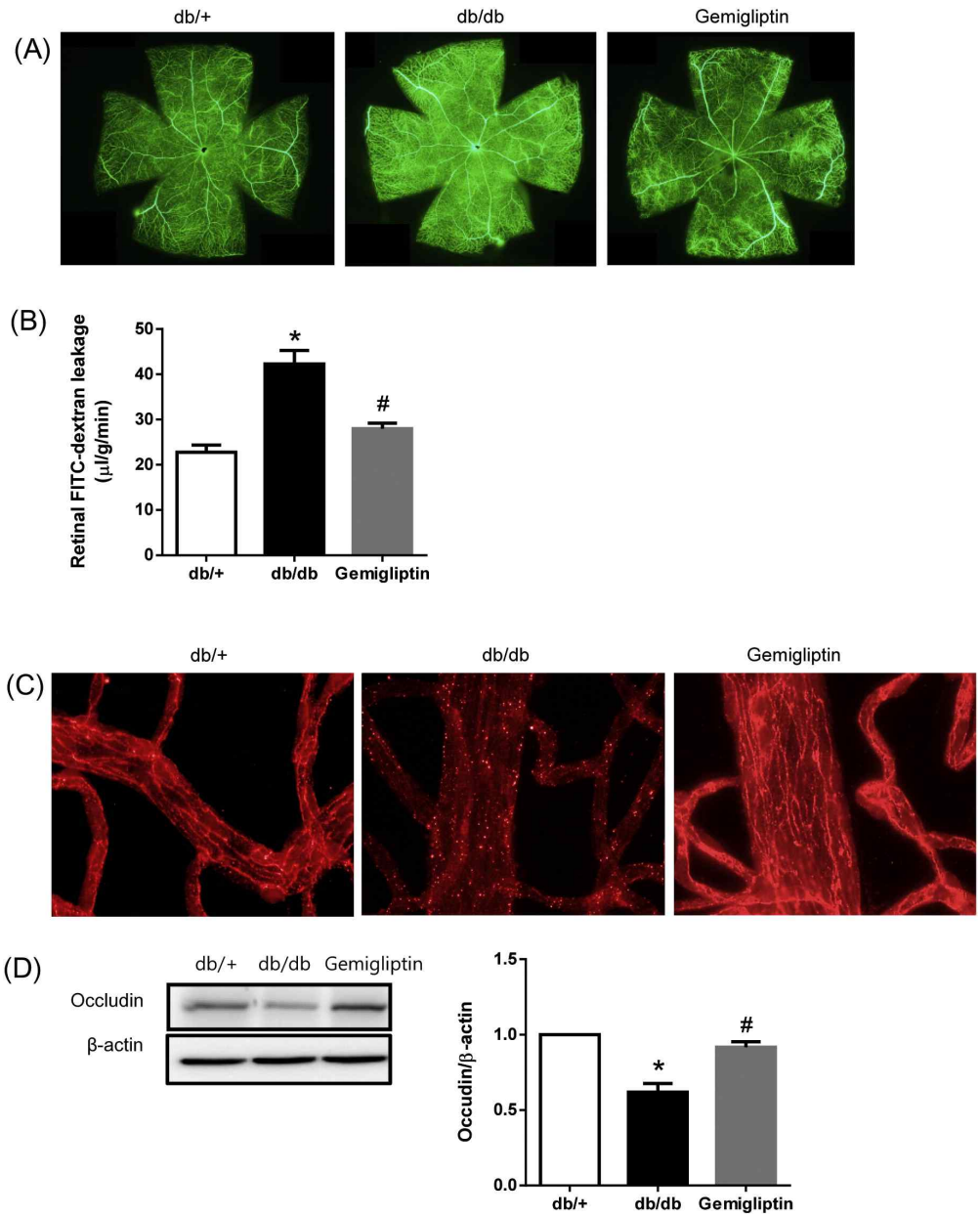


Figure 3. Inhibitory effects of gemigliptin on diabetes-induced blood-retinal barrier breakdown (A) FITC-dextran angiography on retinal flat mounts. (B) Quantitative analysis of retinal vascular permeability. (C) Immunofluorescence staining for occludin protein in

retinal whole mounts. In normal *db/+* mice, immunoreactivity for occludin was continuous at the cell borders, whereas diabetes disrupted occludin immunoreactivity at the cell border and increased intracellular punctate labeling. Gemigliptin prevented the changes of occludin in *db/db* mice. (D) Western blot analysis for occludin in retinal tissues. Values in the bar graphs represent the mean ± SEM, n = 10. *P < 0.05 vs. *db/+* mice, #P < 0.05 vs. *db/db* mice.

3.3 Gemigliptin attenuates retinal neovascularization in OIR mice

The above results indicate that the effects of gemigliptin on retinal vascular alterations can be achieved without a reduction of hyperglycemia in *db/db* mice. To confirm whether gemigliptin's effects on diabetic retinopathy occurred via the direct effects on the retina that independently affected blood glucose, nondiabetic OIR mice were used. Gemigliptin was administered intraperitoneally once a day for 5 days from P12 in OIR mice, which is a model of ischemia-induced retinopathy. Fluorescein angiography at P17 indicated that the gemigliptin-treated retinas developed less retinal vascular leakage and neovascularization (Fig. 4A) compared with vehicle-treated OIR retinas. The quantification of preretinal neovascular tufts using isolectin B4 staining showed that the retinas treated with gemigliptin developed significantly fewer preretinal neovascular tufts, relative to the vehicle-treated OIR mice (Fig. 4B-D), supporting the notion that gemigliptin has an anti-angiogenic effect. In addition, the retinal vascular permeability assay showed that gemigliptin also significantly reduced retinal vascular leakage compared with vehicle-treated OIR mice (Fig. 4E).

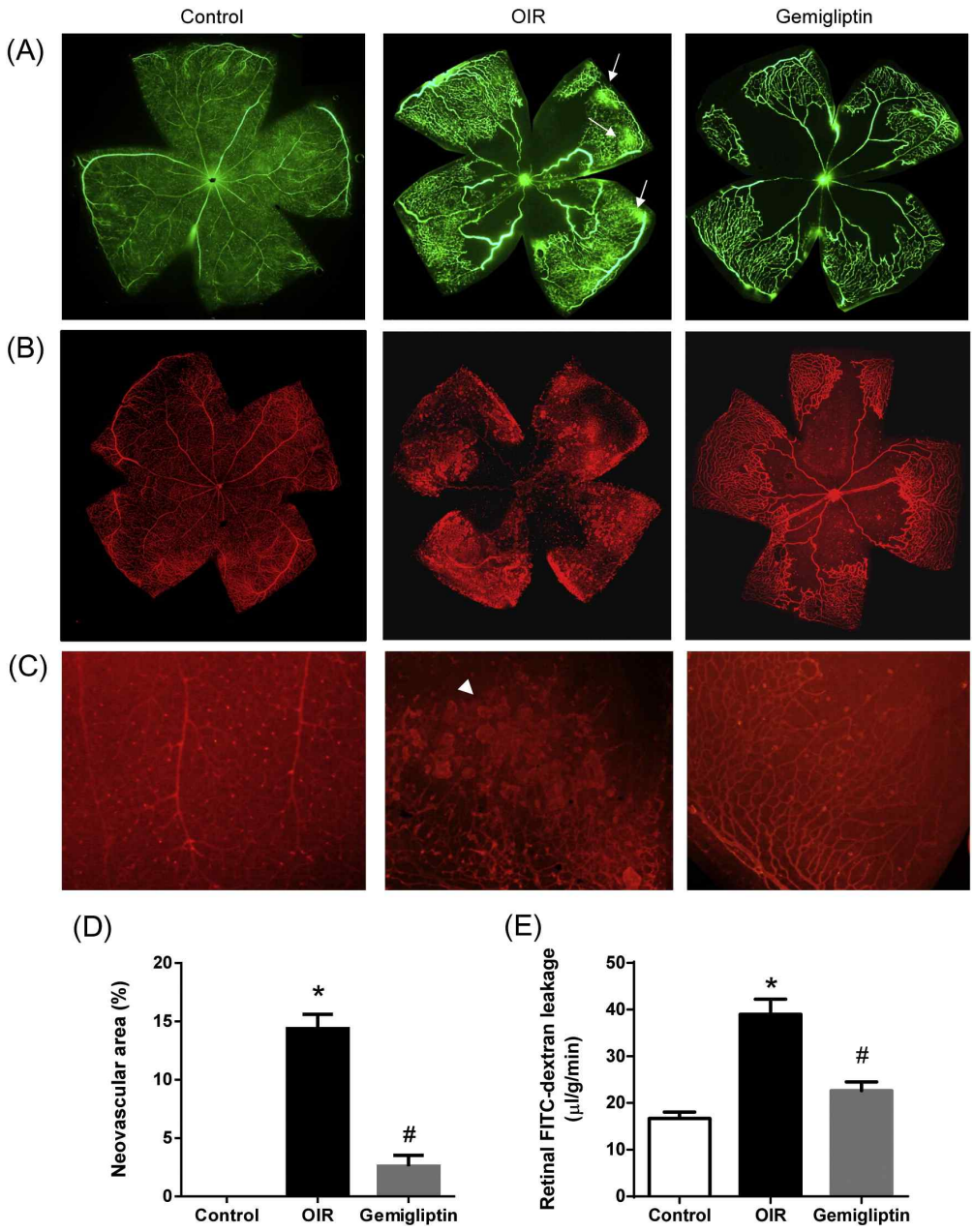


Fig. 4 Effect of gemigliptin on ischemic-induced retinal neovascularization Mice were exposed to 75% oxygen from P7 to

P12. The mice were returned to room air and received an intraperitoneal injection of 50 mg/kg/day of gemigliptin for 5 days. (A) Retinal fluorescein angiography at P17. The arrow indicates the area of retinal vascular leakage. (B) Isolectin B4-stained retinal whole-mounts. (C) High-magnification images for isolectin B4-stained retinas (x100). The arrowhead indicates the area of neovascular tufts. (D) The preretinal neovascular area was measured and normalized to the total retinal area. (E) Quantitative analysis of retinal vascular permeability. Values in the bar graphs represent the mean ± SEM, n = 10. *P < 0.05 vs. normal control mice, #P < 0.05 vs. OIR mice.

3.4 Gemigliptin regulates the expression of angiogenesis-related factors

We investigated the expression levels of 55 angiogenesis-related factors in the retinas using a protein array to evaluate the direct effects of gemigliptin on retinal neovascularization. As shown in Fig. 5, gemigliptin decreased the expression of pro-angiogenic factors [stromal cell-derived factor 1 (SDF-1), PAI-1, monocyte chemoattractant protein-1 (MCP-1), placental growth factor-2 (PGF-2), and VEGF] in the OIR mice compared with the vehicle-treated mice. Among these pro-angiogenic factors, PAI-1 and MCP-1 displayed a >2-fold up-regulation in the OIR group and a <2-fold down-regulation in the gemigliptin group. The expression of insulin-like growth factor binding protein-1 (IGFBP-1), IGFBP-3 and fibroblast growth factor-2 (FGF-2) was significantly increased in the vehicle-treated OIR mice, but these pro-angiogenic factors remained unaffected by gemigliptin treatment. Additionally, platelet factor-4 (PF-4), an anti-angiogenic factor, was also down-regulated by gemigliptin compared to vehicle-treatment in the OIR mice. The up-regulation of anti-angiogenic factors in vehicle-treated OIR mice may have been due to a protective feedback mechanism against angiogenesis. These results indicate that gemigliptin might exert anti-angiogenic effects by inhibiting the expression of SDF-1, PAI-1, MCP-1, PGF-2 and VEGF.

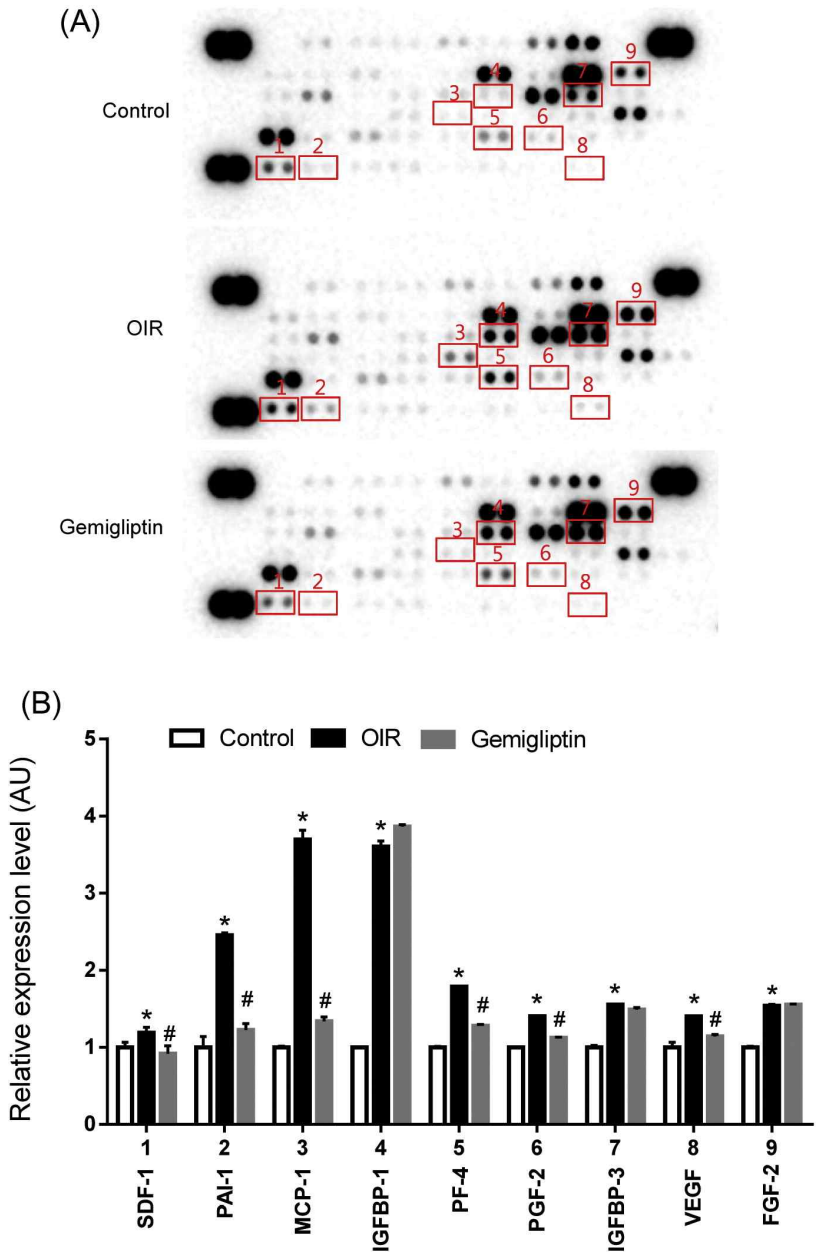


Fig. 5 Effect of gemigliptin on the expression levels of 55 angiogenesis-related proteins The positive controls are located in three corners of the arrays, and the negative control is located in the

lower right corner of the arrays. Modulated proteins in retinas treated with gemigliptin are highlighted with squares and indicated by numbers. The values in the bar graph represent the mean ± SEM, n = 4. *P < 0.05 vs. normal control mice, #P < 0.05 vs. OIR mice.

3.5 Gemigliptin inhibits high glucose-induced pericyte apoptosis *in vitro*

Pericytes were cultured in 30 mmol/L glucose for 1 week with various concentrations of gemigliptin to determine whether gemigliptin directly protects pericytes from apoptosis. The number of apoptotic pericytes was increased under high glucose conditions, but gemigliptin inhibited pericyte apoptosis in a dose-dependent manner (Fig. 6A). Pericyte apoptosis was further examined using TUNEL staining. The TUNEL-positive pericytes were detectable under high glucose conditions, whereas gemigliptin inhibited high-glucose-induced pericyte apoptosis (Fig. 6B). These results support our *in vivo* observation that gemigliptin inhibits pericyte injury in *db/db* mice.

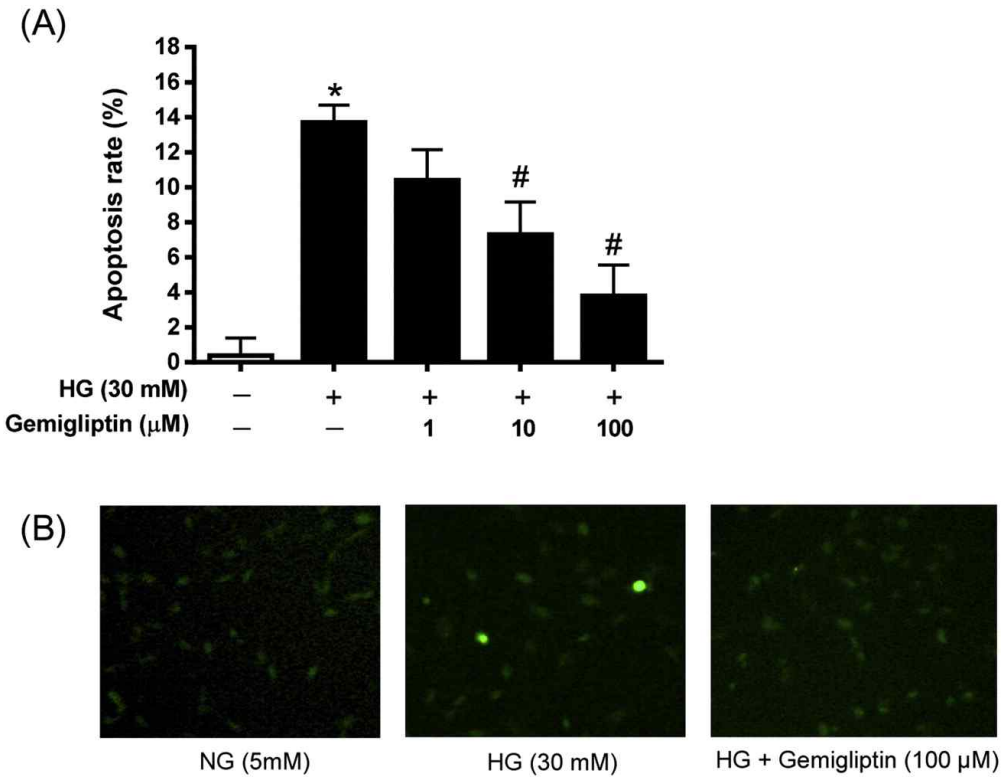


Fig. 6 Gemigliptin inhibits high glucose-induced pericyte apoptosis (A) Primary human retinal pericytes were exposed to high glucose (HG, 30 mmol/L) or normal glucose (NG, 5 mmol/L) for 1 week with or without gemigliptin (0, 1, 10 and 100 μmol/L). Apoptotic cells were detected using an FITC-labeled annexin V protein and flow cytometry. (B) TUNEL staining at 100x magnification. Each bar represents the mean ± SEM from three independent experiments. *P<0.05 vs. normal glucose group, #P<0.05 vs. high glucose group.

3.6 High glucose-induced pericyte apoptosis is synergistically suppressed by a combination treatment using gemigliptin and DPP-4 siRNA

To determine the effect of gemigliptin on DPP-4, plasma DPP-4 activity and retinal DPP-4 protein levels were examined in *db/db* mice. Gemigliptin decreased the activity of plasma DPP-4 to 40% of normal *db/+* mice, corresponding to a 50% decrease compared to *db/db* mice (Fig. 7A). Retinal DPP-4 protein levels were also reduced by gemigliptin treatment compared to vehicle treatment in *db/db* mice (Fig. 7B). However, the retinal DPP-4 protein levels in *db/db* mice might be affected by the extravasation of the soluble form of the protein because retinal vascular permeability was significantly increased in the *db/db* mice and was suppressed by gemigliptin. Moreover, the increased presence of extravasated serum albumin into retinal parenchyma was also observed in *db/db* mice (Fig. 7B).

We next examined DPP-4 protein levels in primary human retinal pericytes under high glucose conditions with or without gemigliptin treatment. DPP-4 is expressed at low levels under normal conditions. However, high glucose induced pericyte apoptosis along with elevated DPP-4 protein levels (Fig. 7C and D). When pericytes were treated with control siRNA or DPP-4 siRNAs, the DPP-4 siRNAs markedly down-regulated DPP-4 expression (Fig. 7C) and prevented high glucose-induced pericyte apoptosis (a 59% reduction compared to the high glucose group, Fig. 7D). Interestingly, a combination of DPP-4 siRNA with gemigliptin induced the enhanced apoptosis inhibition (an 87% reduction compared with the high glucose group) more than either DPP-4 siRNA or gemigliptin alone (a 71% reduction), although

treatment with gemigliptin alone or a combination of siRNA and gemigliptin did not reduce the expression of DPP4 protein more than that of siRNA treatment alone (Fig. 7C and D). These results suggest that the inhibitory effect of gemigliptin on high glucose-induced pericyte apoptosis might be caused not only by its ability to inactivate DPP-4 but also by its off-target effects.

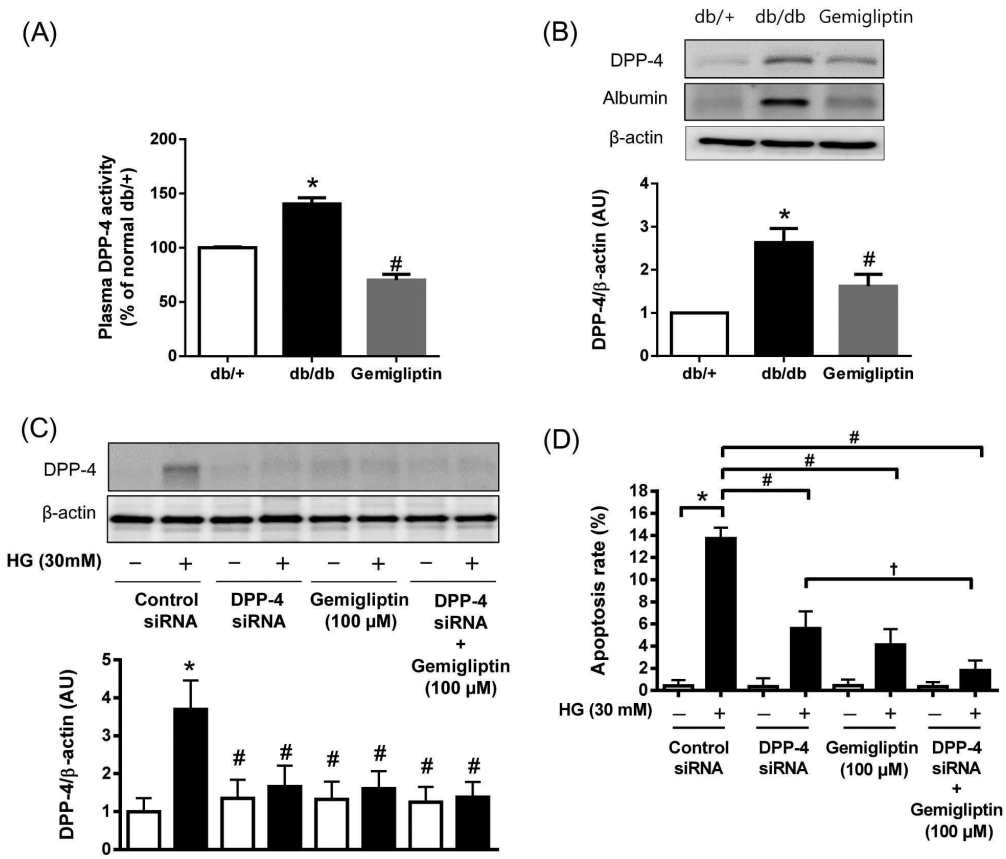


Fig. 7 High glucose-induced pericyte apoptosis is synergistically suppressed by a combination treatment using gemigliptin and DPP-4 siRNA. (A) DPP-4 activity was measured in the plasma. (B) Retinal protein levels of DPP-4 were analyzed by western blotting. Values in the bar graphs represent the mean ± SEM, n = 4. *P<0.05 vs. *db/+* mice, #P<0.05 vs. *db/db* mice. (C) After the pericyte transfection with control siRNA or DPP4 siRNA, Western blot analysis for DPP-4 was performed in pericytes under high glucose (30 mmol/L) with or without gemigliptin (100 μmol/L). (D) Apoptotic cell counts were assessed using FACS analysis. Data represent three

independent experiments. Each bar represents the mean ± SEM from three independent experiments. *P<0.05 vs. normal glucose group, #P<0.05 vs. high glucose group, † P<0.05 vs. DPP-4 siRNA group

3.7 Gemigliptin suppressed PAI-1 expression, which inhibited high glucose-induced pericyte apoptosis

A protein array indicated that gemigliptin markedly suppressed the expression of PAI-1 and MCP-1. MCP-1 expression is increased in both diabetic and ischemic retinopathy. However, despite the pro-angiogenic properties of MCP-1, MCP-1 deficiency did not prevent retinal neovascularization in MCP-/- mice with ischemic retinopathy (Davies et al., 2008). MCP-1 has been shown to contribute to the recruitment of inflammatory cells into the diabetic retina (Funatsu et al., 2005) and indirectly induces apoptosis in retinal pigment epithelial cells by infiltrating inflammatory cells (Yang et al., 2011). Based on these findings, we can exclude the notion of MCP-1 directly promoting retina neovascularization and diabetes-induced pericyte apoptosis. Thus, we examined whether PAI-1 is involved in the inhibitory role of gemigliptin in high glucose-induced pericyte apoptosis to determine the underlying mechanisms by which gemigliptin suppresses diabetic retinopathy. In *db/db* mice, PAI-1 protein was increased 2.3-fold in retinal tissues compared with normal *db/+* mice retinal tissues. Gemigliptin treatment significantly decreased retinal PAI-1 levels (Fig. 8A). Additionally, we examined the localization of PAI-1 in the retinal vasculature using immunostaining to determine whether pericytes are the cellular source of PAI-1. As expected, PAI-1 and NG-2 double-positive pericytes were observed in *db/db* mice (Fig. 8B).

Based on the *in vivo* experiments, we also observed high glucose-induced PAI-1 expression in primary human retinal pericytes (Fig. 8C). When pericytes were transfected with control siRNA or PAI-1 siRNA, the PAI-1 siRNA effectively down-regulated PAI-1

expression (Fig. 8C) and also attenuated high glucose-induced pericyte apoptosis (Fig. 8D). This PAI-1 overexpression and pericyte apoptosis induced by high glucose was also significantly inhibited by gemigliptin (Fig. 8C and D). These data suggest that the effect of gemigliptin on high glucose-induced pericyte apoptosis may be mediated at least in part through the inhibition of PAI-1.

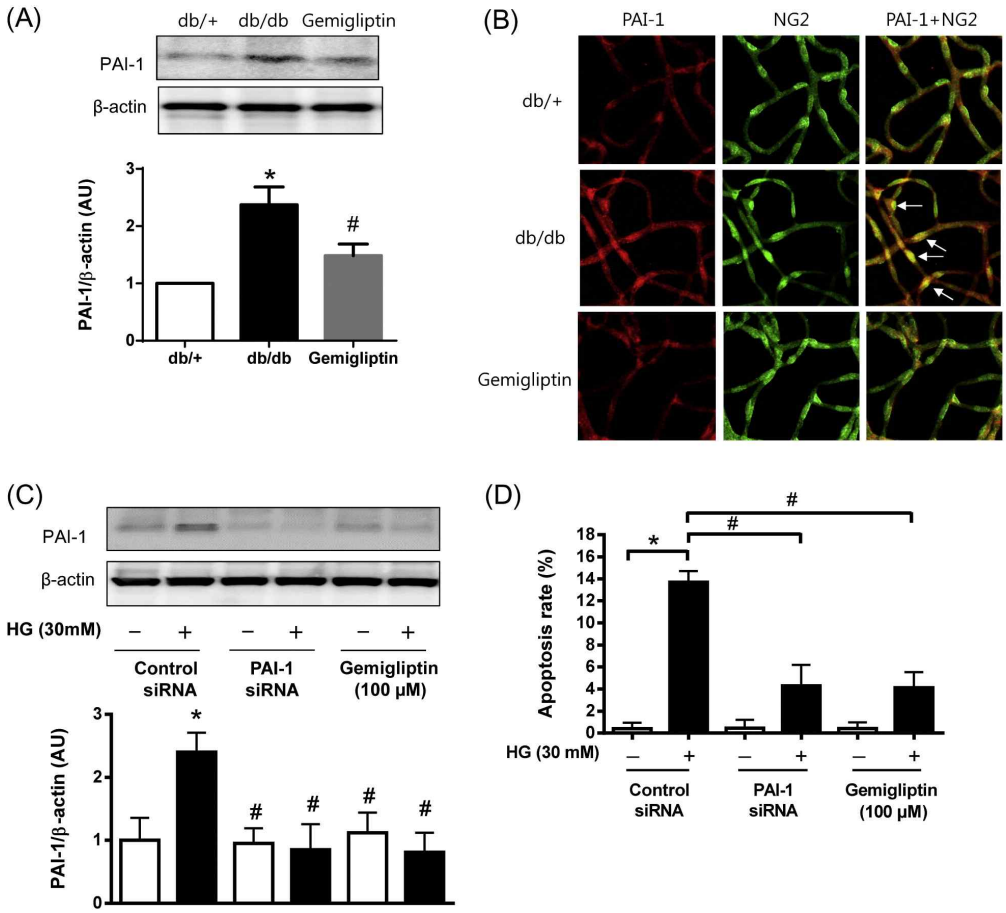


Fig. 8 Gemigliptin suppressed PAI-1 expression, which inhibited high glucose-induced pericyte apoptosis. (A) Retinal protein levels of PAI-1 were analyzed by Western blotting. Values in the bar graphs represent the mean ± SEM, n = 4. *P<0.05 vs. *db/+* mice, #P<0.05 vs. *db/db* mice. (B) Immunofluorescence staining for PAI-1 (red) and NG2 (green) in retinal vessels. White arrows indicate PAI-1-expressed pericytes. (C) After the pericyte transfection with control siRNA or PAI-1 siRNA, Western blot analysis for PAI-1 was performed in pericytes under high glucose (30 mmol/L) with or

without gemigliptin (100 μmol/L). (D) Apoptotic cell counts were assessed by FACS analysis. Data represent three independent experiments. Each bar represents the mean ± SEM from three independent experiments. *P<0.05 vs. normal glucose group, #P<0.05 vs. high glucose group.

4. Discussion

Few experimental studies have demonstrated the effect of DPP-4 inhibitors on diabetic retinopathy. One small clinical study in type 2 diabetic patients without retinopathy demonstrated that a DPP-4 inhibitor improved retinal capillary blood flow and vasodilation (Ott et al., 2014). Here, we provide the first evidence that gemigliptin has preventive effects on diabetes-induced pericyte injury and ischemia-induced retinal neovascularization. Furthermore, we demonstrated that the effects of gemigliptin on retinal vascular alterations can be achieved without a reduction of hyperglycemia in *db/db* mice or nondiabetic OIR mice, suggesting that gemigliptin's protective effects are independent of its effects on glucose homeostasis. More importantly, our results suggest that the preventive effects of gemigliptin on diabetic retinopathy occur through a PAI-1 dependent mechanism.

In the present study, gemigliptin did not reduce blood glucose levels in *db/db* mice. Similarly, a previous report showed that alogliptin did not exhibit beneficial effects on blood glucose in *db/db* mice aged between 8 and 16 weeks. This ineffectiveness of DPP-4 inhibitors on hyperglycemia in this animal model can be explained by the reduction in GLP-1 receptor expression in pancreas (Kawashima et al., 2011). Therefore, we investigated the retinoprotective possibility of gemigliptin in this animal model of diabetes independent of glucose levels.

DPP-4 inhibition has beneficial effects on the vasculature (Shah et al., 2011), heart (Bostick et al., 2014) and brain (Kosaraju et al., 2013). Recently, Goncalves et al. reported that DPP-4 inhibition also has beneficial effects in retinal vessels and neurons (Goncalves et al., 2014). DPP-4 is widely expressed in various cells types (Barnett,

2006) and in vascular endothelial cells (Ludwig et al., 2002) and pericytes (Avolio et al., 2015). Regarding the retina, the expression of DPP-4 protein was enhanced in the retinal tissues of STZ-induced diabetic rats, and immunofluorescence staining showed that it was mainly expressed in ganglion cells (Goncalves et al., 2014). Avolio et al. reported that DPP-4 mRNA was expressed at relatively low levels in pericytes under normal conditions (Avolio et al., 2015). Consistent with this report, our results showed that retinal pericytes have relatively low expression levels of DPP-4 under normal conditions, but high glucose induces a significant increase in DPP-4. Gemigliptin prevented the increase in DPP-4 protein in pericytes with high glucose. However, it has been claimed that the beneficial effect of DPP4 inhibitors in the retina is mainly due to the inhibition of DPP-4. Our study clearly showed that high glucose-induced pericyte apoptosis was prevented by siRNA-mediated DPP-4 reduction. In particular, a combination of DPP-4 siRNA with gemigliptin is more effective than either DPP-4 siRNA or gemigliptin alone, although gemigliptin alone or a combination of siRNA and gemigliptin did not reduce the expression of DPP4 proteins more than that of siRNA treatment alone. Although we could not measure DPP-4 enzyme activity in the pericytes due to the technical limitations of the assay sensitivity (data not shown), our results provide the initial evidence that gemigliptin exerts a DPP-4 inhibitory action as well as additional off-target activities in retinas.

The retina has the highest pericyte density among all vascular beds (Sims, 2000). Vasoregression in diabetic retinopathy starts with pericyte loss (Hammes et al., 2011). Moreover, the loss of pericytes leads to increased vascular permeability resulting in vascular leakage and macular edema (Shin et al., 2014). A loss of pericytes in diabetic

retinopathy also triggers the development of acellular capillaries and capillary nonperfusion, which leads to retinal ischemia and sight-threatening neovascularization (Arboleda-Velasquez et al., 2015). Pericyte loss also occurs in diabetic rodent models. Although it has been known that pericytes begin to die relatively early in the course of diabetic retinopathy, the time of onset of pericyte loss in *db/db* mice is a controversial issue. Twenty three-week-old *db/db* mice have been shown to exhibit an increase in E/P ratio and acellular capillaries (Midena et al., 1989), and a more than 25% decrease in pericyte density was observed after 15 months of diabetes in *db/db* mice (Cheung et al., 2005). TUNEL-positive apoptotic pericytes were found in 18-week-old *db/db* mice (Zhang et al., 2013). In this study, we terminated gemigliptin treatment in *db/db* mice at 20 weeks of age. Similar to previous studies, pericyte loss was already visible at this early time point.

Pericytes may be a valid target for the treatment of diabetic retinopathy. Here, we hypothesized that the reduction in PAI-1 by gemigliptin may contribute to the inhibition of pericyte loss and retinal neovascularization. To test the hypothesis, we examined the pathogenic role of PAI-1 in human primary retinal pericytes under high glucose conditions in the presence or absence of gemigliptin. The current study demonstrates that both diabetic retinas in *db/db* mice and ischemic retinas in OIR mice exhibited the PAI-1 overexpression. Gemigliptin treatment restored its expression to near-normal levels in these animals, in parallel with a marked inhibition in pericyte injury, vascular leakage and retinal neovascularization. In addition, the down-regulation of PAI-1 with siRNA ameliorated high glucose-induced pericyte apoptosis in vitro. Consistent with these results, the reduction of PAI-1 by gemigliptin

treatment also attenuated pericyte injury under high glucose conditions. These findings provide evidence that overexpressed PAI-1 in retinal tissues confers its pro-apoptotic and angiogenic effects in the retina, and gemigliptin inhibits the up-regulation of PAI-1, which may account for its beneficial effect in diabetic retinopathy and ischemia-induced retinopathy.

PAI-1 is a primary regulator of fibrinolysis and is a biosynthetic product of retinal endothelial cells (Munjal et al., 1994) and pericytes (Canfield et al., 1989). PAI-1 plays an important role in the development of diabetic retinopathy. High levels of PAI-1 have been observed in serum (Mansouritorghabe et al., 2013), vitreous (Hattenbach et al., 1999) and retinal microvasculature (Grant et al., 1996) of patients with diabetes. Furthermore, the retinal vasculature of transgenic mice that overexpress PAI-1 exhibited an increase in the basal membranes and E/P ratio, similar to diabetic retinopathy (Grant et al., 2000). In an animal model of laser-induced choroidal neovascularization, PAI-1 deficiency inhibited subretinal neovascularization in PAI-$1^{-/-}$ mice, and the restoration of PAI-1 expression by an adenoviral vector expressing human PAI-1 cDNA induced subretinal neovascularization (Lambert et al., 2001). This finding is similar to the results of ischemia-induced retinal neovascularization. The loss of PAI-1 reduced retinal neovascularization in PAI-$1^{-/-}$ mice with OIR (Basu et al., 2009). In contrast, the intravitreal injection of exogenous human PAI-1 protein in rats with retinopathy of prematurity inhibited retinal neovascularization (Penn and Rajaratnam, 2003). This paradoxical role of PAI-1 in angiogenesis is dependent on the cell type and PAI-1 concentration (Balsara and Ploplis, 2008). High levels (micromolar) of PAI-1 prevented angiogenesis, whereas low levels (physiological

levels; nanomolar) of PAI-1 conversely facilitated angiogenesis (Devy et al., 2002). Taken together with results observed previously in proliferative retinopathy models, our results confirm that PAI-1 has pro-angiogenic activity in retinal tissues under pathological conditions, such as diabetes and hypoxia.

Additionally, apart from modulating angiogenesis, experimental studies have shown that PAI-1 has both deleterious and beneficial effects on apoptosis. PAI-1 induces apoptosis in vascular endothelial cells (Al-Fakhri et al., 2003), whereas anti-apoptotic effects also have been observed in these cells (Chen et al., 2004). However, to date, there have been no reports on the exact role of PAI-1 in retinal pericytes. We demonstrated that pericyte loss occurred with PAI-1 increases in diabetic mice retinas, indicating that pericyte loss may be partially correlated with PAI-1. Consistent with a previous report (Kane et al., 2005), we also showed that high glucose induced an up-regulation of PAI-1 in retinal pericytes. The down-regulation of PAI-1 with siRNA suppressed high glucose-induced pericyte apoptosis. These results suggest that PAI-1 down-regulation might be helpful for the prevention of diabetic retinopathy. Although we did not provide concrete evidence of whether PAI-1 deficiency actually ameliorates diabetic retinopathy in animal models, the down-regulation of PAI-1 by gemigliptin reduced high-glucose-induced pericyte apoptosis both in vitro and in vivo.

Gemigliptin has been used clinically to treat type 2 diabetes. Our surprising finding of direct ocular effects of gemigliptin on diabetic retinopathy and ischemia-induced retinopathy provides evidence that the oral administration of gemigliptin may be a promising therapeutic approach to diabetic retinopathy. An oral dose of 50 mg/day gemigliptin is recommended for glycemic control in diabetic patients.

Although we used a relatively high dose of gemigliptin in animal models, a dose of 50 mg/day gemigliptin may be sufficient to obtain the same relative beneficial effects on diabetic retinopathy in human subjects. The elimination half-life of gemigliptin was shown to be 17.1 h (Lim et al., 2008). The elimination half-life of gemigliptin in *db/db* mice was 1.5 h (our unpublished data). Due to its short half-life in *db/db* mice, we selected an oral dose of 100 mg/kg gemigliptin for this animal model. Moreover, *db/db* mice are obese, and their body surface area is approximately 2 times larger than that of normal C57BL/6 mice (Cheung et al., 2009). Thus, we also selected an oral dose of 50 mg/kg gemigliptin for the OIR mice.

In summary, our study demonstrated that gemigliptin has both anti-apoptotic and anti-angiogenic effects in the retinas of diabetic mice and ischemia-induced retinopathy mice through the down-regulation of PAI-1. These novel findings provide insight into the retinoprotective effects of gemigliptin that are independent of glycemic control.

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국문 초록

DPP-4 억제제의 당뇨 합병증에서 다양한 혈당 비의존성 기전 연구

정 은 수
서울대학교 수의과대학
수의학과 수의병인생물학 및 예방수의학(독성학) 전공

2006년 Januvia의 미국 허가를 시작으로, incretin 기반의 2형 당뇨 치료제는 매해 큰 성장을 거듭하며 당뇨 치료를 위한 처방 2차 약제로 널리 사용되고 있다. DPP-4 (dipeptidyl peptidase-4) 억제제는 해당 효소를 억제하여, DPP-4 에 의해 분해되는 GLP-1 의 기능 연장을 이용한 혈당강하 효과를 기반으로 하는데, DPP-4 억제제들은 혈당강하 효능 외에도 당뇨 합병증에 대한 긍적적인 효능이 일부 보고되었다. 따라서 본 연구에서는 DPP-4 억제제중 하나인 gemigliptin을 이용하여 혈당 강하 약효와 무관한 직접적인 당뇨병성 신증 및 망막증의 예방 효능을 확인하였으며, 나아가 gemigliptin의 혈당강하 외 신규 작용 기전을 규명하였다.

첫 번째 보호 기전은, 고혈당 상태에서 형성되는 advanced glycation end product 형성을 gemigliptin이 직접적으로 억제하였다. Methyglyoxal modified AGE-bovine serum albumin formation, AGE-collagen cross-linking assay 에서 기존 알려진 AGE 형성 억제제 (aminoguanidine)보다 강력한 효과가 확인되었다. 제2형 당뇨모델 (*db/db* 마우스)에서도 gemigliptin 12주 투약 후 AGE serum level은 줄어들었다.

두 번째로는 제2형 당뇨모델(*db/db*마우스)에서 gemigliptin 12주 투약 후 혈당 강하와 상관없이 당뇨병성 신증을 보호하는 효과를 확인하였다. 당뇨병성 신증에서 확인되는 albuminuria 억제, DPP-4 효소 활성 및 발현 억제, podocyte apoptosis (8-hydroxydeoxyguanosine 감소)가 억제되었고, 보호 기전으로 oxidative damage 억제(advanced oxidation protein products 감소), RAGE 와 ILK 발현 억제 (receptor for advanced glycation end products, integrin-linked kinase)가 확인되었다.

세 번째로는 당뇨병성 망막증의 여러 요인 중, 2형 당뇨 모델 *db/db* 마우스에서 retinal vascular leakage를 억제하는 것과 OIR (oxygen induced retinopathy) 모델에서 망막 신생혈관생성을 억제하는 기전을 밝혔다. 고혈당 상태인데도 gemigliptin을 투약한 그룹에서는 retinal pericyte apoptosis 와 vascular leakage 가 감소되었고, OIR 모델에서는 여러angiogenesis related protein의 발현이 줄어드는 것을 확인하였는데, 특히 PAI-1 (plasminogen activator inhibitor-1)의 발현이 gemigliptin에 의해 현저히 억제되는 것을 확인하였다. 또한 *in vitro* cultured pericyte 에서 high-glucose에 의한 pericyte의 apoptosis가 PAI-1 siRNA에 의해 억제되는 것을 확인하였으며, 이러한 현상이 gemigliptin에 의해서도 동일하게 관찰되었다. 따라서 본 연구를 통해 gemigliptin의 anti-angiogenic, anti-apoptotic activities를 처음 규명하였다.

Gemigliptin의 이러한 microvascular 에서의 anti-glycation, anti-oxidant, anti-apoptotic, anti-angiogenic 기능은 당뇨환자에서의 고혈당으로 인한 vascular damage의 많은 부분을 보호할 수 있는 기전으로 생각되고, 혈당을 줄이는 1차 endpoints 외에 추가적인 장점이 될 수 있다. 따라서, 본 연구 결과를 바탕으로 많은 임상 시험들을 통해, 당뇨 환자에서 실질적인 benefits으로 구현될 수 있기를 기대한다.

핵심 단어: 2형 당뇨, DPP-4 억제제, 당뇨 합병증, 당뇨병성 신증, 당뇨병성 망막증
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A Dissertation
for the Degree of Doctor of Philosophy

Glucose independent pleiotropic effects of a dipeptidyl peptidase-4 inhibitor on diabetic complications

DPP-4 억제제의 당뇨 합병증에서
다양한 혈당 비의존성 기전 연구

February 2016

By
Eunsoo Jung
정 은 수

Department of Veterinary Pathobiology and Preventive Medicine (Toxicology)
Graduate School of Seoul National University

Glucose independent pleiotropic effects of a dipeptidyl peptidase-4 inhibitor on diabetic complications

Supervised by Professor Myung-Haing Cho

A Dissertation submitted to the Faculty of the Graduate School of Seoul National University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Veterinary Pathobiology and Preventive Medicine (Toxicology)

Supervisory Committee Approval of Thesis submitted by December 2015

Chairman: _____
Chan-Hee Chae, D.V.M., Ph.D.

Assistant chairman: _____
Myung-Haing Cho, D.V.M., Ph.D.

Member: _____
Youngchan Park, D.V.M., Ph.D.

Member: _____
Choongman Hong, D.V.M., Ph.D.

Member: _____
Hwan-Goo Kang, D.V.M., Ph.D.

ABSTRACT

Dipeptidyl peptidase-4 (DPP-4) inhibitors are widely used as anti-diabetic agents in clinical practice. Gemigliptin, a new and selective DPP-4 inhibitor, has shown robust blood-glucose lowering effects in type 2 diabetic patients, but its effects on diabetic complications have not yet been reported.

This study evaluated the inhibitory effects of gemigliptin, a highly selective dipeptidyl peptidase-4 inhibitor, on the formation of advanced glycation end products (AGEs) and AGE cross-links with proteins in *in vitro* as well as in type 2 diabetic *db/db* mice. In *in vitro* assay, gemigliptin dose-dependently inhibited methylglyoxal-modified AGE-bovine serum albumin (BSA) formation (IC_{50} = 11.69 mM). AGE-collagen cross-linking assays showed that gemigliptin had a potent inhibitory effect (IC_{50} = 1.39 mM) on AGE-BSA cross-links to rat tail tendon collagen, and its activity was stronger than aminoguanidine (IC_{50} = 26.4 mM). In addition, gemigliptin directly trapped methylglyoxal in a concentration-dependent manner *in vitro*. To determine whether gemigliptin inhibits the *in vivo* glycation processes, gemigliptin (100 mg/kg/day) was orally administered into type 2 diabetic *db/db* mice for 12 weeks. Elevated serum levels of AGEs in *db/db* mice were suppressed by the administration of gemigliptin. These inhibitory effects of gemigliptin on the glycation process in both *in vitro* and *in vivo* suggest its therapeutic potential for ameliorating AGE-related diabetic complications.

Podocytes participate in the formation and regulation of the glomerular filtration barrier. Loss of podocytes occurs during the early stages of diabetic nephropathy and impairs glomerular filtration. Dipeptidyl peptidase-4 (DPP-4) inhibitors are widely used as

anti-diabetic agents in clinical practice. In this study, we showed that gemigliptin, a novel DPP-4 inhibitor, reduced podocyte apoptosis in type 2 diabetic *db/db* mice without reducing hyperglycemia. Gemigliptin (100 mg/kg/day) was administered orally for 12 weeks in db/db mice. Blood glucose levels and albuminuria were measured. The renal cortex was collected for histological examination, and molecular assays were used to detect 8-hydroxydeoxyguanosine, advanced oxidation protein products (AOPP), the receptor for advanced glycation end products (RAGE), and integrin-linked kinase (ILK). Type 2 diabetic *db/db* mice exhibited albuminuria, renal histopathological changes, and podocyte loss. Administration of gemigliptin to *db/db* mice suppressed albuminuria, enzyme activity and expression of DPP-4, and podocyte apoptosis. The effect of gemigliptin on diabetes-induced podocyte loss was associated with the suppression of oxidative damage, AOPP accumulation, RAGE expression, and ILK expression. These results indicate the possible benefits of using gemigliptin in diabetes patients to treat renal impairment without affecting glycemic control.

Retinal pericyte loss and neovascularization are characteristic features of diabetic retinopathy. Gemigliptin, a dipeptidyl peptidase-4 (DPP-4) inhibitor, has shown robust blood-glucose lowering effects in type 2 diabetic patients, but its effects on diabetic retinopathy have not yet been reported. We evaluated the efficacy of gemigliptin on retinal vascular leakage in *db/db* mice, which is an animal model for type 2 diabetes, and neovascularization in oxygen-induced retinopathy (OIR) mice, which is an animal model for ischemic proliferative retinopathy.

Gemigliptin (100mg/kg/day) was orally administered to the *db/db* mice for 12 weeks. C57BL/6 mice on postnatal day 7 (P7) were

exposed to 75% hyperoxia for 5 days, followed by exposure to room air from P12 to P17 to induce OIR. Gemigliptin (50 mg/kg/day) was intraperitoneally injected daily from P12 to P17. Retinal neovascularization was analyzed in flat-mounted retinas on P17. We determined the efficacy and possible mechanism of gemigliptin on high glucose-induced apoptosis of primary human retinal pericytes. The oral administration of gemigliptin for 4 months significantly ameliorated retinal pericyte apoptosis and vascular leakage in the *db/db* mice. Gemigliptin also ameliorated retinal neovascularization in the OIR mice. Gemigliptin attenuated the overexpression of plasminogen activator inhibitor-1 (PAI-1) in the retinas of diabetic and OIR mice. Gemigliptin and PAI-1 siRNA significantly inhibited pericyte apoptosis by inhibiting the overexpression of PAI-1, which is induced by high glucose. Our results suggest that gemigliptin has potent anti-angiogenic and anti-apoptotic activities via suppressing DPP-4 and PAI-1, and the results support the direct retinoprotective action of gemigliptin.

Keywords: Diabetic mellitus, Dipeptidyl peptidase-4 inhibitor, Diabetic complications, Diabetic nephropathy, Diabetic retinopathy
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General Introduction

1. Diabetes mellitus

Diabetes mellitus (DM) is a metabolic disorder characterized by a loss of glucose homeostasis with disturbances of carbohydrate, lipid and protein metabolism due to an impaired insulin secretion or decrease in the biological activity of insulin (Schneider et al., 2003). Diabetes is due to either the β cells of the pancreas not producing enough insulin (type 1), or the peripheral cells not responding properly to the insulin produced (type 2). The prevalence of DM for all age-groups worldwide is increasing with estimated projection of approximately 300 million patients by year 2025 (King et al., 1998).

2. Complications of diabetes mellitus

Uncontrolled hyperglycemia in both type 1 and type 2 diabetes lead to the development of complications (Weiss and Sumpio, 2006). Diabetic complications include cardiovascular diseases, hypertension, chronic kidney disease, retinal injury and nerve damage. Chronic hyperglycemia elicits an enhanced production of mitochondrial reactive oxygen species (ROS), which contribute to the development of diabetic complications in several organs (Weiss and Sumpio, 2006). The metabolic pathways activated by hyperglycemia include the formation of advanced glycation end product (AGE), polyol pathway, hexosamine pathway and the protein kinase C (PKC) pathway (Fig. 1).

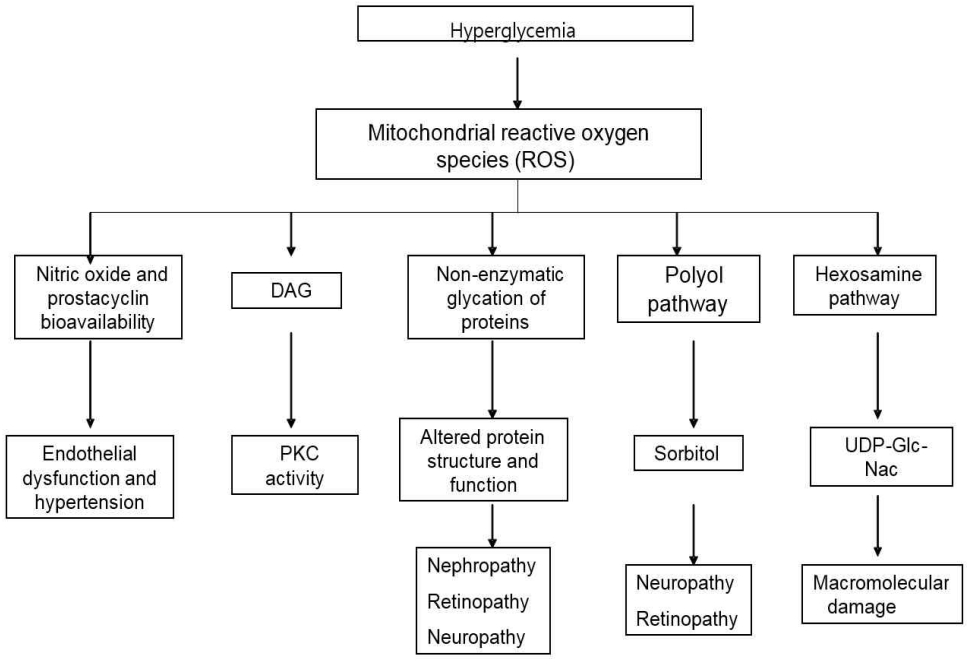

Figure 1. Metabolic pathways activated by chronically elevated blood glucose levels as well as the resultant long term complications of diabetes mellitus

2.1. Diabetic nephropathy

The hallmark of diabetic nephropathy is increased urinal excretion of proteins, mainly albumin. The progression of diabetic nephropathy is a descending path from normo-albuminuria to micro-albuminuria and overt proteinuria and eventually to end-stage renal disease (Fig. 2). Micro-albuminuria is defined as urinal albumin excretion rate (AER) 20-200 mg/min in overnight urine specimens (Mogensen et al., 1985). A urinal albumin levels above this value is called macro-albuminuria and considered a typical symptom of diabetic nephropathy.

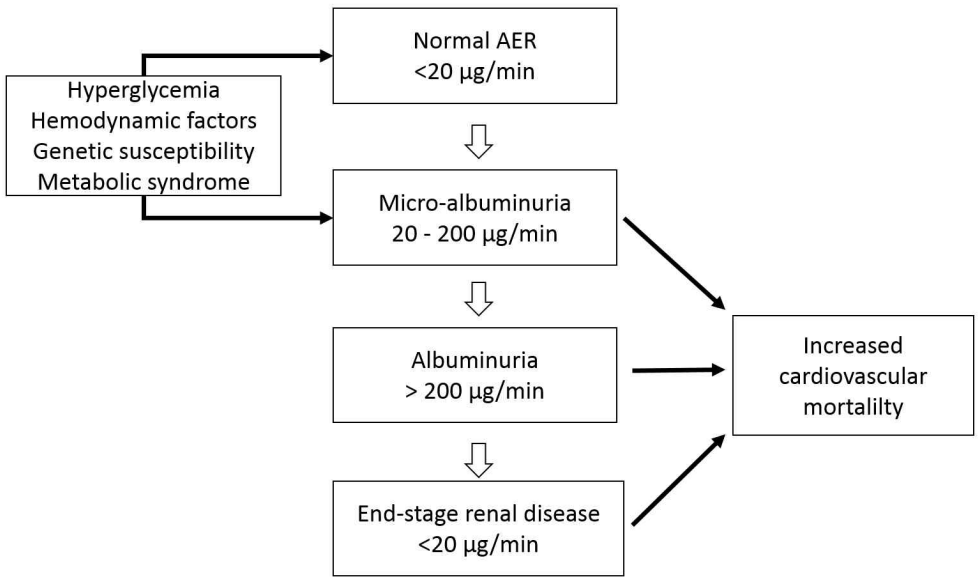

Figure 2. The progression of albumin excretion (AER), risk factors and prognosis

The incidence of diabetic nephropathy have increased rapidly after ten years of diabetes (Andersen et al., 1983). Approximately 35 ~ 45% of diabetic patients developed diabetic nephropathy during forty years of diabetes (Krolewski et al., 1985). About 50% of patients after duration of 20 years of diabetes had micro-albuminuria. (Krolewski et al., 1985). In patients with type 1 diabetes, micro-albuminuria is rare during the first years. However, micro-albuminuria is often observed in persons first diagnosed with type 2 diabetes (Uusitupa et al., 1987). This high incidence of micro-albuminuria in patients with type 2 diabetes can result from a difficulty in determining exact time at the onset of type 2 diabetes (Parving et al., 1992). Moreover, in patients with the metabolic syndrome, micro-albuminuria has been

also associated with a more generalized vascular damage (Nannipieri et al., 1997).

2.2. Diabetic retinopathy

Diabetes leads to formation of typical lesions in the retinal microvessels, such as vascular leakage, a formation of microaneurysms and hemorrhages. These vascular changes are defined as non-proliferative diabetic retinopathy (NPDR). NPDR do not threaten visual acuity unless they are located in the macular area, where they can cause macular edema. However, in advanced stage of diabetic retinopathy, capillary closure can lead to areas of impaired perfusion, which can result in retinal ischemia and neovascularization, defined as proliferative diabetic retinopathy (PDR) (Fig. 3). Retinal neovascularization can be responsible for intraocular hemorrhage and visual impairment. The formation of fibrous tissue in endovascular areas may eventually cause retinal detachment and blindness (Kohner, 1993).

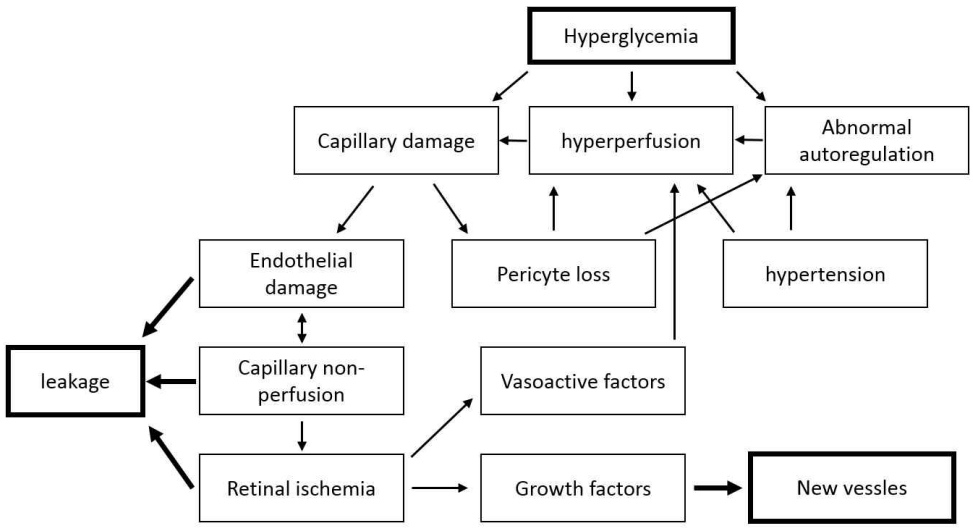

Figure 3. Role of hyperglycemic in the progression of diabetic retinopathy.

Age, duration of diabetes, blood glucose levels and treatment for glycemic control can contribute the prevalence of diabetic retinopathy (Agardh et al., 1989; Henricsson et al., 1996; Klein et al., 1984). The Wisconsin Epidemiological Study of Diabetic Retinopathy (WESDR) (Klein et al., 1984) reported that younger-onset group (diagnosed before 30 years of age) had no retinopathy during the first 5 years of disease, but more than 90% of the patients had some degree of retinopathy after 15 years of diabetes. About 20% of the older onset patients (diagnosed after 30 years of age) had some signs of retinopathy during the first 5 years and after a long duration still 20% of the patients did not have diabetic retinopathy. The incidence of PDR increased by 50% of the younger-onset patients and by 10 ~ 30 % in the older-onset patients after 25 years of diabetes. The United Kingdom Prospective Diabetes Study (UKPDS) showed that diabetic retinopathy was observed in about one third of first diagnosed type 2 diabetic patients (Kohner et al., 1998).

3. Dipeptidyl peptidase 4 (DPP-4) inhibitor

Medical nutrition therapy including increased physical activity and reduced food intake is the traditional first line of treatment for type 2 diabetes followed by the addition of oral anti-diabetes therapies and exogenous insulin as required (DeFronzo, 1999). A lot of attempts have been made to get near normal glycemic control and variety of drugs are now available (Takei and Kasatani, 2004). As a new therapeutic class of anti-diabetic drugs, the numerous DPP-4 inhibitors, often called as 'gliptins', have been developed in varying stages of clinical development with seven already approved in Korea.

Table 1. Approved DPP-4 inhibitors

Name	Approval (Company)
Sitagliptin (Januvia)	FDA approved in 2006 (Merck & Co)
Vildagliptin (Galvus)	EU approved in 2007 (Novartis)
Saxagliptin (Onglyza)	FDA approved in 2009 (BMS)
Linagliptin (Tradjenta)	FDA approved in 2011 (BI & Eli lily)
Gemigliptin (Zemiglo)	Korea approved in 2012 (LG Life Sciences)
Anagliptin (Suiny)	Japan approved in 2012 (Sanwa and Kowa)
Teneligliptin (Tenelia)	Japan approved in 2012 (Mitsubishi Tanabe)
Alogliptin (Nesina)	FDA approved in 2013 (Takeda)
Trelagliptin (Zafatek)	Japan approved in 2015 (Takeda)
Omarigliptin (Marizev)	Japan approved in 2015 (Merck & Co)
Evogliptin	Korea approved in 2015 (Dong-A ST)

3.1. Mode of actions of DPP-4 inhibitors

DPP-4 is an integral membrane serine protease that cleaves dipeptides from the N-terminus of proteins and substrate peptides that containing proline or alanine in the second amino terminal position (Huang et al., 2012; Silveira et al., 2013). DPP-4 is expressed on the surface of most cells and also exists as a soluble form in plasma. DPP-4 participates in numerous biological processes. Proteolytic activity of DPP-4 leads to inactivation or activation of some peptides, cytokines and growth factors (Cohen et al., 2004; Cunningham and O'Connor, 1997; Lambeir et al., 2003). DPP-4 also cleave many hormones, including two major incretin hormones glucagon-like peptide- 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which enhance the insulin secretion (Flatt et al., 2008). As shown in Fig. 4, the incretin hormones, such as GLP-1 and GIP, are synthesized in the small intestine and secreted in response to food (Velarde-Salcedo et al., 2013). Incretin hormones elicit glucose dependent insulin secretion in the pancreatic α-cells (Silveira et al., 2013), which leads to a lowering of the blood glucose levels (Flatt et al., 2008). It has been estimated that the incretin hormones, especially GLP-1 and GIP are responsible for over 50% of the total insulin secretion after a meal (Silveira et al., 2013; Velarde-Salcedo et al., 2013). However, DPP-4 cleaves and inactivates GLP-1 and GIP within a few minutes, thereby losing their insulinotropic activities (Li-Chan et al., 2012). The inactivation of GLP-1 and GIP by DPP-4 cannot stimulate insulin-release in the pancreas (Flatt et al., 2008). DPP-4 inhibitors have been developed to inhibit this rapid inactivation of GLP-1 and GIP, thus leading to prolonged incretin action and improving glucose tolerance and can thereby be used as a drug to treatment type 2 diabetes (Flatt et al.,

2008).

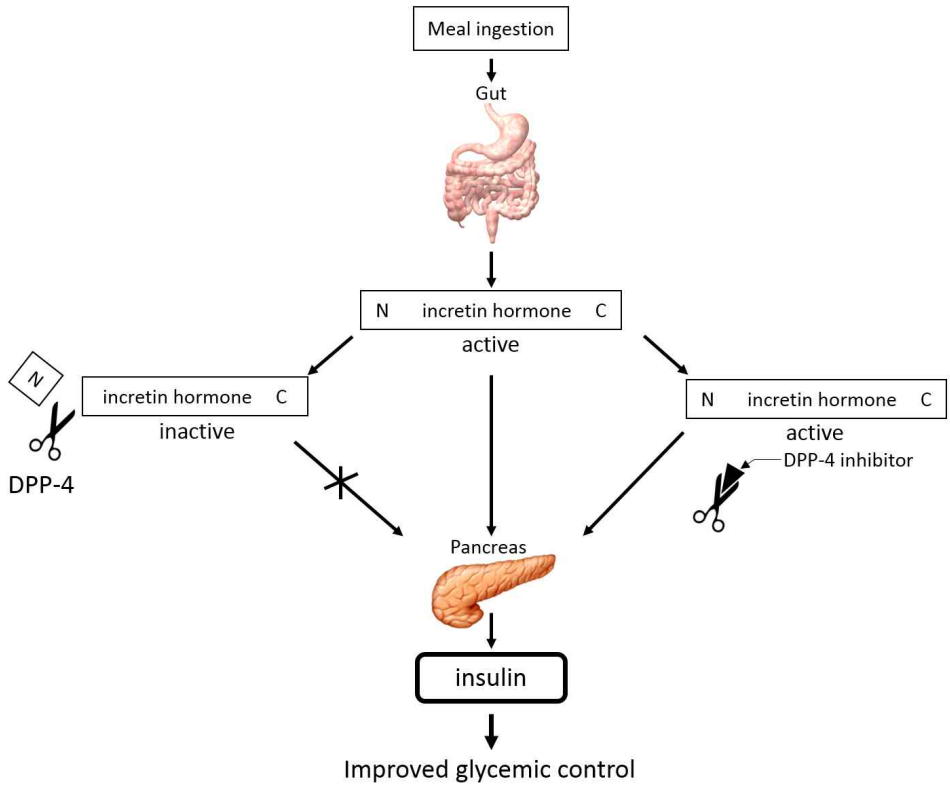

Figure 4. Overview of DPP-4 regulation in relation to the glucose level (Flatt et al., 2008).

3.2. Pleiotropic effect of DPP-4 inhibitors

Several biological molecules apart from the incretin hormones are potentially substrates for DPP-4 (Mentlein, 1999). The inhibition of DPP-4 enzyme activity may exert pleiotropic effects through activation or inactivation of biological molecules that are substrates for DPP-4. Stromal-derived factor (SDF)-1α is a small peptide chemokine that is a substrate for DPP-4 since its active form is

rapidly degraded to an inactive form by DPP-4 (Proost et al., 1998). A recent study demonstrated that SDF-1α is also expressed in pancreatic islets, and its expression is enhanced by cellular injury. SDF-1α increases the expression of prohormone convertase-1 in α-cells, which increases the production of GLP-1 in islet (Liu et al., 2011). In pancreatic damage by oxidative stress or glucolipotoxicity, SDF-1α expression is increased with increased production of GLP-1. Therefore, DPP-4 inhibition facilitates further the intra islet GLP-1 production when inactivation of SDF-1α by DPP-4 is inhibited.

Recently, Krijnenet al. observed a marked decrease of microvascular endothelial DPP-4 expression in recently infarcted human hearts (Krijnen et al., 2012). DPP-4 can prevent fibrin polymerization and clot formation via cleaving N-terminal Gly-Pro from the fibrin α-chain, (Mentlein and Heymann, 1982). The treatment of diprotin A, a DPP-4 inhibitor, in human umbilical cord vein endothelial cells (HUVECs) increased the expression of endothelial tissue factor and consequently induced adherence of platelets to the ECs, although platelet aggregation was not increased (Krijnen et al., 2012). Sitagliptin has been shown to inhibit platelet aggregation through its inhibitory effects on thrombin-induced intracellular free calcium accumulation and on thrombin-induced tyrosine phosphorylation of multiple proteins in human platelets (Gupta et al., 2012). This finding suggests that DPP-4 has an antithrombotic effect.

Sitagliptin promotes reverse cholesterol transport through reduced intestinal cholesterol absorption in a mouse model (Briand et al., 2012). Vildagliptin was also shown to decrease the level of hepatic mRNA transcript for farnesyl diphosphate transferase in dual incretin receptor knockout mice (Flock et al., 2007). Farnesyl diphosphate is a substrate for the synthesis of dolichol, coenzyme Q and cholesterol.

Although the postprandial lipid lowering effect of DPP-4 inhibitor is dependent on GLP-1 (Hsieh et al., 2010), DPP-4 inhibition may have a direct effect on cholesterol metabolism by affecting the isoprenoid pathway (Choe et al., 2014; Monami et al., 2012).

Several previous studies have shown that many DPP-4 inhibitors improve endothelial function in diabetic patients (van Poppel et al., 2011) in GLP-1 dependent as well as independent manners (Yoon and Lee, 2011). Alogliptin induced a dose-dependent vasorelaxation through Src-Akt-endothelial nitric oxide synthase pathway in a mouse model (Shah et al., 2011). In a recent study, linagliptin treatment led to a decrease in lipid and protein oxidation in a rat model of renovascular hypertension (Chaykovska et al., 2013). DPP-4 can bind mannose-6-phosphate/insulin-like growth factor II receptor (M6P/IGF-IIR) which plays an important roles in two distinct biological processes; protein trafficking and transmembrane signal transduction (Hawkes and Kar, 2004). The interaction between DPP-4 and M6P/IGF-IIR is responsible for T cell activation (Busek et al., 2004). DPP-4 can directly contribute to stimulate ROS production and receptor for advanced glycation end products (RAGE) gene expression via the interaction with M6P/IGF-IIR in HUVECs (Ishibashi et al., 2013). In addition, linagliptin inhibited AGEs-induced soluble DPP-4 production, ROS generation and gene expressions of RAGE, intercellular adhesion molecule-1 and plasminogen activator inhibitor-1 in HUVECs (Ishibashi et al., 2013). Soluble DPP-4 proteins stimulated the proliferation of vascular smooth muscle cells (VSMCs), but DPP-4 inhibitor suppressed the proliferationof VSMCs by inhibiting ERK phosphorylation (Ervinna et al., 2013). Sitagliptin treatment suppressed proliferation of VSMCs, promoted apoptosis of VSMCs and reduced inflammation and MMP-2 and MMP-9 expressions in

the injured artery (Lim et al., 2012). Short-term treatment with vildagliptin prevented left ventricular hypertrophy induced by continuous infusion of isoproterenol through the amelioration of perivascular fibrosis and expression of genes associated with glucose uptake (GLUT4) and inflammation (TNF-α and IL-6) (Miyoshi et al., 2014).

In renal tissues of diabetic mice, the DPP-4 protein levels were enhanced as compared with normal kidneys. Diabetic glomerulosclerosis and tubulointerstitial fibrosis are associated with increased both DPP-4 protein levels and enzyme activity and increased transforming growth factor-β2 signaling pathway (Kanasaki et al., 2014). Linagliptin treatment ameliorated these pathophysiologic changes in the diabetic kidney (Kanasaki et al., 2014).

Many studies have provided the strong evidence to support the anti-atherosclerotic and cardiovascular and renal protective effects of DPP-4 inhibitors (Shah et al., 2011). Recent meta-analysis of clinical trial data have shown more favorable CV outcomes with DPP-4 inhibitors than with other classes of anti-diabetic agents (Cameron-Vendrig et al., 2014; Monami et al., 2013; Wu et al., 2014).

4. Aim of the study

Gemigliptin is a selective and long-lasting DPP-4 inhibitor, and it is approved by the Korea Ministry of Food and Drug Safety for clinical use in patients with type 2 diabetes (Lim et al., 2009). Most research on gemigliptin has focused on the normalization of blood glucose in patients with type 2 diabetes (Kim et al., 2013). However, this is the first study demonstrating that gemigliptin has protective effects in the diabetic nephropathy and retinopathy by a mechanism independent of lowered blood glucose. In addition, we investigate the possible mechanism of action whereby gemigliptin exert its blood glucose pleiotropic effects. The study had the following objectives.

1) To evaluate the inhibitory effects of gemigliptin on the formation of AGEs and the cross-linking between preformed AGE-BSA and proteins *in vitro*. In addition, we also investigated whether the inhibitory activity of gemigliptin on the glycation process is due to its reactivity with reactive dicarbonyl compounds. Moreover, because various classes of structurally different DPP-4 inhibitors are currently available, we also compared the effectiveness of gemigliptin with that of the other DPP-4 inhibitors, vildagliptin and saxagliptin. Subsequently, we compared AGE formation and cross-links in type 2 diabetic *db/db* mice with and without administration of gemigliptin.

2) To determine whether gemigliptin reduced diabetic nephropathy independently of its glucose-lowering property, we have studied the effect of gemigliptin diabetes-induced podocyte injury in type 2 diabetic *db/db* mice.

3) To the best of our knowledge, there have been no reports on the effects of DPP-4 inhibitors on retinal pericyte injury and neovascularization. Therefore, we studied whether gemigliptin inhibits retinal pericyte injury through the modulation of apoptotic cascades in type 2 diabetic *db/db* mice. Additionally, we evaluated whether gemigliptin effectively prevents retinal neovascularization in oxygen-induced retinopathy (OIR) mice.

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Part I.

Gemigliptin, a novel dipeptidyl peptidase-4 inhibitor, exhibits potent anti-glycation properties *in vitro* and *in vivo*

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Abstract

This study evaluated the inhibitory effects of gemigliptin, a highly selective dipeptidyl peptidase-4 inhibitor, on the formation of advanced glycation end products (AGEs) and AGE cross-links with proteins in *in vitro* as well as in type 2 diabetic *db/db* mice. In *in vitro* assay, gemigliptin dose-dependently inhibited methylglyoxal-modified AGE-bovine serum albumin (BSA) formation (IC_{50} = 11.69 mM). AGE-collagen cross-linking assays showed that gemigliptin had a potent inhibitory effect (IC_{50} = 1.39 mM) on AGE-BSA cross-links to rat tail tendon collagen, and its activity was stronger than aminoguanidine (IC_{50} = 26.4 mM). In addition, gemigliptin directly trapped methylglyoxal in a concentration-dependent manner *in vitro*. To determine whether gemigliptin inhibits the *in vivo* glycation processes, gemigliptin (100 mg/kg/day) was orally administered into type 2 diabetic *db/db* mice for 12 weeks. Elevated serum levels of AGEs in *db/db* mice were suppressed by the administration of gemigliptin. These inhibitory effects of gemigliptin on the glycation process in both *in vitro* and *in vivo* suggest its therapeutic potential for ameliorating AGE-related diabetic complications.

1. Introduction

The glycation process is a spontaneous non-enzymatic chemical reaction (called glycosylation) of sugar molecules with proteins, DNA and lipids to form Amadori product in a biological environment. The Amadori product irreversibly undergoes a variety of dehydration and rearrangement reactions, resulting in the formation of complex group known as advanced glycation end products (AGEs) (Brownlee et al., 1988). AGEs accumulate in all tissues and on plasma lipoproteins and bind to a specific receptor for AGE (RAGE) (Moritoh et al., 2009). Enhanced binding of AGEs to RAGE have been shown in diabetic patients, and the AGE/RAGE interaction has an important role to develop diabetes related complications (Sato et al., 2006) Moreover, AGEs can covalently cross-link with proteins, which changes the biochemical structures and functions of those proteins. Intracellular AGEs are also generated from sugar-derived dicarbonyl precursors. Methylglyoxal, a reactive dicarbonyl metabolite, is physiologically produced as an intermediate in glycolysis. The levels of methylglyoxal are increased in the plasma and tissue of patients and animals with diabetes (Haik et al., 1994). Methylglyoxal is more chemically reactive than blood sugars. Thus, methylglyoxal easily cross-links with free amino acid residues in proteins, which leads to the generation of stable end products (Bourajjaj et al., 2003). Evidence for methylglyoxal-derived modifications in human and animal tissues has been reported (Horan et al., 2007 Miyata et al., 1997 Shamsi et al., 1998).

It has been suggested that suppression of the glycation reaction may prevent the progress of diabetic complications. Aminoguanidine, a nucleophilic hydrazine compound, could inhibit the glycation reaction

in vitro as well as *in vivo* (Hammes et al., 1991 Kumari et al., 1991). The mechanism of action of aminoguanidine may involve trapping dicarbonyl metabolites, such as methylglyoxal (Lo et al., 1994). The roles of potential glycation reaction inhibitors have not yet been investigated thoroughly. Gemigliptin (previously identified as LC15-0444) is a novel and selective dipeptidyl peptidase-4 (DPP-4) inhibitor used for the treatment of type 2 diabetes (Pennock et al., 2013). Although the conformational structure of gemigliptin differs from that of aminoguanidine, a very recent report has shown that another chemically similar DDP-4 inhibitor, sitagliptin, reduces serum levels of glycated albumin in type 2 diabetic subjects (Shima et al., 2014) and reduces the AGE content in lenses in streptozotocin-induced diabetic rats (Pandit et al., 2013). However, it remains unclear whether DPP-4 inhibitors have inhibitory effects on the glycation processes and cross-linking with proteins.

The purpose of this study was to evaluate the inhibitory effects of gemigliptin on the formation of AGEs and the cross-linking between preformed AGE-BSA and proteins *in vitro*. In addition, we also investigated whether the inhibitory activity of gemigliptin on the glycation process is due to its reactivity with reactive dicarbonyl compounds. Moreover, because various classes of structurally different DPP-4 inhibitors are currently available, we also compared the effectiveness of gemigliptin with that of the other DPP-4 inhibitors, vildagliptin and saxagliptin. Subsequently, we compared AGE formation and cross-links in type 2 diabetic *db/db* mice with and without administration of gemigliptin.

2. Materials and Methods

2.1 *In vitro* tests

2.1.1 Inhibitory activity on AGEs formation

Bovine serum albumin (Sigma Chemicals, MO, USA) was incubated at 4 °C for 7 days with methylglyoxal (5mM) in sodium phosphate buffer (0.1 M, pH 7.4). All of the reagent and samples were sterilized by filtration through 0.2 mm membrane filters. The reaction mixture was then mixed with gemigliptin (LG Life Sciences, Seoul, Korea, 99.2% pure by HPLC analysis), vildagliptin (Beijing HuiKang BoYuan Chemical Tech, Beijing, China, 99.4% pure by HPLC) or saxagliptin (Beijing HuiKang BoYuan Chemical Tech, Beijing, China, 99.0% pure by HPLC). Aminoguanidine (Sigma Chemicals, MO, USA) was used as a positive inhibitor. The levels of AGE were determined by measuring AGE-specific fluorescence using a spectrofluorometer (excitation at 370 nm and emission at 440 nm, Synergy HT, BIO-TEK, VT, USA). We calculated the 50% inhibition concentration (IC_{50}) of AGE formation.

2.1.2 AGE cross-linking assay

The ability of DPP-4 inhibitors to inhibit cross-linking of preformed AGE-BSA with collagen was examined. Preformed AGE-BSA (TransGenic Inc, Kobe, Japan) was mixed with DPP-4 inhibitors or aminoguanidine. This mixture was incubated in a rat tail tendon collagen-coated 96-well plate (Sigma, MO, USA) for 4 h at 37 °C. The cross-linked complexes of preformed AGE-BSA-collagen were detected using a mouse anti-AGE antibody (TransGenic, Kobe, Japan) and horseradish peroxidase-conjugated secondary antibody (Santa

Cruz, CA, USA). Peroxidase activity was quantified using tetramethylbenzidine. Inhibition of preformed AGE-BSA and collagen cross-linking was expressed as the percentage of optical density.

2.1.3 Scavenging of carbonyl intermediates of AGE formation

We evaluated the ability of DPP-4 inhibitors to interact with methylglyoxal *in vitro* according to our previously reported method (Kim et al., 2011). Aminoguanidine was used as a positive control to determine the relative concentration of the remaining methylglyoxal.

2.2 *In vivo* tests

2.2.1 Animals

All mice were handled according to the approved procedure (LGMD13-083) by Institutional Animal Care and Use Committee of LG Life Sciences. Seven week-old male C57BL/KsJ-*db/db* mice (*db/db,* SLC, Shizuoka, Japan) and their lean littermates (*db/+*, normal) were randomly assigned to three (n = 10) groups. One group of *db/db* mice was orally administered gemigliptin (100 mg/kg body weight) and another group was administered the same amount of vehicle via oral gavage for 12 weeks. Non-diabetic littermates received the same vehicle treatment. Blood glucose level and body weight were measured weekly.

2.2.2 Quantification of serum AGEs levels

At necropsy, serum samples were collected and serum AGE levels were analyzed using an AGE ELISA kit (MyBioSource Inc, CA,

USA) according to the manufacturer's instruction.

2.2.3 RBC-IgG assay

Immunoglobulin G (IgG) is cross-linked to membrane protein of red blood cells (RBCs). RBC-IgG are formed before other AGE cross-links *in vivo*. The amount of RBC-IgG can be used to estimate protein cross-linking levels (Vasan et al., 1996). To test the inhibitory effect of gemigliptin on AGE cross-links, RBCs from heparinized whole blood were collected and RBC-IgG levels were determined using an anti-IgG ELISA.

2.3 Statistical analysis

All results are expressed as the mean ± standard error of the mean (S.E.M.). The IC_{50} values were determined by interpolation from the concentration-inhibition curve. Differences between groups were assessed by Student's t-test for single comparisons or by one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. Differences were considered significant at $P<0.01$. The statistical differences and IC_{50} values were determined using Prism 4.0 program (Graphpad, CA, USA).

3. Results

3.1 Inhibitory effect of DPP-4 inhibitors on AGEs formation *in vitro*

Three DPP-4 inhibitors were examined to evaluate inhibitory effects on AGE-BSA formation. As shown in Fig. 1, gemigliptin dose-dependently inhibited the formation of AGE-BSA (IC_{50} = 11.69 ± 0.13 mM). The inhibitory activity of gemigliptin was less than aminoguanidine (IC_{50} = 2.69 ± 0.06 mM) and other two DPP-4 inhibitors did not show anti-AGE formation activity.

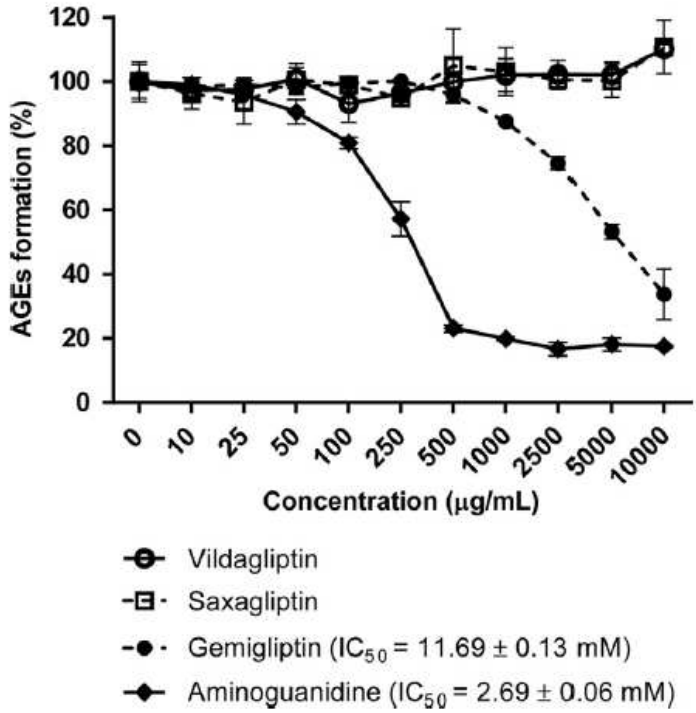

Figure 1. Inhibitory effect of DPP-4 inhibitors and aminoguanidine on AGEs formation. Data are expressed as the mean ± S.E.M., n=4. The IC_{50} values were determined from the plotted graph of AGE inhibition activity.

3.2 Inhibitory effect of DPP-4 inhibitors on cross-linking of preformed AGE-BSA with rat tail tendon collagen *in vitro*

The inhibition of cross-links between preformed AGE-BSA and collagen under various concentrations of DPP-4 inhibitors was examined (Fig. 2). Gemigliptin dose-dependently suppressed the cross-linking of preformed AGE-BSA with rat tail tendon collagen (IC_{50} = 1.39 ± 0.10 mM), and its inhibitory activity was greater than that of aminoguanidine (IC_{50} = 26.4 ± 1.20 mM). Additionally, other two DPP-4 inhibitors did not show inhibitory effects on AGE cross-linking with collagen.

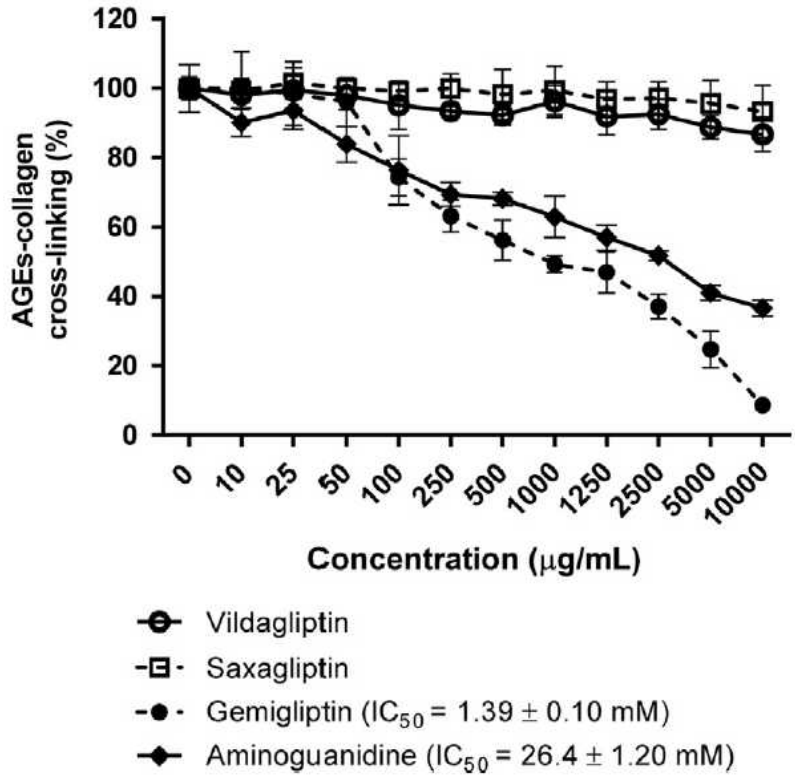

Figure 2. Inhibitory effect of DPP-4 inhibitors on the cross-links of preformed AGE-BSA with rat tail tendon collagen *in vitro* All results are expressed the mean ± S.E.M., n = 4. The IC_{50} values were determined from the plotted graph of cross-link inhibition activity.

3.3 Methylglyoxal scavenging effect of DPP-4 inhibitors

Many AGE inhibitors can interact with reactive dicarbonyl metabolites during the glycation process (Edelstein and Brownlee, 1992). Therefore, we tested whether three DPP-4 inhibitors can also interact with methylglyoxal *in vitro* (Fig. 3). Gemigliptin dose-dependently scavenged methylglyoxal (IC_{50} = 1.15 ± 0.02 mM), and its activity was greater than aminoguanidine (IC_{50} = 7.02 ± 0.16 mM).

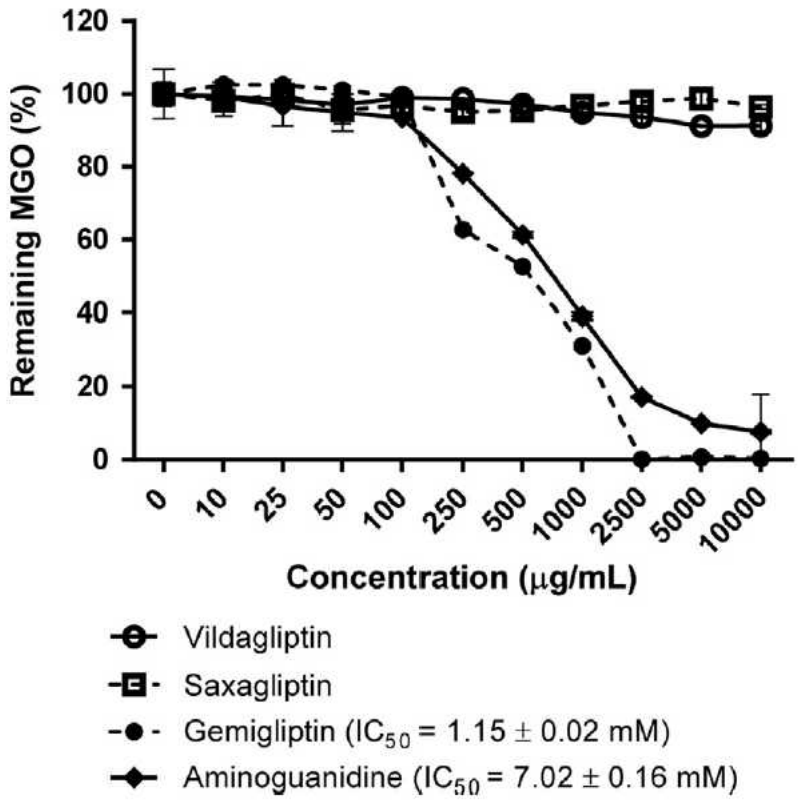

Figure 3. Carbonyl trapping activity of DPP-4 inhibitors and aminoguanidine All results are expressed as the mean ± S.E.M., n = 4. The IC_{50} values were determined from the plotted graph of scavenging activity.

3.4 Gemigliptin inhibits AGEs formation and AGE cross-links *in vivo*

The *in vivo* inhibitory effect of gemigliptin on AGEs formation in diabetic animal models was examined. After a 12 week-treatment, the body weight of *db/db* mice was greater than normal *db/+* mice. Severe hyperglycemia was observed in *db/db* mice ($P<0.01$ vs. normal *db/+* mice). Gemigliptin did not reduce blood glucose levels below that of *db/db* mice (Table 1). At the end of the study, *db/db* mice showed significantly increased serum AGE levels compared to normal *db/+* mice, but gemigliptin significantly reduced circulating AGE levels by 44.5 % in serum compared to vehicle-treated *db/db* mice (Fig. 4A). We next conducted the RBC-IgG assay to evaluate AGE cross-links. As shown in Fig. 4B, the RBC-IgG content of normal *db/+* mice was 0.21±0.01 and the content of vehicle-treated *db/db* mice was 0.61±0.04 ($P<0.01$). However, treatment with gemigliptin significantly reduced RBC-IgG content by 38.6% (0.41±0.06; $P<0.01$) compared with vehicle-treated *db/db* mice.

Table 1. Metabolic and physical parameters

		NOR	DM	Gemigliptin
Body weight (g)	Initial	25.1±0.8	34.61.9[a]	34.6±3.7
	Final	29.0±1.2	35.4±1.9[a]	33.2±8.0
Blood glucose (mg/dl)	Initial	133.4±17.0	459.4±46.2[a]	413.8±93.9
	Final	139.8±27.7	531.6±42.9[a]	510.81±115.4

NOR, normal *db/+* mice; DM, *db/db* mice; Gemigliptin, *db/db* mice treated with gemigliptin (100 mg/kg/day). All data are expressed as the mean ± S.E.M. [a]$P<0.01$ vs. NOR group.

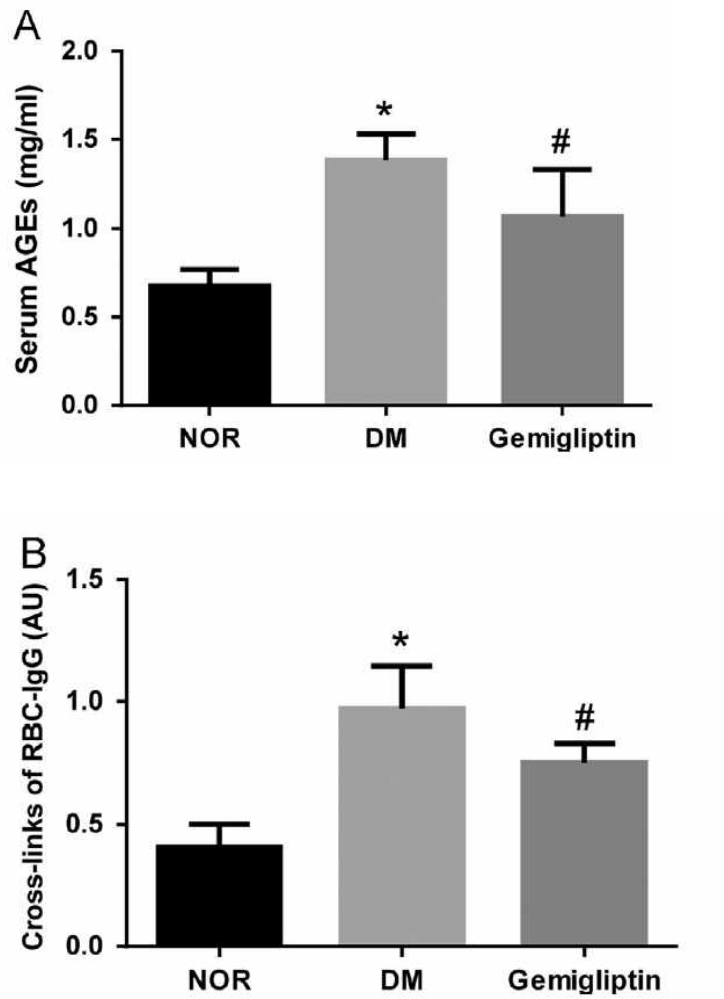

Figure 4. Effect of gemigliptin on AGE formation (A) and IgG cross-linked to the RBC surface (B) in blood of *db/db* mice. The values in the bar graph represent mean ± S.E.M., n = 10. *P<0.01 vs. normal *db/+* mice, # P<0.01 vs. vehicle-treated *db/db* mice.

4. Discussion

Many previous studies have reported that the formation of AGEs and their accumulation in tissues and cells play a crucial role in the pathogenic processes of diabetic complications (Sato et al., 2006). The cross-linked complex of AGEs with target proteins, including collagen, is permanent and irreversible. In the present study, we showed that a novel DPP-4 inhibitor, gemigliptin, possessed the potent activities of an AGE inhibitor and a carbonyl scavenger. Although a relatively high concentration (1 to 10 mM) of gemigliptin was required to block the glycation process *in vitro*, gemigliptin may be more potent than aminoguanidine as an AGE cross-linking inhibitor and methylglyoxal scavenger.

The cytotoxic effects of AGEs under diabetic conditions have been shown in many previous studies (Brownlee, 1995). Human body does not have any enzymes to break AGE structures. Thus, the importance of inhibiting AGE formation has been shown using a number of AGE inhibitors. Aminoguanidine prevented AGE formation resulting in inhibition of diabetic nephropathy, retinopathy and neuropathy in numerous diabetic animal models (Thornalley, 2003). However, because of safety concerns about its pro-oxidant activities (Suji and Sivakami, 2006) and inhibition of NO synthase (Tilton et al., 1993), aminoguanidine has currently not been used to treat diabetic complications (Turgut and Bolton, 2010). New antiglycating agents with higher levels of efficacy and safety in human are continually pursued.

Gemigliptin is an oral anti-diabetic agent that is approved by the Korean Food and Drug Administration and has an excellent safety record (Pennock et al., 2013). The detailed mechanism of action of gemigliptin as an AGE-inhibitor is still unclear. AGE inhibitors,

including aminoguanidine, pyridoxamine and 2-Isopropylidenehydrazono -4-oxo-thiazolidin-5-ylacetanilide (OPB-9195), suppress AGE formation through interaction with the reactive dicarbonyl species and acting as dicarbonyl scavenger. Aminoguanidine reacts with the α -dicarbonyls of reactive dicarbonyl species to form triazine derivatives (Lo et al., 1994 Thornalley et al., 2000). Pyridoxamine interacts with glyoxal, glyoxalaldehyde and methylglyoxal, resulting in the formation of adducts (Nagaraj et al., 2002). Our *in vitro* study showed that gemigliptin directly trapped methylglyoxal, and its activity was more potent than aminoguanidine. It is suggested that the scavenging activity of gemigliptin could participate in the inhibition of AGE formation and may prevent or delay the development of AGE-related diabetic complications.

Interestingly, among the three DPP-4 inhibitors used in this study, gemigliptin is the most potent anti-AGE inhibitor and methylglyoxal scavenger. Vildagliptin and saxagliptin had no inhibitory effects on the glycation processes. Consistent with our result, vildagliptin did not suppress circulating serum AGE levels in type 2 diabetic OLETF rats (Matsui et al., 2011). DPP-4 inhibitors include diverse structural types. Structurally, DPP-4 inhibitors are divided into two main classes, those that interact covalently with DPP-4 and those that do not (Horan et al., 2007). Vildagliptin and saxagliptin are covalent DPP-4 inhibitors and cyanopyrrolidine derivatives. Gemigliptin is a non-covalent DPP-4 inhibitor and piperazine derivative (Safavi et al., 2013). Vardhan et al. reported that piperazine analogues are potential AGE inhibitors (Vardhan et al., 2013). A cognition-enhancing drug tenilsetam, (+)-3-(2-thienyl)-2-piperazine, acts as an inhibitor of AGE formation and also methylglyoxal scavenger. Although the mechanisms of action of tenilsetam have yet to be explained,

tenilsetam inhibits the protein cross-linking by acting with sugars and glycated proteins (Rahbar and Figarola, 2003). In addition, recent *in vivo* studies showed that sitagliptin, a piperazine derivate, reduced the serum levels of glycated albumin in type 2 diabetic subjects (Shima et al., 2014) and reduced the AGE contents in lenses in streptozotocin-induced diabetic rats (Pandit et al., 2013). These results suggest that a piperazine derivative, such as gemigliptin, is among numerous DPP-4 inhibitors that can act as both AGE inhibitor and methylglyoxal scavenger.

In our *in vivo* study, gemigliptin induced only a minor decrease in blood glucose levels. Consistent with our observation, sitagliptin and vildagliptin also failed to improve hyperglycemia in similarly designed *db/db* mice studies (Moritoh et al., 2009). In addition, DPP-4 inhibitor, valine-pyrrolidide improved glucose intolerance in 6-week-old *db/db* mice in early stage of diabetes but not in 23-week-old *db/db* mice (Nagakura et al., 2003), which suggests that the glucose-lowering effect of DPP-4 inhibitors is more effective in the presence of healthy pancreatic β-cells. In our present study, gemigliptin has an inhibitory effect on the glycation processes despite continued hyperglycemia in *db/db* mice. These results suggest that even in hyperglycemia, it is possible to attenuate AGE-related tissue injury by gemigliptin.

In conclusion, our study showed that gemigliptin is a potent inhibitor of AGE formation and its cross-linking reaction with proteins. These activities may be explained in large part by its scavenging effect on methylglyoxal. Our results indicate the potential utility of gemigliptin as an AGE inhibitor and provide a possibility for treating diabetic complications associated with AGEs. Further clinical studies are needed to assess the value and possible application of gemigliptin as

an anti-glycation agent in diabetic patients.

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Part II.

Gemigliptin improves renal function and attenuates podocyte injury in mice with diabetic nephropathy

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Abstract

Podocytes participate in the formation and regulation of the glomerular filtration barrier. Loss of podocytes occurs during the early stages of diabetic nephropathy and impairs glomerular filtration. Dipeptidyl peptidase-4 (DPP-4) inhibitors are widely used as anti-diabetic agents in clinical practice. In this study, we showed that gemigliptin, a novel DPP-4 inhibitor, reduced podocyte apoptosis in type 2 diabetic *db/db* mice without reducing hyperglycemia. Gemigliptin (100 mg/kg/day) was administered orally for 12 weeks in *db/db* mice. Blood glucose levels and albuminuria were measured. The renal cortex was collected for histological examination, and molecular assays were used to detect 8-hydroxydeoxyguanosine, advanced oxidation protein products (AOPP), the receptor for advanced glycation end products (RAGE), and integrin-linked kinase (ILK). Type 2 diabetic *db/db* mice exhibited albuminuria, renal histopathological changes, and podocyte loss. Administration of gemigliptin to *db/db* mice suppressed albuminuria, enzyme activity and expression of DPP-4, and podocyte apoptosis. The effect of gemigliptin on diabetes-induced podocyte loss was associated with the suppression of oxidative damage, AOPP accumulation, RAGE expression, and ILK expression. These results indicate the possible benefits of using gemigliptin in diabetes patients to treat renal impairment without affecting glycemic control.

1. Introduction

Diabetic nephropathy represents one of the most frequent and serious microvascular complications that develop in patients with diabetes (White and Bilous, 2000). It is characterized by albuminuria and a decreased glomerular filtration rate (Ruggenenti et al., 2004). The association between the onset of albuminuria and progressive loss of renal function is well described, but the underlying pathophysiological mechanisms are still unclear. Albuminuria could be the result of impaired function of the glomerular filtration barrier or tubular reabsorption (Gorriz and Martinez-Castelao, 2012). The glomerular filtration barrier consists of a highly specialized structure with three layers: the endothelium, basement membrane, and podocytes (Siddiqi and Advani, 2013). Damage or loss of podocytes is a primary cause of the decreased ability of the barrier to filter out albumin. The density of podocytes per glomerulus is markedly reduced in patients with diabetes (Drummond and Mauer, 2002 Pagtalunan et al., 1997). In animal models of diabetes, podocyte depletion represents one of the earliest cellular injuries affecting the kidney (Susztak et al., 2006). Podocytes are highly differentiated cells with limited potential for proliferation (Wiggins, 2007). Thus, podocytes that are lost from the glomerulus are slowly replenished leading to podocytopenia (Spurney and Coffman, 2008). This has focused attention on podocytes as the primary target for developing therapies against diabetic nephropathy (Mathieson, 2012).

Currently, there are no commercially available drugs that specifically target podocytes. However, many agents originally developed for other clinical applications are used to treat glomerular disease by preserving the structural integrity of podocytes (Meliambro et al.,

2013). Glucocorticoids (Ransom et al., 2005), abatacept (Yu et al., 2013), thiazolidinedione (Kanjanabuch et al., 2007), angiotensin-converting -enzyme inhibitors (Mifsud et al., 2001), and rituximab (Fornoni et al., 2011) have been successfully repurposed to treat renal podocyte injury. Dipeptidyl peptidase-4 (DPP-4) inhibitors are a major new class of anti-diabetic drugs that increase the level of a gastrointestinal incretin hormone, glucagon-like peptide-1 (GLP-1), by inhibiting its cleavage. DPP-4 inhibitors show glucose-dependent activity by increasing insulin secretion, inhibiting glucagon secretion, and increasing β-cell mass, thereby resulting in lower blood glucose levels (Baetta and Corsini, 2011). Several recent studies have suggested that DPP-4 inhibitors owe their protective effects against diabetic nephropathy to a therapeutic off target action. Vildagliptin reduced diabetic renal injury by reducing the production of transforming growth factor-β1 in the kidney of streptozotocin (STZ)-induced diabetic rats (Liu et al., 2012). Linagliptin reduced renal oxidative stress and also reduced albuminuria in STZ-induced diabetic mice (Nakashima et al., 2014). Sitagliptin prevented diabetes-induced renal inflammation and apoptosis in Zucker diabetic fatty rats (Marques et al., 2014). It is noteworthy that vildagliptin and linagliptin both reduced diabetic nephropathy independently of their glucose-lowering properties. However, the precise mechanisms, beyond the effect of DPP-4 inhibitors on blood glucose, have not yet been elucidated. To the best of our knowledge, there are no reports on the effects of DPP-4 inhibitors on podocytes under hyperglycemic conditions. To address this, we have studied the effect of gemigliptin, a novel DPP-4 inhibitor, on diabetes-induced podocyte injury in type 2 diabetic *db/db* mice.

2. Materials and Methods

2.1 Animals and experimental design

Five-week-old male C57BL/KsJ-Lepr$^{db/db}$ diabetic mice (*db/db*) and nondiabetic heterozygous littermates (*db/+*) were purchased from Jung-Ang Lab Animal Inc. (Seoul, Korea). After 2 weeks of acclimatization, the *db/+* and *db/db* mice were divided into the following three groups: *db/+* group (NOR, n = 10), *db/db* group (DM, n = 10), and *db/db* + gemigliptin group (Gemigliptin, n = 10). Gemigliptin (100 mg/kg body weight) or an equal amount of vehicle (0.5% methylcellulose solution) was administered to diabetic *db/db* mice by oral gavage once a day for 12 weeks. Non-diabetic littermates received the same amount of methylcellulose solution. Body weight and blood glucose levels were recorded every week. Approval for animal studies was obtained from the Animal Welfare Review Board of LG Life Sciences (LGMD13-083; approval date 01/07/2014).

2.2 Measurement of albuminuria and histological analysis

After 12 weeks of treatment, urine samples were collected using metabolic cages for a 24 h period (Techniplast, Buguggiate, Italy). Urinary albumin level was measured using a mouse albumin enzyme-linked immunosorbent assay (ELISA) kit (Abnova, Taipei, Taiwan). After killing the mice, both kidneys were collected, and the kidney cortex was fixed in a 10% formalin solution and embedded in paraffin. Cortical tissue slices (4 μm thickness) were dewaxed and stained with periodic acid-Schiff.

2.3 Detection of 8-hydroxydeoxyguanosine and nephrin in urine samples

Urinary samples were collected over 24h and stored until analysis at -80°C under nitrogen to prevent the formation of artificial adducts. Urinary 8-hydroxydeoxyguanosine (8-OHdG) and nephrin levels were determined using an 8-OHdG Check ELISA kit (Japan Institute for the Control of Aging, Fukuroi, Japan) and a mouse nephrin ELISA kit (Exocell, Philadelphia, PA, USA), respectively. The concentrations of 8-OHdG and nephrin were calculated as ng per mg of total protein.

2.4 Renal DPP-4 enzymatic activity assay

Frozen renal cortex samples were weighed and homogenized in assay buffer (25 mM HEPES, 140 mM NaCl, 80 mM $MgCl_2$, pH 7.8) with Triton X-100 (1%, v/v). DPP-4 enzymatic activity was assessed using a DPP-4 Activity Assay Kit (Biovision, Milpitas, CA, USA) according to the manufacturer's instructions. DPP-4 activity was calculated using the cleavage rate of 7-amino-4-methylcoumarin per tissue weight (μM/min per g tissue).

2.5 Renal advanced oxidation protein products assay

Frozen renal cortex tissue (200 mg) was homogenized using a Teflon homogenizer in 2 ml of 100 mM KH_2PO_4 - K_2HPO_4buffer (pH7.4) containing digitonin (0.1%, v/v). Advanced oxidation protein products (AOPP) were measured using an OxiSelect™ AOPP Assay Kit (Cell Biolabs, San Diego, CA, USA) according to the

manufacturer's instructions. The levels of AOPP were expressed as the amount of chloramine per mg of protein (μM/mg total protein).

2.6 Immunostaining

Immunostaining was conducted according to a previously described protocol. (Park et al., 2013) For immunohistochemical analysis, the antibodies used were rabbit anti-8-OHdG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-receptor for advanced glycation end product (anti-RAGE, Abcam, MA, USA), and mouse anti-integrin-linked kinase (anti-ILK, Santa Cruz Biotechnology). Their expression was detected using an EnVision Plus detection system (DAKO, Carpinteria, CA, USA) and developed using 3,3′-diaminobenzidine tetrahydrochloride chromogen solution. The antibody used for immunofluorescence analysis was rabbit anti-synaptopodin (Santa Cruz Biotechnology). To detect synaptopodin, the slides were probed with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit immunoglobulin G (Santa Cruz Biotechnology). Negative control slides were incubated with non-immune normal serum as the primary antibody. Immunostaining intensity was evaluated quantitatively in 40 glomeruli per animal using cellSens software (Olympus, Tokyo, Japan).

2.7 Double immunofluorescence staining

To directly observe the apoptosis of renal podocytes, tissue sections were double stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and Wilms tumor antigen-1 (WT-1) according to a previously published procedure. (Park et al., 2013) Briefly, the sections were first labeled using the DeadEnd TUNEL system (Promega, Madison, WI, USA) and FITC-conjugated

streptavidin (Santa Cruz Biotechnology). The second sequence of staining using rabbit anti-WT-1 antibody (Santa Cruz Biotechnology) was performed on the same section with rhodamine-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology). To achieve dual-labeling for synaptopodin and integrin-linked kinase (ILK), a sequence of two single immunofluorescent staining procedures was performed. To prevent any unwanted reactions between the two staining sequences, sections were blocked with normal serum from the same species as the labeled antibody following the first staining. The number of cells positive for WT-1 was quantified from 40 glomeruli per animal using cellSens software (Olympus, Tokyo, Japan).

2.8 Western blotting analysis

Protein lysate (20 μg) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Biorad, Hercules, CA, USA). The membrane was labeled with mouse anti-DPP-4 (Santa Cruz Biotechnology), mouse anti-RAGE antibody (Abcam, Cambridge, MA, USA), and mouse anti-ILK antibody (Santa Cruz Biotechnology). The immunoreactive bands were detected using an enhanced chemiluminescence kit (Amersham, Piscataway, NJ, USA) and the density of the bands-of-interests was further measured using a LAS-3000 (Fujifilm, Tokyo, Japan).

2.9 Statistical analysis

Data are expressed as the mean ± standard error of the mean (S.E.M.). Differences between groups were assessed by one-way analysis of variance (ANOVA) with post hoc analysis using Prism

4.0 program (GraphPad, La Jolla, CA, USA). Differences were considered statistically significant with $P < 0.01$.

3. Results

3.1 Body weight and blood glucose

Compared to non-diabetic controls, *db/db* mice had an increased body weight, and this change was unaffected by gemigliptin administration (Table 1). Blood glucose and glycated hemoglobin (HbA1c) levels were significantly higher in *db/db* mice ($P < 0.01$ vs. normal *db/+*mice), but no significant reduction of these values was observed after gemigliptin treatment (Table 1).

Table 1. Body weight, blood glucose, and HbA1c levels in experimental animals

	NOR	DM	Gemigliptin
Body weight (g)	29.0 ± 1.2	35.4 ± 1.9[a]	33.2 ± 8.0
Blood glucose (mg/dl)	139.8 ± 27.7	531.6 ± 42.9[a]	510.81 ± 115.4
HbA1c (%)	4.80 ± 1.82	12.45 ± 1.99[a]	12.99 ±2.89

NOR, normal *db/+* mice; DM, *db/db* mice; Gemigliptin, *db/db* mice treated with gemigliptin (100 mg/kg/day); HbA1c, glycated hemoglobin. Data are expressed as the mean ± S.E.M.

[a] $P < 0.01$ vs. NOR group.

3.2 Gemigliptin inhibits diabetes-induced podocyte injury

Our first aim was to determine whether gemigliptin could prevent diabetes-induced renal impairment. Fig. 1A and 1B show diffuse mesangial expansion with sclerotic lesions (arrow) ad albuminuria in *db/db* mice. Compared to the vehicle-treated *db/db* mice, gemigliptin-treated mice showed reduction of diabetes-induced histopathological changes and albuminuria ($P<0.01$).

Next, to determine podocyte loss from renal tissue, urine specimens were tested for the urinary podocyte-specific protein, nephrin. At the end of the treatment period, urinary levels of nephrin had increased significantly in *db/db* mice (Fig. 2A, $P<0.01$ vs. *db/db* mice). To more accurately determine the extent of podocyte loss, podocytes were probed with anti-WT-1 and anti-synaptopodin antibodies (Park et al., 2013), and the mean number of positive-stained cells in a particular glomerulus were determined. In *db/db* mice, the number of WT-1-positive or synaptopodin-positive cells (red and green, respectively) significantly decreased compared to that in *db/+* mice (Fig. 2B to 2D). Interestingly, TUNEL and WT-1 double-positive cells (arrow, Fig.2B) were rare or absent in *db/+* mice. In contrast, the number of apoptotic podocytes increased in *db/db* mice with higher nephrinuria. However, gemigliptin ameliorated this apoptotic podocyte loss with low-grade nephrinuria (Fig.2B). Taken together, these results suggest that gemigliptin prevents diabetes-induced podocyte apoptosis and reduces albuminuria.

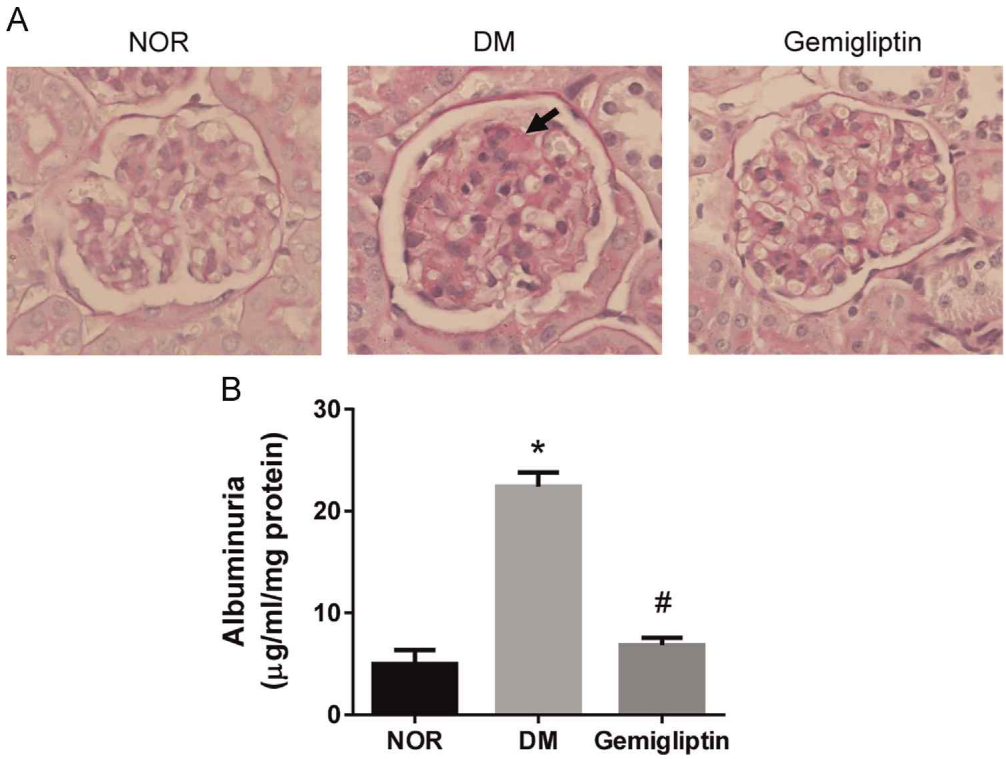

Figure 1. Renal histopathology and function. (A) Periodic acid-Schiff staining of glomeruli. x400 magnification. Arrows indicate sclerotic lesions. (B) Albuminuria. NOR, normal *db/+* mice; DM, *db/db* mice; Gemigliptin, *db/db* mice treated with gemigliptin (100 mg/kg/day). Data are expressed as mean ± S.E.M. (n = 10). *P < 0.01 vs. NOR group, #P < 0.01 vs. DM group.

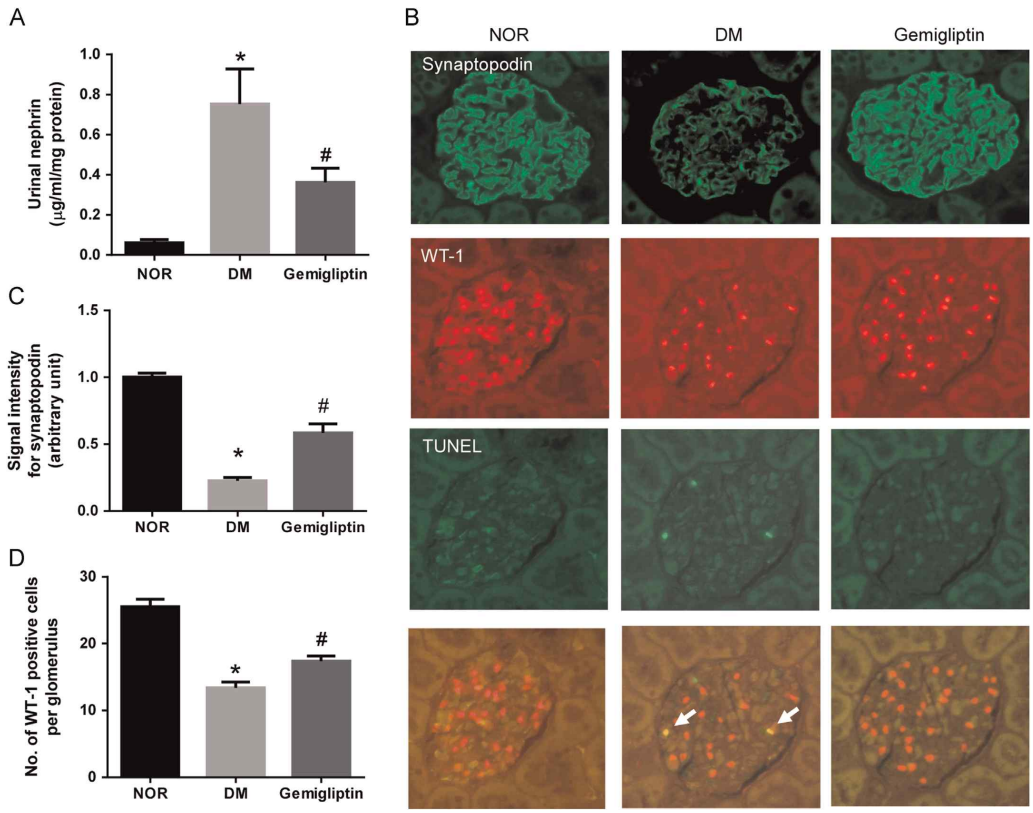

Figure 2. Inhibitory effects of gemigliptin on diabetes-induced podocyte loss. (A) ELISA analysis of urinary nephrin. (B) Immunofluorescence staining for synaptopodin (green) and a dual labeling for WT-1 (red) and TUNEL (green). x400 magnification. The renal cortex was stained using specific antibodies for synaptopodin and WT-1 (specific markers of podocytes). Double positive cell (arrow) indicates co-localization of the podocyte marker and apoptosis. Quantitative analyses of (C) signal intensity of synaptopodin, (D) and numbers of WT-1-positive cells. Data are expressed as mean ± S.E.M. (n = 10). *P < 0.01 vs. NOR group, #P < 0.01 vs. DM group.

3.3 Gemigliptin inhibits renal DPP-4 activity and protein expression

DPP-4 enzyme activity was significantly enhanced in the renal tissue of *db/db* mice compared to that in *db/+* mice ($P<0.01$, Fig.3A). Treatment with gemigliptin normalized enzyme activity in *db/db* mice. Similarly, diabetes led to increased DPP-4 protein levels compared to those in *db/+* mice, while gemigliptin prevented the increase in renal DPP-4 protein levels in *db/db* mice (Fig. 3B).

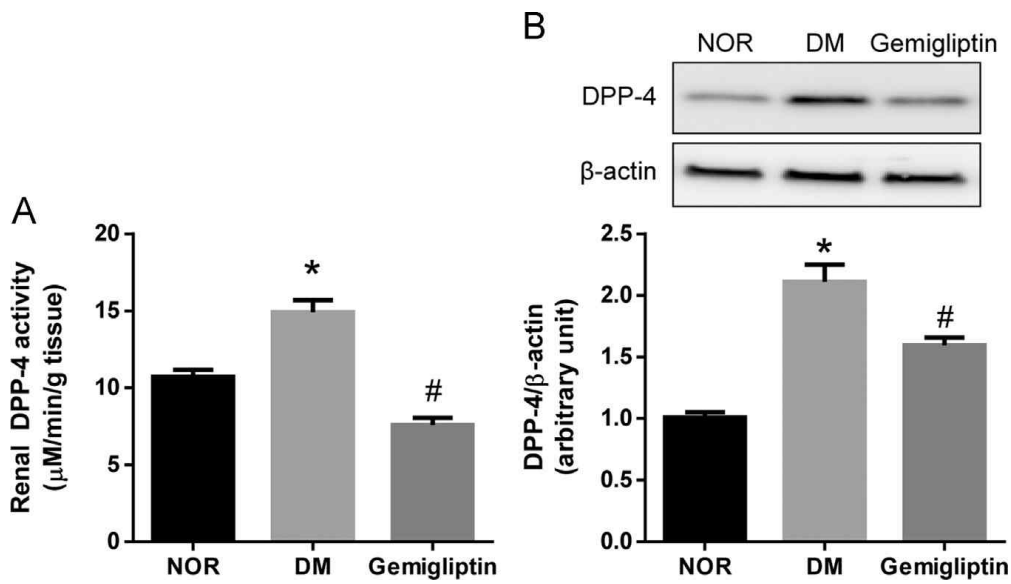

Figure 3. Renal DPP-4 activity and protein expression. (A) DPP-4 enzyme activity was measured by the cleavage of a fluorescent substrate. (B) The protein levels of DPP-4 were assessed by western blot analysis. The bands were detected and quantified using an image analyzer (LAS-3000, Fujifilm), and DPP-4 protein levels were normalized to β-actin levels. Data are expressed as mean ± S.E.M. (n = 10). *P < 0.01 vs. NOR group, #P < 0.01 vs. DM group.

3.4 Gemigliptin prevents the accumulation of AOPP and expression of its receptor in renal tissues

AOPP has been identified as a novel family of protein adducts that are cross-linked by dityrosine. They are known to occur more frequently in diseases associated with increased oxidative stress, including chronic renal disease and diabetes (Witko-Sarsat et al., 1998). AOPP induces apoptosis of podocyte (Zhou et al., 2009) and causes inflammatory response in endothelial cells via binding to RAGE (Guo et al., 2008). Thus, we performed immunostaining and a urinary ELISA for 8-OHdG to examine oxidative damage and measure renal AOPP and RAGE protein expression in *db/db* mice in the absence or presence of gemigliptin. Urinary excretion of 8-OHdG was significantly greater in the DM group than in the NOR group (Fig. 4A). However, this observed increase in urinary 8-OHdG was markedly reduced by treatment with gemigliptin (db/db mice, 11.64 ± 3.31 ng/ml/mg protein; gemigliptin-treated *db/db* mice, 7.94 ± 1.78 ng/ml/mg protein). A similar pattern was observed in urinary levels of 8-OHdG and albumin. As shown in Fig. 4B, the number of cells stained positively for 8-OHdG (arrow) was significantly higher in the DM group than in the NOR group. However, significantly fewer cells tested positive for 8-OHdG in *db/db* mice treated with gemigliptin. In addition, AOPP clearly accumulated in the renal tissue of *db/db* mice; it was significantly higher in the DM group than in the NOR group (Fig. 4C). However, AOPP accumulation was reduced in renal tissue of gemigliptin-treated *db/db* mice.

Next, we conducted immunostaining and immunoblotting assays for RAGE. Immunostaining demonstrated a significant increase in RAGE protein (arrow) in the glomeruli of *db/db* mice compared to that in

non-diabetic controls, but RAGE expression was down-regulated by gemigliptin (Fig. 5A). In the western blot analysis, increased levels of RAGE proteins were noted in the renal cortex of *db/db* mice. Gemigliptin significantly lowered the expression of RAGE proteins (Fig. 5B).

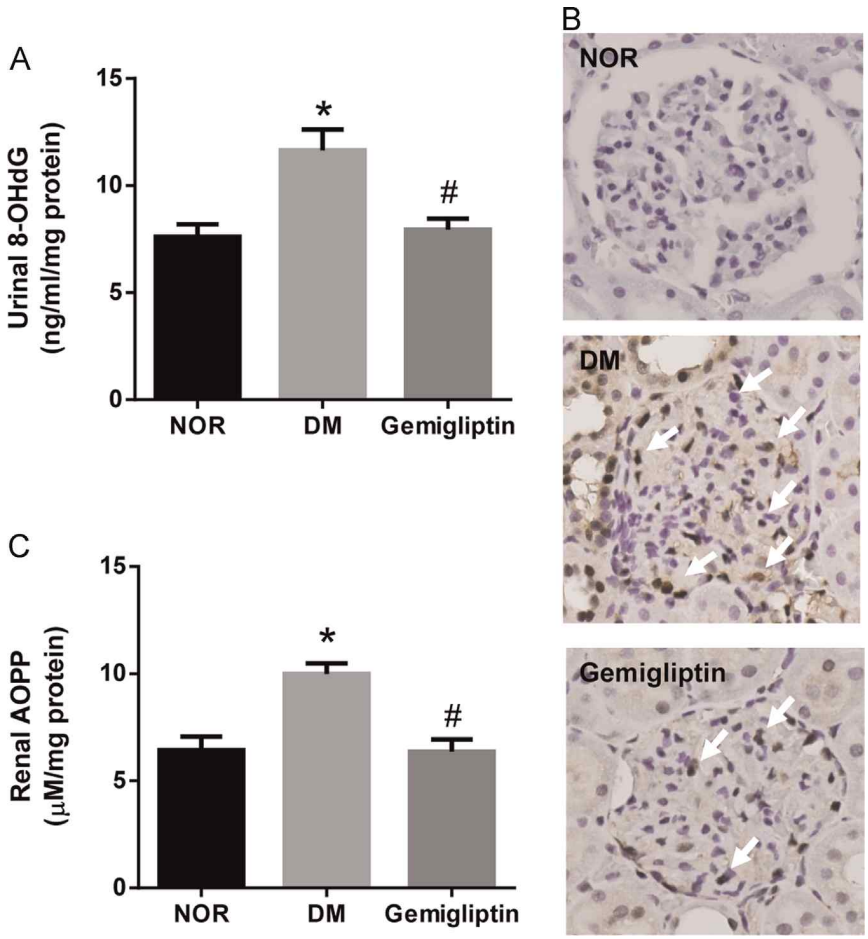

Figure 4. Effect of gemigliptin on 8-OHdG formation and AOPP accumulation in renal tissues. (A) Urinary 8-OHdG excretion levels. (B) Immunohistochemical staining for 8-OHdG. Arrows indicate 8-OHdG - positive nuclei in the glomerulus. (C) Renal AOPP levels. Data are expressed as mean ± S.E.M. (n = 10). *P < 0.01 vs. NOR group, #P < 0.01 vs. DM group.

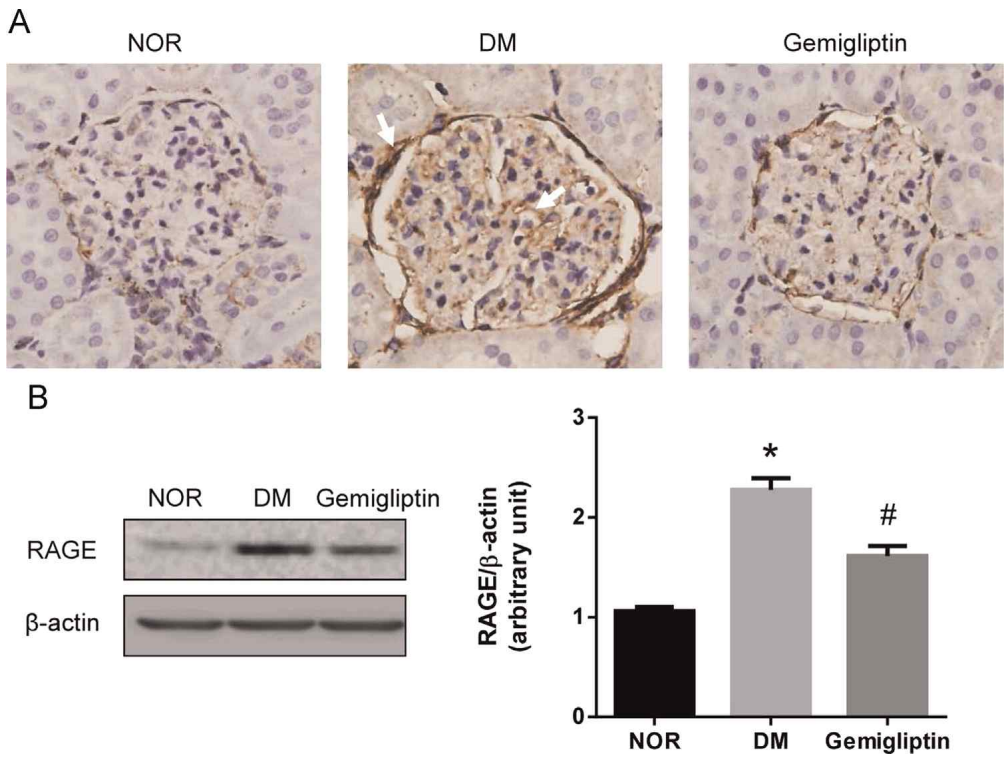

Figure 5. Effect of gemigliptin on RAGE protein expression in renal tissues. (A) Immunohistochemical staining for RAGE. Arrows indicate the area of RAGE expression. (B) Representative immunoblots of RAGE in protein extracts. Quantitative analysis of protein expression. RAGE protein levels were normalized to β-actin levels. Data are expressed as mean ± S.E.M. (n = 10). *P < 0.01 vs. NOR group, #P < 0.01 vs. DM group.

3.5 Gemigliptin inhibits ILK expression

ILK, a 59-kDa Ser/Thr kinase, is important for the control of podocyte-matrix adhesion and podocyte survival. Advanced glycation end products (AGEs) have been shown to reduce podocyte adhesion by up-regulating ILK expression (Cheng et al., 2013). Moreover, AGEs are structurally similar to AOPP and elicit similar biological responses (Kalousova et al., 2002). These findings suggest that there may be a link between AOPP accumulation, ILK regulation, and podocyte loss. Thus, we evaluated the effect of gemigliptin on ILK expression in *db/db* mice. As shown in Fig.6A, immunohistochemical staining showed little ILK protein present in the glomeruli of *db/+* mice. In *db/db* mice, ILK expression was up-regulated in the glomeruli (arrow). Double immunostaining for synaptopodin (green) and ILK (red) clearly confirmed ILK expression in podocytes. Although the number of podocytes reduced, co-localization (arrowhead) of synaptopodin and ILK was clearly observed in *db/db* mice (Fig. 6B). However, gemigliptin decreased ILK protein expression in the glomeruli of *db/db* mice. Similarly, we observed changes in the quantity of ILK protein using western blot analysis. The expression of ILK was consistent with their immunohistochemical response (Fig. 6B).

Compared to ILK expression in *db/+* mice, ILK expression was significantly higher in *db/db* mice, and gemigliptin treatment reduced ILK expression significantly. These observations suggested that the protective effect of gemigliptin on podocytes might be caused by down-regulation of ILK protein expression.

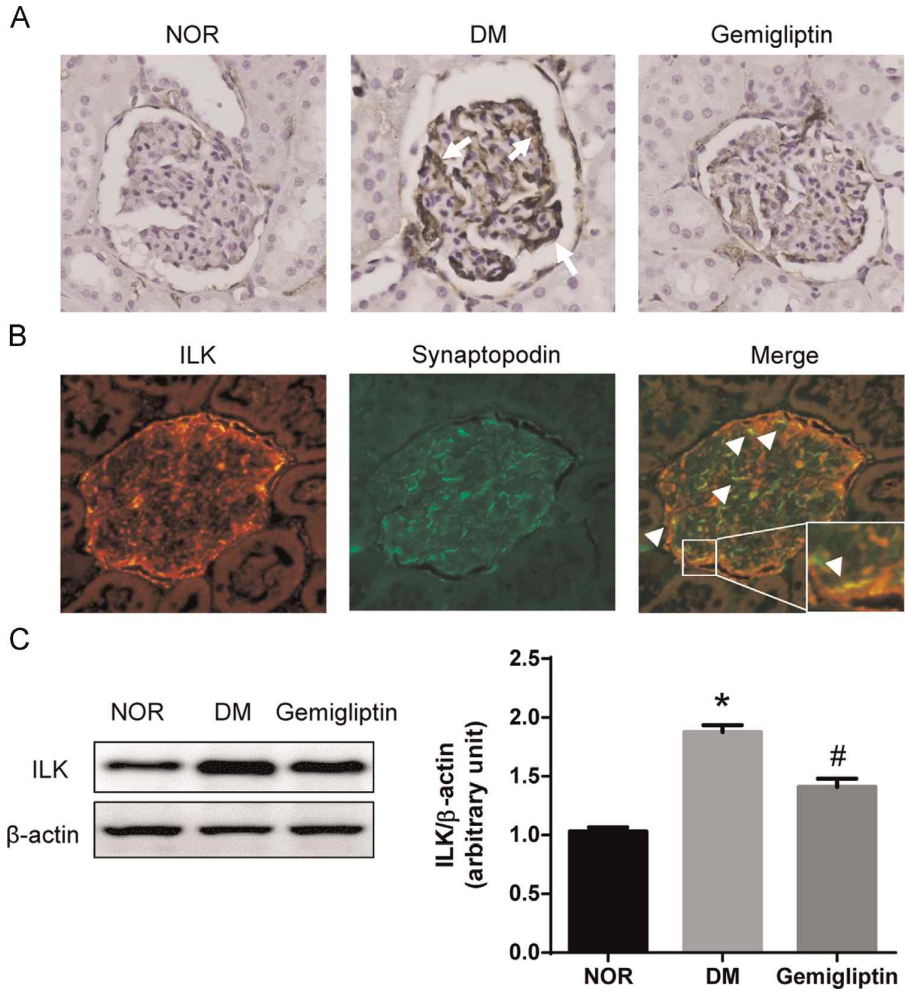

Figure 6. Gemigliptin inhibited the protein expression of ILK in renal tissue. (A) Immunohistochemical staining for ILK. Arrows indicate the area of ILK expression (B) Double immunofluorescence staining for ILK (red) and synaptopodin (green) to confirm the expression of ILK in podocytes. The specific co-staining (arrowhead) for ILK and synaptopodin is shown in the merged picture as yellow. (C) The protein expression of ILK was also examined by western blot. ILK protein levels were normalized to β-actin levels. Data are

expressed as mean ± S.E.M. (n = 10). *P < 0.01 vs. NOR group, #P < 0.01 vs. DM group.

4. Discussion

Gemigliptin is a selective and long-lasting DPP-4 inhibitor, and it is approved by the Korea Ministry of Food and Drug Safety for clinical use in patients with type 2 diabetes (Lim et al., 2009). Most research on gemigliptin has focused on the normalization of blood glucose in patients with type 2 diabetes (Kim et al., 2013). However, this is the first study demonstrating that gemigliptin has protective effects in the renal podocytes of diabetic animals by a mechanism independent of lowered blood glucose.

Podocytes, highly specialized epithelial cells found in the glomerulus, contribute to the formation of the glomerular filtration barrier. Increased renal podocyte loss is a well-established consequence of diabetes, and is one of the first detectable signs of diabetic nephropathy(Pagtalunan et al., 1997). Importantly, the reduction of podocyte number is observed even in patients with a relatively recent onset of diabetes, and podocyte injury could even occur before albuminuria (Steffes et al., 2001). Moreover, podocyte apoptosis and depletion correspond with the development of glomerulosclerosis in a time-dependent manner (Kriz and Lemley, 1999). Therefore, improving the health of podocytes is a reasonable therapeutic approach to prevent renal function impairment in the event of diabetic nephropathy (Leeuwis et al., 2010; Mathieson, 2012). Interestingly, gemigliptin successfully prevented podocyte apoptosis through the inhibition of AOPP accumulation and ILK up-regulation in *db/db* mice, despite continued hyperglycemia. These results indicate that gemigliptin has direct effects on podocytes that are independent of its anti-hyperglycemic effect.

It was previously reported that DPP-4 inhibitors were unable to

improve hyperglycemia in a late-stage animal disease model (Nagakura et al., 2003). Kawashima et al. reported that alogliptin did not show beneficial effects on blood glucose and β-cells in *db/db* mice aged between 8 and 16 weeks. Their research suggests that the ineffectiveness of DPP-4 inhibitors might be owing to the reduction of GLP-1 receptor expression in *db/db* mice (Kawashima et al., 2011). Consistent with previous reports, gemigliptin failed to reduce blood glucose levels in *db/db* mice. Therefore, this animal model is suitable for evaluating the effects of gemigliptin in the diabetic kidney independent of its glucose-lowering effects.

An oral dose of 50 mg/day gemigliptin is recommended for glycemic control in patients with diabetes. However, our recent study showed that oral administration of 100 mg/kg/day gemigliptin reduced plasma AGE levels in *db/db* mice (Jung et al., 2014). Min et al. also showed that oral consumption of 150 mg/kg/day gemigliptin in a mouse model of ureteral obstruction prevented renal interstitial fibrosis (Min et al., 2014). On the basis of these results, we selected a dose of 100 mg/kg gemigliptin for daily oral administration in *db/db* mice. Our finding regarding the effective dose of gemigliptin on kidney tissues in *db/db* mice is consistent with that of the Min et al. (Min et al., 2014). In a previous report, the average peak plasma concentration of gemigliptin was 62.7 ng/ml in humans after oral administration of a single 50 mg dose, and the area under the plasma concentration time curve (AUC) was 743.1 ng/ml • h^{-1}. The elimination half-life of gemigliptin was 17.1 h. Inhibition of DPP-4 activity was maintained for 24 h (Lim et al., 2008). However, in *db/db* mice, the peak plasma concentration of gemigliptin after oral administration of 100 mg/kg gemigliptin was 2614 ng/ml, but the elimination half-life of gemigliptin was 1.5 h. Thus, the AUC value of gemigliptin was 4510

ng/ml • h^{-1} in *db/db* mice (unpublished data). Although, this AUC value of gemigliptin after oral administration in human was 6-folds lower than that observed in mice, the elimination half-life of gemigliptin in human were 11-folds longer than in mice. Moreover, due to its short half-life in *db/db* mice, the DPP-4 inhibitory activity of gemigliptin for 24 h was equivalent to that seen in *db/db* mice after oral administration of 100 mg/kg gemigliptin. For this reason, gemigliptin may have renoprotective effect at a relatively lower dose in diabetic patients.

DPP-4 is a serine exopeptidase that cleaves Xaa-Ala or Xaa-Pro dipeptides at the N-terminus (Lambeir et al., 2003). Several studies have demonstrated the renoprotective effects of DPP-4 inhibitors. Linagliptin reduced renal fibrosis in STZ-induced diabetic mice (Kanasaki et al., 2014). Alogliptin ameliorated albuminuria in type 2 diabetes patients with early stage of nephropathy (Fujita et al., 2014), and vildagliptin attenuated renal sclerosis in Zucker diabetic fatty rats (Vavrinec et al., 2014). An important aspect of these studies is that DPP-4 inhibitors demonstrate renoprotective effects independent of glycemic control. Although the mechanism of action of DPP-4 inhibitors in diabetes-induced renal injury is associated with GLP-1, the enzymatic or non-enzymatic functions of DPP-4 may also regulate the progression of diabetic renal disease (Hocher et al., 2012). DPP-4 is widely expressed in the entire body (Barnett, 2006), and expressed at the highest level per organ weight in renal tissue. (Mentlein, 1999) It is also found in podocytes (Machado et al., 2012). In the present study, gemigliptin normalized renal DPP-4 activity. Similarly, vildagliptin normalized renal DPP-4 activity in STZ-induced diabetic rats, and significantly prevented albuminuria, oxidative DNA damage, and renal cell apoptosis (Vavrinec et al., 2014). The

amelioration of renal DPP-4 activity by gemigliptin may contribute to the significant renal protection observed. Moreover, gemigliptin suppressed DPP-4 activity as well as protein expression in renal tissue. This result agrees with that of other research showing that sitagliptin and linagliptin also inhibited renal DPP-4 protein expression in diabetic animals (Kanasaki et al., 2014; Marques et al., 2014). In our present study, gemigliptin inhibited renal DPP-4 activity than that of *db/+* mice, although DPP-4 protein level in gemigliptin-treated db/db mice was higher than normal *db/+* mice. Similarly, linagliptin also highly inhibited renal DPP-4 activity in STZ-induced diabetic mice than normal control mice. Linagliptin inhibited DPP-4 protein expression, but its protein level was higher than normal control mice (Kanasaki et al., 2014). This discrepancy between the protein amount and its enzyme activity could be explained by a mechanism of action of gemigliptin. Gemigliptin is a highly selective DPP-4 inhibitor and directly bind to DPP-4 in a reversible manner, which reduces its enzyme activity (Kim et al., 2013). Moreover, it was reported that DPP-4 protein expression was regulated by microRNA 29s in STZ-induced diabetic mice, not by DPP-4 inhibitor. Linagliptin restored microRNA 29s, leading to the suppression of DPP-4 protein levels (Kanasaki et al., 2014). Further studies are necessary to evaluate whether gemigliptin increases microRNA 29s in the kidney of diabetic animal models.

DPP-4 directly increased reactive oxygen species generation in endothelial cells (Ishibashi et al., 2013). Its deficiency inhibited H_2O_2-induced intracellular reactive oxygen species production in cardiomyocytes (Ku et al., 2013). Additionally, it has been demonstrated that reactive oxygen species overproduction induced by high-glucose leads to podocyte apoptosis in vitro and to progressive

podocytopenia in experimental animals, leading to diabetic nephropathy (Susztak et al., 2006). Although podocyte-specific reactive oxygen species generation by DPP-4 and podocyte-specific inhibition of reactive oxygen species production by gemigliptin was not shown in this study, we demonstrated that gemigliptin led to the reduction of renal DPP-4 protein expression and prevented renal AOPP accumulation, enhanced 8-OHdG formation and enhanced reactive oxygen species-mediated podocyte apoptosis in *db/db* mice. These findings suggest that DPP-4 inhibition by gemigliptin could reduce oxidative stress-related podocyte injury and reduce the risk of cell death.

There is increasing evidence to suggest that ILK has an important role in the onset and progression of diabetic nephropathy (Chen et al., 2014). Changes to the cytoskeletal architecture lead to weakening of the adhesion between podocytes and the glomerular basement membrane. ILKs are reported to be involved in these processes (Wu and Dedhar, 2001). ILK proteins are highly expressed in mesangial cells and glomerular epithelial cells of diabetes patients (Guo et al., 2001) and diabetic rats (Dai et al., 2012). ILK activation leads to the reduction of integrin α3β1 expression and impaired cell-cell contact of podocytes under high-glucose conditions (Chen et al., 2000). Moreover, it was reported that AGEs reduced podocyte adhesion via up-regulation of ILK expression (Cheng et al., 2013). The effects caused by AGEs are because of their interaction with the receptor, RAGE; AOPP is structurally similar to AGE and elicits similar biological responses via interaction with the same receptor (Guo et al., 2008). Blocking RAGE dramatically attenuated the effect of AOPP (Iwao et al., 2008). Moreover, it was recently reported that DPP-4 increases RAGE gene expression and the DPP-4 inhibitor, linagliptin,

prevented up-regulation of RAGE mRNA levels in endothelial cells (Ishibashi et al., 2013). Our results clearly showed that gemigliptin treatment leads to decreased RAGE and ILK protein expression in the kidney of diabetic mice. Although we did not show AOPP-specific up-regulation of ILK in podocytes, our observations suggest that gemigliptin therapy may be useful in regulating AOPP/RAGE interaction and ILK expression in the kidney of individuals with diabetes.

Our study demonstrated that gemigliptin has an anti-apoptotic effect in podocytes that is attributable to inhibition of AOPP accumulation, RAGE expression, and ILK up-regulation in *db/db* mice. These novel findings provide information regarding the renoprotective effects of gemigliptin that are independent of glycemic control.

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Part III.

Gemigliptin, a dipeptidyl peptidase-4 inhibitor, inhibits retinal pericyte injury in db/db mice and retinal neovascularization in mice with ischemic retinopathy

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Abstract

Retinal pericyte loss and neovascularization are characteristic features of diabetic retinopathy. Gemigliptin, a dipeptidyl peptidase-4 (DPP-4) inhibitor, has shown robust blood-glucose lowering effects in type 2 diabetic patients, but its effects on diabetic retinopathy have not yet been reported. We evaluated the efficacy of gemigliptin on retinal vascular leakage in *db/db* mice, which is an animal model for type 2 diabetes, and neovascularization in oxygen-induced retinopathy (OIR) mice, which is an animal model for ischemic proliferative retinopathy. Gemigliptin (100 mg/kg/day) was orally administered to the *db/db* mice for 12 weeks. C57BL/6 mice on postnatal day 7(P7) were exposed to 75% hyperoxia for 5 days, followed by exposure to room air from P12 to P17 to induce OIR. Gemigliptin (50 mg/kg/day) was intraperitoneally injected daily from P12 to P17. Retinal neovascularization was analyzed in flat-mounted retinas on P17. We determined the efficacy and possible mechanism of gemigliptin on high glucose-induced apoptosis of primary human retinal pericytes. The oral administration of gemigliptin for 4 months significantly ameliorated retinal pericyte apoptosis and vascular leakage in the *db/db* mice. Gemigliptin also ameliorated retinal neovascularization in the OIR mice. Gemigliptin attenuated the overexpression of plasminogen activator inhibitor-1 (PAI-1) in the retinas of diabetic and OIR mice. Gemigliptin and PAI-1 siRNA significantly inhibited pericyte apoptosis by inhibiting the overexpression of PAI-1, which is induced by high glucose. These results suggest that gemigliptin has potent anti-angiogenic and anti-apoptotic activities via its ability to suppress DPP-4 and PAI-1, and the results support the direct retinoprotective action of gemigliptin.

1. Introduction

Diabetic retinopathy is a common and serious microvascular complication of diabetes (Fong et al., 2002). The normal function of retinal vessels requires interactions between the inner endothelial cells and surrounding pericytes (Hammes et al., 2002). Pericyte loss is one of the earliest detectable lesions in the diabetic retina (Hammes, 2005) and may be responsible for the retinal vascular leakage and sight-threatening neovascularization (Arboleda-Velasquez et al., 2015). Pericytes express several angiogenic growth factors, which are critical for determining whether vessels are stable or will undergo angiogenesis (Tomasek et al., 2006). The current management strategy for diabetic retinopathy is focused on early detection and tight glycemic control to slow the progression of the disease. However, the persistence of hyperglycemic stress despite glucose normalization has been observed in the diabetic retina (Engerman and Kern, 1987; Ihnat et al., 2007). Previous studies have shown that vascular endothelial cells and retinal pigment epithelial cells previously exposed to high glucose continued to display a reactive oxygen species (ROS)-mediated cellular stress despite subsequent normalization of glucose concentration in the media (Ihnat et al., 2007). Indeed, although many classes of glucose-lowering oral agents have been available for clinical use, the prevalence of diabetic retinopathy is increasing (Girach et al., 2006).

Dipeptidyl peptidase-4 (DPP-4) inhibitors are a major new class of anti-diabetic drugs that increase the levels of a gastrointestinal incretin hormone, glucagon-like peptide-1 (GLP-1), by inhibiting its cleavage. DPP-4 inhibitors show glucose-dependent activity by increasing insulin secretion, inhibiting glucagon secretion, and increasing β-cell mass, thereby producing lower blood glucose levels

(Baetta and Corsini, 2011). Recently, Blaslov et al. reported that circulating DPP-4 activity is associated with the progression and severity of diabetic retinopathy (Blaslov et al., 2015). Several DPP-4 inhibitors were shown to have protective effects against diabetic retinopathy. Sitagliptin inhibited retinal vascular leakage, inflammation and neuronal apoptosis in both type 1 and type 2 diabetic rats (Goncalves et al., 2012; Goncalves et al., 2014). Vildagliptin down-regulated the gene expression levels of angiogenic growth factors, such as vascular endothelial growth factor (VEGF), intercellular adhesion molecule-1 (ICAM-1), plasminogen activator inhibitor-1 (PAI-1) and pigment epithelium-derived factor (PEDF) in the retinas of obese type 2 diabetic rats (Maeda et al., 2013). Saxagliptin improved retinal capillary flow in diabetic patients without the clinical signs of microvascular alteration (Ott et al., 2014). However, the precise mechanisms of DPP-4 inhibitors against diabetic retinopathy beyond its glucose-lowering role are still not well understood.

Gemigliptin is a highly selective DPP-4 inhibitor developed in Korea and has been clinically used as an oral agent for type 2 diabetes (Kim et al., 2013). Previous *in vitro* and *in vivo* data suggest that gemigliptin also exerts a number of additional activities, including anti-glycation, anti-inflammation and renoprotective effects (Hwang et al., 2015; Hwang et al., 2014; Jung et al., 2014; Min et al., 2014). To the best of our knowledge, there have been no reports on the effects of DPP-4 inhibitors on retinal pericyte injury and neovascularization. Therefore, we studied whether gemigliptin inhibits retinal pericyte injury through the modulation of apoptotic cascades in type 2 diabetic *db/db* mice. Additionally, we evaluated whether gemigliptin effectively prevents retinal neovascularization in oxygen-induced retinopathy

(OIR) mice.

2. Materials and Methods

2.1. Animals

Six-week-old male C57BL/KsJ-Leprdb/db diabetic mice (*db/db*) and nondiabetic heterozygous littermates (*db/+*) were purchased from Japan SLC (Shizuoka, Japan). C57BL/6 mice were purchased from Orient Bio (Seoul, Korea). The care and use of the animals were approved by the Animal Welfare Review Board of LG Life Sciences (LGMD13-083; approval date 01/07/2014).

2.2. Type 2 diabetic *db/db* mice

After 2 weeks of acclimatization, the *db/+* and *db/db* mice were divided into the following three groups: the *db/+* group (n = 10), the *db/db* group (n = 10) and the gemigliptin group (n = 10). Gemigliptin (100 mg/kg body weight; LG Life Sciences, Seoul, Korea) or an equal amount of vehicle (0.5% methylcellulose solution) was administered to diabetic db/db mice by oral gavage daily for 12 weeks. Non-diabetic littermates received the same amount of methylcellulose solution. The blood glucose levels were recorded every week.

2.3. OIR mice and analysis of retinal neovascularization

OIR was induced in C57BL/6 mice. On postnatal day 12 (P12), after the mice were exposed to 75 ± 2% oxygen for 5 days (P7-P12), they were returned to room air and randomly assigned to two groups: vehicle-treated OIR mice and gemigliptin-treated (50 mg/kg/day) OIR mice. The normal control mice were maintained under room conditions from birth until postnatal day 17 (P17). Gemigliptin was diluted with 5% dimethyl sulfoxide (Sigma, St Louis, MO, USA) in

saline. The mice were injected intraperitoneally with 100 μL of this solution daily for 5 days. The vehicle solution was injected in the normal control group. On P17, the mice were anesthetized and sacrificed. Fluorescein retinal angiography and Bandeiraea simplicifolia isolectin B4 staining for the quantification of preretinal neovascular tufts were performed according to a method described previously (Connor et al., 2009) Briefly, preretinal neovascularization areas were outlined and quantified in each quadrant of the retina as a percentage of total area of retina analyzed.

2.4. Trypsin digest preparation to isolate retinal vascular beds

Each retina was carefully isolated under a microscope. After fixation in 4% paraformaldehyde for 24 h, the retinas were incubated in 3% trypsin (Sigma, St Louis, MO, USA) in sodium phosphate buffer containing 100 mmol/L NaF for 1 h to inhibit the DNase activity. The retinal vascular beds were separated from the retinal tissue by gentle rinsing in phosphate-buffered saline (PBS) and transferred to microscope slides.

2.5. Determination of the endothelial cells/pericytes (E/P) ratio

The retinas (n=10) from each group were isolated, and the trypsin-digested retinal vessels were stained with periodic acid Schiff base reagent. Pericytes and endothelial cells were identified based on the morphology and relative location to the capillaries. The E/P ratio was calculated using a previously established method (Midena et al., 1989). To determine the E/P ratio, the total numbers of pericytes and endothelial cells were counted in 10 randomly selected areas

(magnification 400x) in the middle one-third of the retinal capillary area. Samples were examined by 3 ophthalmic pathologists in a blinded fashion.

2.6. TUNEL assay and immunofluorescence staining

The retinal digests were incubated with a mouse anti-NG2 antibody (Chemicon, Temecula, CA, USA), a mouse anti-PAI-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and a TUNEL fluorescein kit (Promega, Madison, WI, USA). Nuclei were counterstained with diamidinophenylindole (DAPI, Sigma). For the quantification of pericyte density, 4 fields of view (mm^2) were randomly collected in the retinal digests from each mouse. The number of NG-2-positive was counted, and the number of pericytes was standardized by the number of DAPI-positive cells (number of NG2-positive cells per 100 DAPI-positive cells). TUNEL-positive cells were determined by counting per mm^2 of capillary area.

2.7. Retinal fluorescein isothiocyanate-dextran leakage

Mice were deeply anesthetized with zolazepam (Virbac, Carros, France). Then, a solution of 50 mg/kg of fluorescein isothiocyanate (FITC)-dextran (a molecular weight of 4.4 kDa, Sigma) in PBS (pH 7.4) was injected into the left ventricle. After circulation for 10 min, the retinas were isolated and transferred onto a microscope slide. Pictures were taken using a fluorescence microscope (Olympus, Tokyo, Japan). To quantify retinal vascular permeability, the mice were perfused with PBS (500 ml/kg body weight) for 6 min. Plasma was also collected before perfusion. The retina of each eye was isolated, weighed and homogenized in 200 μL of distilled water. The homogenate was centrifuged at 15,000 rpm for 20 min, and the

supernatant was collected. The fluorescence in each 100 μL sample was measured using a spectrofluorophotometer (Bio-Tek, Winooski, VT, USA). The amount of FITC-dextran leakage into the retinal tissues was calculated using the following equation: retinal FITC-dextran leakage = [retinal FITC-dextran (μg)/retinal weight (g)]/[plasma FITC-dextran (μg/μL) x circulation time (min)]

2.8. Immunofluorescence staining for occludin in retinal whole mounts

The whole retinas from each group were fixed with 4% paraformaldehyde for 24 h. The retinas were blocked and permeabilized in 10% normal donkey serum with 0.3% Triton in PBS for 1 h. The retinas were incubated with mouse anti-occludin antibody (Invitrogen, Carlsbad, CA, USA) in block solution for 48 h at 4°C. The retinas were washed for 30 min at room temperature and then incubated for 2 h at 4°C with rhodamine-conjugated donkey anti-mouse immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, CA, USA). All retinas were flat mounted and viewed with a fluorescence microscope (Olympus, Tokyo, Japan).

2.9. Protein array

On P17, the mice were anesthetized and sacrificed. Each retina was carefully isolated under a microscope. The retinas were homogenized in PBS using protease inhibitors and centrifuged at 10000 x g for 5 minutes, and the total protein concentrations were quantified. To determine the expression levels of angiogenesis-related factors, protein arrays were performed using a kit (Proteome Profiler™ Mouse Angiogenesis Antibody Array Kit, R&D Systems, Abingdon, UK). Optical density measurements were performed using ImageJ software

(NIH, Bethesda, MD, USA). A list of the 55 factors can be found on the manufacturer's webpage.

2.10. DPP-4 enzymatic activity assay

Plasma DPP-4 enzymatic activity was assessed using a DPP-4 Activity Assay Kit (Biovision, Milpitas, CA, USA) according to the manufacturer's instructions. DPP-4 activity was calculated using the cleavage rate of 7-amino-4-methylcoumarin per min per mL.

2.11. Primary human retinal pericyte culture

Primary human retinal pericytes (Cell Systems, Kirkland, WA, USA) were maintained in the specified Pericyte Media (PromoCell, Heidelberg, Germany). Pericytes were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were plated in 6-well plates and were used in experiments when the cells were approximately 80% confluent.

2.12. Apoptosis assay using flow cytometry

Pericytes were treated with normal glucose (5 mmol/L) and high glucose (30 mmol/L) in the presence or absence of gemigliptin at various concentrations (0, 1, 10 and 100 mmol/L) for 1 week. Mannitol (30 mmol/L) was used as an osmotic control. To determine the effect of DPP-4 and PAI-1 suppression, pericytes were transfected with 40 nmol/L DPP-4 siRNA (Santa Cruz Biotechnology), PAI-1 siRNA (Santa Cruz Biotechnology), or a corresponding scrambled siRNA using Lipofectamine™ 2000 (Invitrogen, Frederick, MD, USA) 24 h before treatment with high glucose. Pericytes were then collected and stained with an Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit (Invitrogen)

according to the manufacturer's instructions. Apoptosis was examined using a flow cytometer (Becton Dickinson, San Jose, CA, USA). The cells that stained positively with annexin V but not with PI were considered apoptotic cells.

2.13. Western blot analysis

Protein lysates were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were labeled with mouse anti-DPP-4 (Santa Cruz Biotechnology) and mouse anti-PAI-1 antibody (Santa Cruz Biotechnology). The immunoreactive bands were detected using chemiluminescence detection reagents (Pierce, Rockford, IL, USA), and the density of the bands-of-interest was further measured using a LAS-3000 machine (Fujifilm, Tokyo, Japan).

2.14. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Differences between groups were assessed using one-way ANOVA and Tukey's multiple comparison test in the Prism 6.0 program (GraphPad, La Jolla, CA, USA), and $P<0.05$ was considered statistically significant.

3. Results

3.1. Gemigliptin inhibits diabetes-induced retinal pericyte injury

The mice with a blood glucose concentration exceeding 300 mg/dL were considered diabetic. In the normal *db/+* mice, blood glucose levels were <150 mg/dL. The blood glucose levels in the *db/db* mice were >300 mg/dL at 8 weeks of age and gradually increased over the treatment period. Interestingly, gemigliptin treatment did not affect the blood glucose levels throughout the treatment period (Fig. 1).

We used retinal digest preparations to determine the presence of pericyte loss. The E/P ratio was calculated to determine pericyte density in the retinal vasculature. The E/P ratio was significantly increased in the *db/db* mice compared to that of normal *db/+* mice, whereas the E/P ratio for the gemigliptin-treated mice was significantly reduced (Fig. 2A and B). To confirm pericyte loss, we performed immunofluorescence analysis for NG2, which is a pericyte marker. As shown in Fig. 2C, the number of NG2-positive cells significantly decreased in the vehicle-treated *db/db* mice compared with normal *db/+* mice. However, gemigliptin significantly inhibited the reduction of NG2-positive cells in *db/db* mice. We performed TUNEL staining to further confirm that the reduction in pericyte density in *db/db* mice was due to apoptosis. The retinal vessels of the *db/db* mice showed many TUNEL-positive pericytes and endothelial cells, whereas normal *db/+* mice and gemigliptin-treated *db/db* mice exhibited few positive cells (Fig. 2D). These findings suggest that the loss of retinal pericytes occurred in *db/db* mice and that gemigliptin might inhibit diabetes-induced pericyte apoptosis.

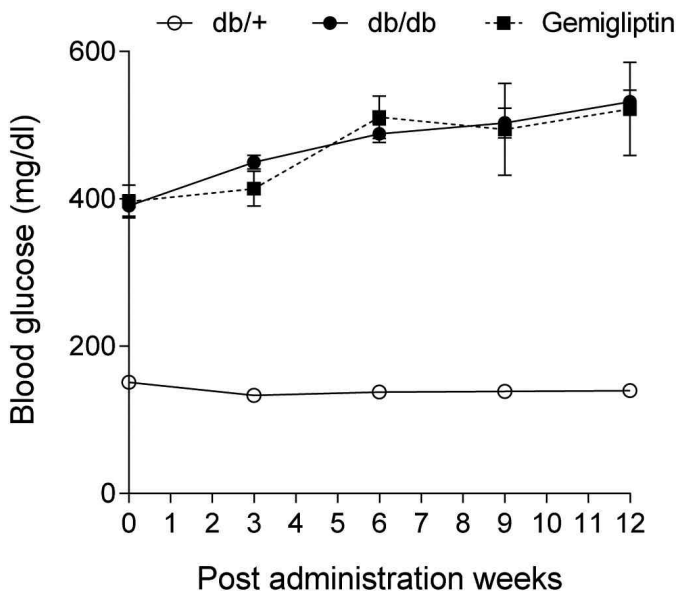
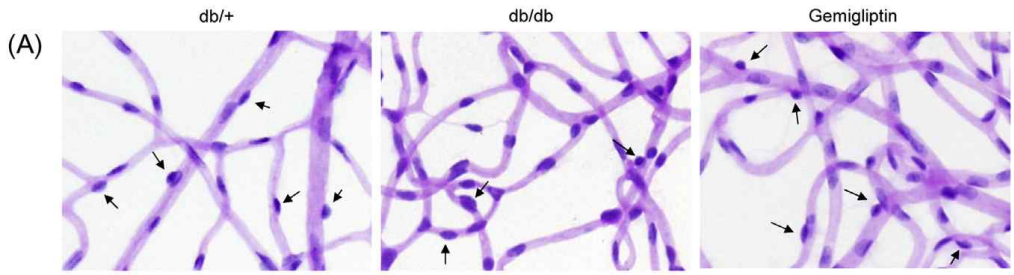
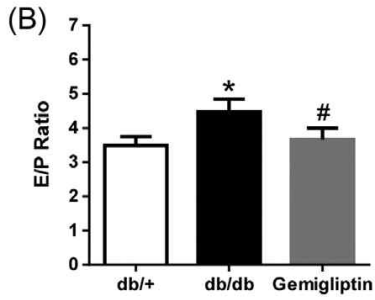
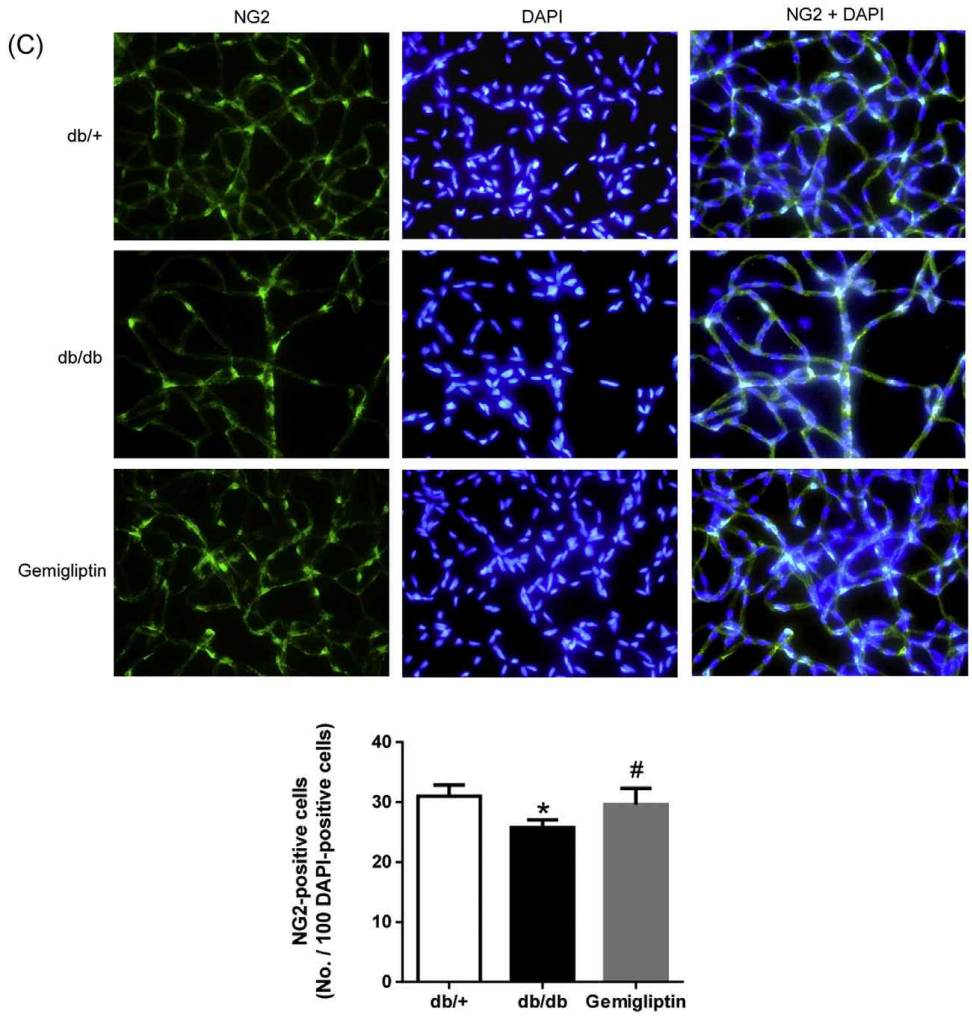


Fig. 1 Blood glucose levels. Values are expressed as the mean ± SEM, n = 10.


(A)
db/+
db/db
Gemigliptin


(B)
E/P Ratio
0
1
2
3
4
5
6
7
*
#
db/+
db/db
Gemigliptin


(C)
NG2
DAPI
NG2 + DAPI
db/+
db/db
Gemigliptin
NG2-positive cells
(No. / 100 DAPI-positive cells)
40
30
20
10
0
*
#
db/+
db/db
Gemigliptin

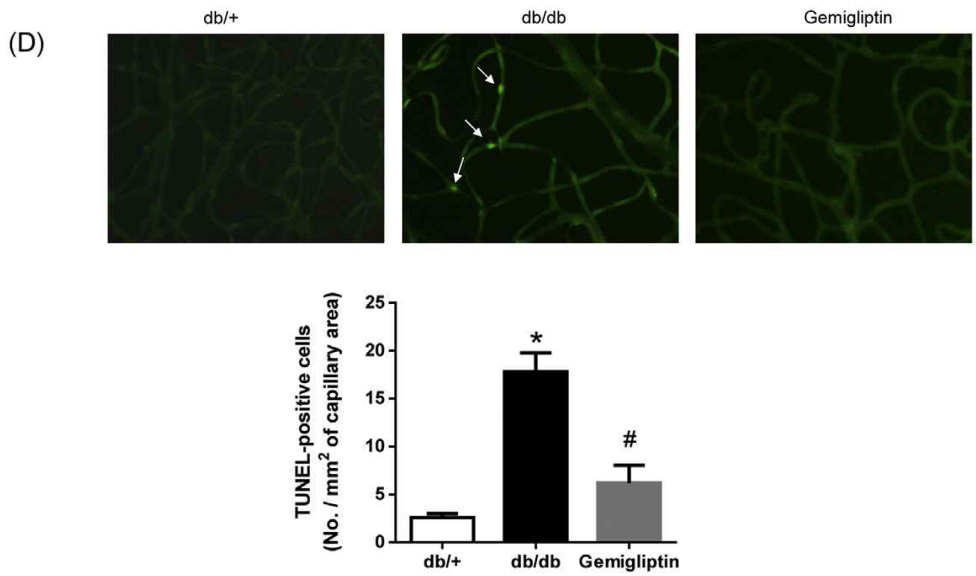


Fig. 2 Inhibitory effects of gemigliptin on diabetes-induced pericyte loss (A) Periodic acid Schiff and hematoxylin-stained retinal digested microvessels. Pericytes were identified in retinal digest preparations based on morphologic criteria (shape, staining intensity, and relative position in the capillary) and quantified. The arrows indicate representative pericytes. (B) The E/P ratio was calculated. (C) Immunofluorescence staining for NG2 (green) in retinal vessels. Vessels were counterstained with DAPI (blue). The number of pericytes was determined by counting the number of NG2 positive cells per mm^2 of capillary area. (D) TUNEL staining. TUNEL-positive nuclei were counted per mm^2 capillary area. Data are expressed as the mean ± SEM, n = 10. *P < 0.05 vs. *db/+* mice, #P < 0.05 vs. *db/db* mice.

3.2 Gemigliptin decreases diabetes-induced BRB disruption

We evaluated retinal vessel dysfunction using FITC-dextran microscopy. Oral gemigliptin treatment significantly reduced retinal vascular leakage in *db/db* mice compared with the vehicle-treated *db/db* mice, to a level that was similar to normal *db/+* mice (Fig. 3A and B). Next, the retinal vessels were immunostained for occludin to examine gemigliptin effects on tight junction protein loss in the retinal microvasculature. Retinal protein extracts were analyzed using Western blot analysis. Unlike the retinal vasculature in normal *db/+* mice, multiple alterations in the subcellular distribution of occludin proteins were observed in the retinal vessels of vehicle-treated *db/db* mice, but gemigliptin significantly restored the decrease in occludin protein levels in *db/db* mice compared with vehicle-treated *db/db* mice to levels that were comparable to the retinas of normal *db/+* mice (Fig. 3C and D).

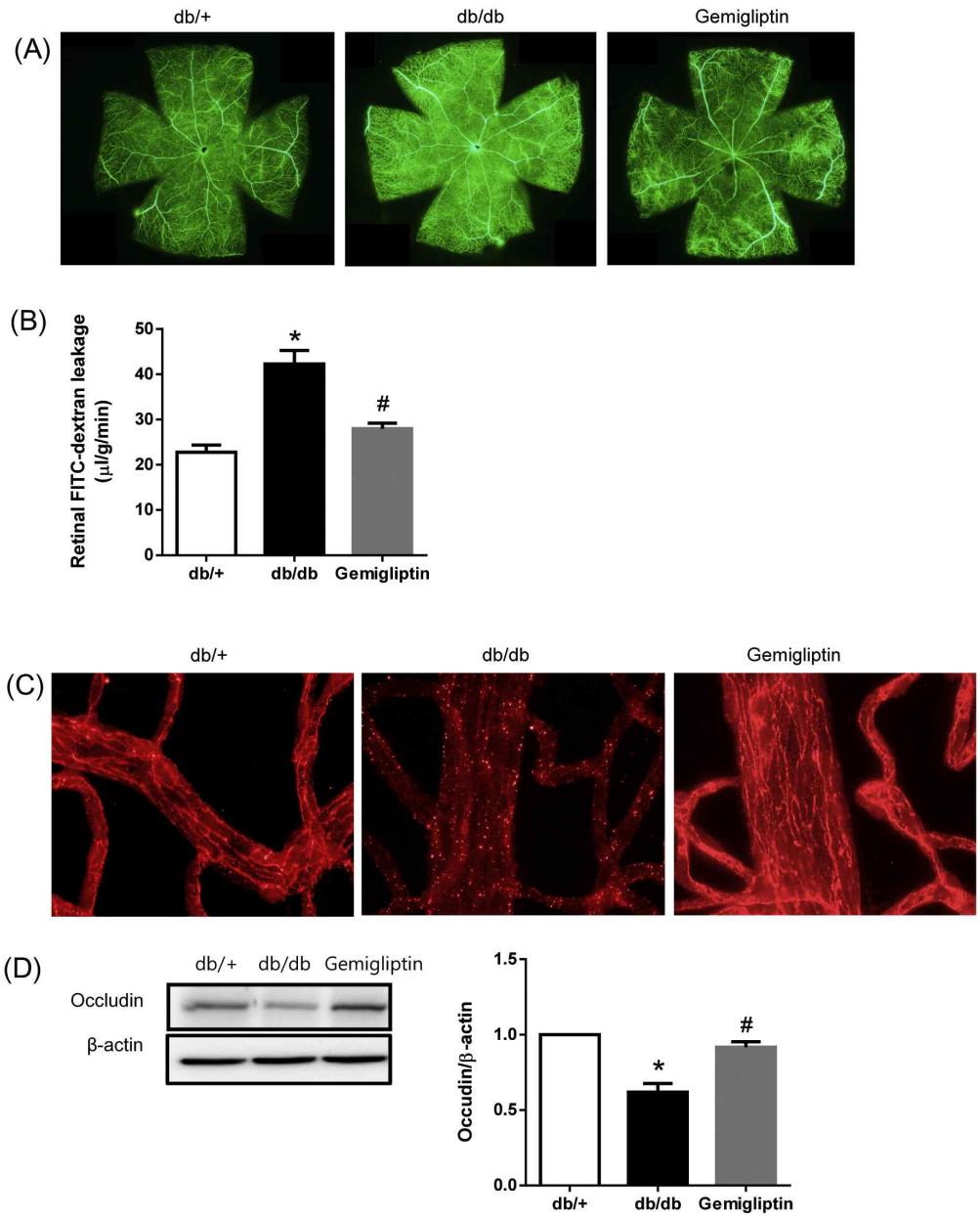


Figure 3. Inhibitory effects of gemigliptin on diabetes-induced blood-retinal barrier breakdown (A) FITC-dextran angiography on retinal flat mounts. (B) Quantitative analysis of retinal vascular permeability. (C) Immunofluorescence staining for occludin protein in

retinal whole mounts. In normal *db/+* mice, immunoreactivity for occludin was continuous at the cell borders, whereas diabetes disrupted occludin immunoreactivity at the cell border and increased intracellular punctate labeling. Gemigliptin prevented the changes of occludin in *db/db* mice. (D) Western blot analysis for occludin in retinal tissues. Values in the bar graphs represent the mean ± SEM, n = 10. *P < 0.05 vs. *db/+* mice, #P < 0.05 vs. *db/db* mice.

3.3 Gemigliptin attenuates retinal neovascularization in OIR mice

The above results indicate that the effects of gemigliptin on retinal vascular alterations can be achieved without a reduction of hyperglycemia in *db/db* mice. To confirm whether gemigliptin's effects on diabetic retinopathy occurred via the direct effects on the retina that independently affected blood glucose, nondiabetic OIR mice were used. Gemigliptin was administered intraperitoneally once a day for 5 days from P12 in OIR mice, which is a model of ischemia-induced retinopathy. Fluorescein angiography at P17 indicated that the gemigliptin-treated retinas developed less retinal vascular leakage and neovascularization (Fig. 4A) compared with vehicle-treated OIR retinas. The quantification of preretinal neovascular tufts using isolectin B4 staining showed that the retinas treated with gemigliptin developed significantly fewer preretinal neovascular tufts, relative to the vehicle-treated OIR mice (Fig. 4B-D), supporting the notion that gemigliptin has an anti-angiogenic effect. In addition, the retinal vascular permeability assay showed that gemigliptin also significantly reduced retinal vascular leakage compared with vehicle-treated OIR mice (Fig. 4E).

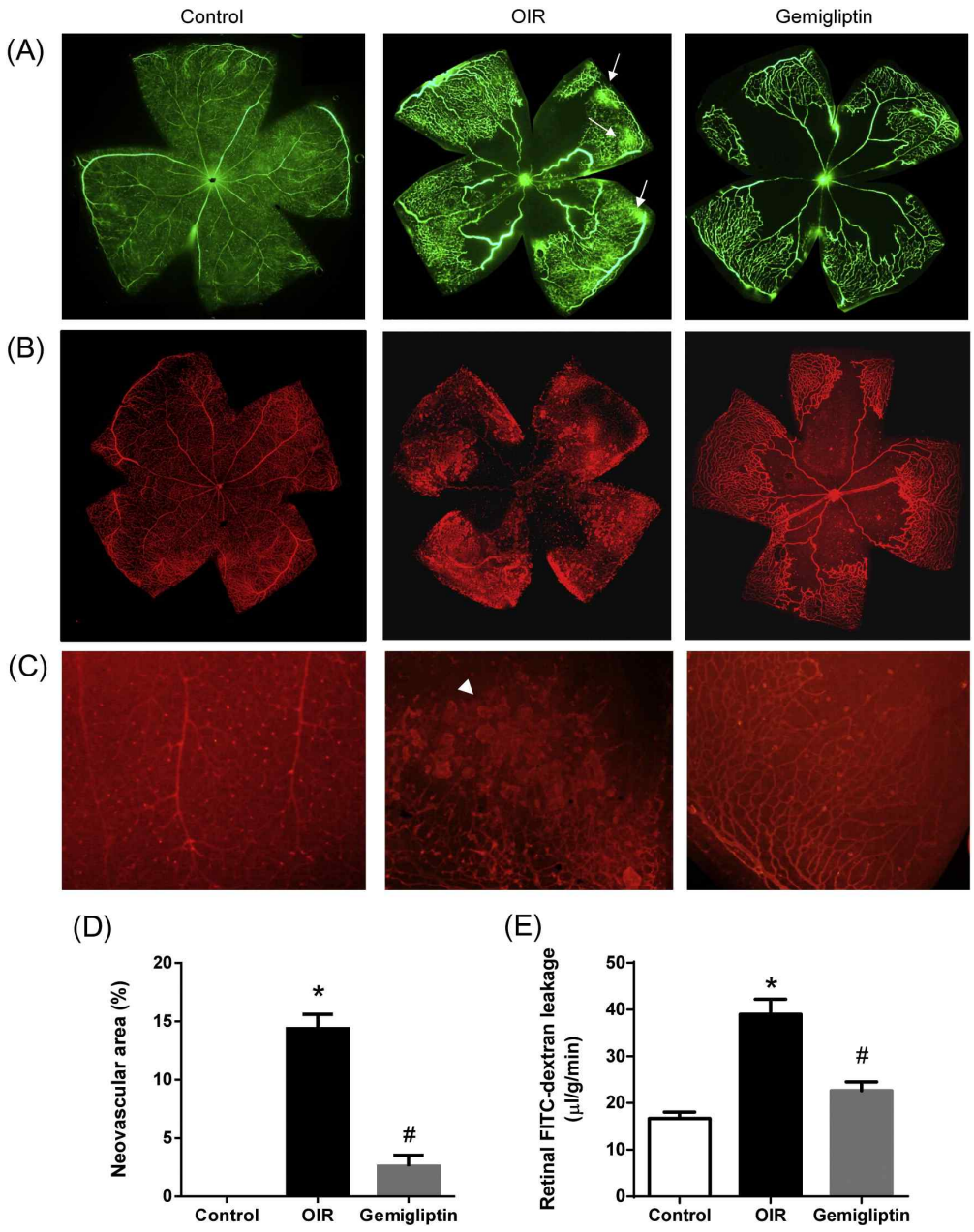


Fig. 4 Effect of gemigliptin on ischemic-induced retinal neovascularization Mice were exposed to 75% oxygen from P7 to

P12. The mice were returned to room air and received an intraperitoneal injection of 50 mg/kg/day of gemigliptin for 5 days. (A) Retinal fluorescein angiography at P17. The arrow indicates the area of retinal vascular leakage. (B) Isolectin B4-stained retinal whole-mounts. (C) High-magnification images for isolectin B4-stained retinas (x100). The arrowhead indicates the area of neovascular tufts. (D) The preretinal neovascular area was measured and normalized to the total retinal area. (E) Quantitative analysis of retinal vascular permeability. Values in the bar graphs represent the mean ± SEM, n = 10. *P < 0.05 vs. normal control mice, #P < 0.05 vs. OIR mice.

3.4 Gemigliptin regulates the expression of angiogenesis-related factors

We investigated the expression levels of 55 angiogenesis-related factors in the retinas using a protein array to evaluate the direct effects of gemigliptin on retinal neovascularization. As shown in Fig. 5, gemigliptin decreased the expression of pro-angiogenic factors [stromal cell-derived factor 1 (SDF-1), PAI-1, monocyte chemoattractant protein-1 (MCP-1), placental growth factor-2 (PGF-2), and VEGF] in the OIR mice compared with the vehicle-treated mice. Among these pro-angiogenic factors, PAI-1 and MCP-1 displayed a >2-fold up-regulation in the OIR group and a <2-fold down-regulation in the gemigliptin group. The expression of insulin-like growth factor binding protein-1 (IGFBP-1), IGFBP-3 and fibroblast growth factor-2 (FGF-2) was significantly increased in the vehicle-treated OIR mice, but these pro-angiogenic factors remained unaffected by gemigliptin treatment. Additionally, platelet factor-4 (PF-4), an anti-angiogenic factor, was also down-regulated by gemigliptin compared to vehicle-treatment in the OIR mice. The up-regulation of anti-angiogenic factors in vehicle-treated OIR mice may have been due to a protective feedback mechanism against angiogenesis. These results indicate that gemigliptin might exert anti-angiogenic effects by inhibiting the expression of SDF-1, PAI-1, MCP-1, PGF-2 and VEGF.

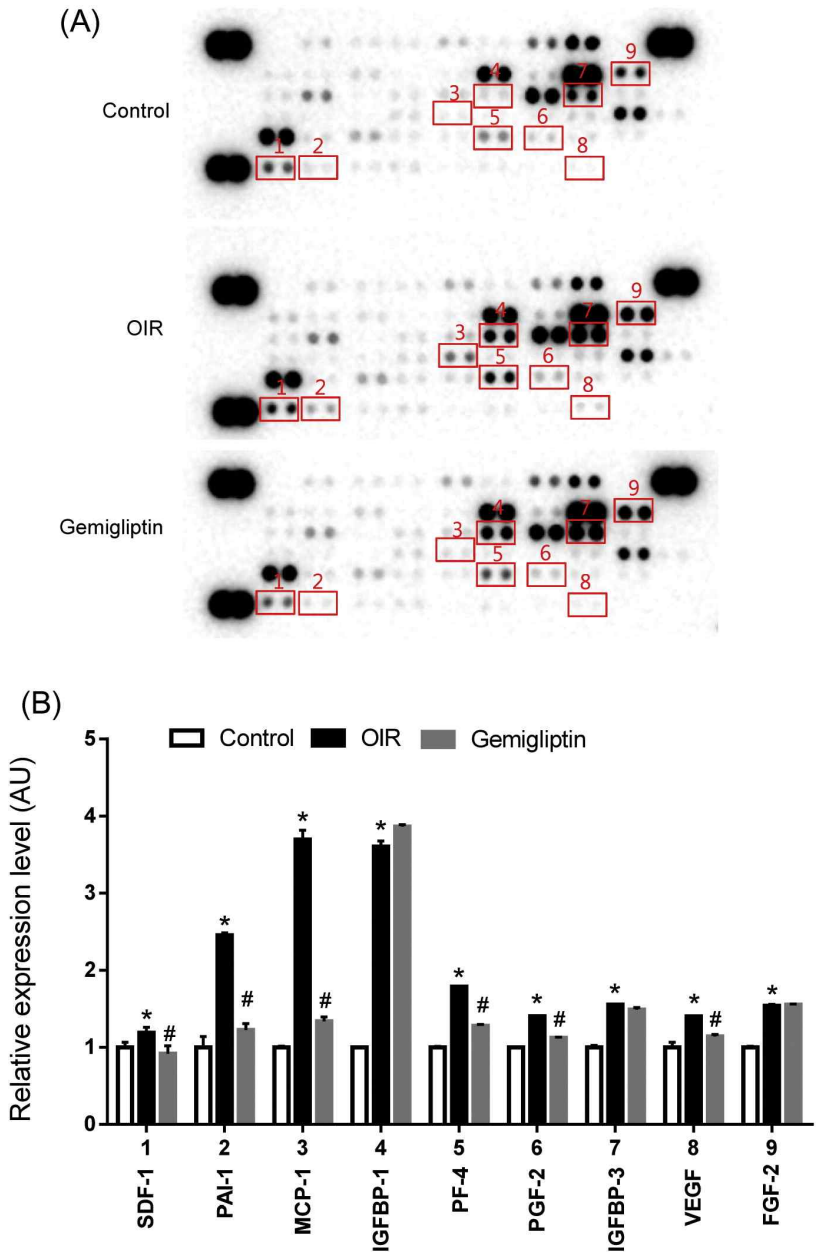


Fig. 5 Effect of gemigliptin on the expression levels of 55 angiogenesis-related proteins The positive controls are located in three corners of the arrays, and the negative control is located in the

lower right corner of the arrays. Modulated proteins in retinas treated with gemigliptin are highlighted with squares and indicated by numbers. The values in the bar graph represent the mean ± SEM, n = 4. *P < 0.05 vs. normal control mice, #P < 0.05 vs. OIR mice.

3.5 Gemigliptin inhibits high glucose-induced pericyte apoptosis *in vitro*

Pericytes were cultured in 30 mmol/L glucose for 1 week with various concentrations of gemigliptin to determine whether gemigliptin directly protects pericytes from apoptosis. The number of apoptotic pericytes was increased under high glucose conditions, but gemigliptin inhibited pericyte apoptosis in a dose-dependent manner (Fig. 6A). Pericyte apoptosis was further examined using TUNEL staining. The TUNEL-positive pericytes were detectable under high glucose conditions, whereas gemigliptin inhibited high-glucose-induced pericyte apoptosis (Fig. 6B). These results support our *in vivo* observation that gemigliptin inhibits pericyte injury in *db/db* mice.

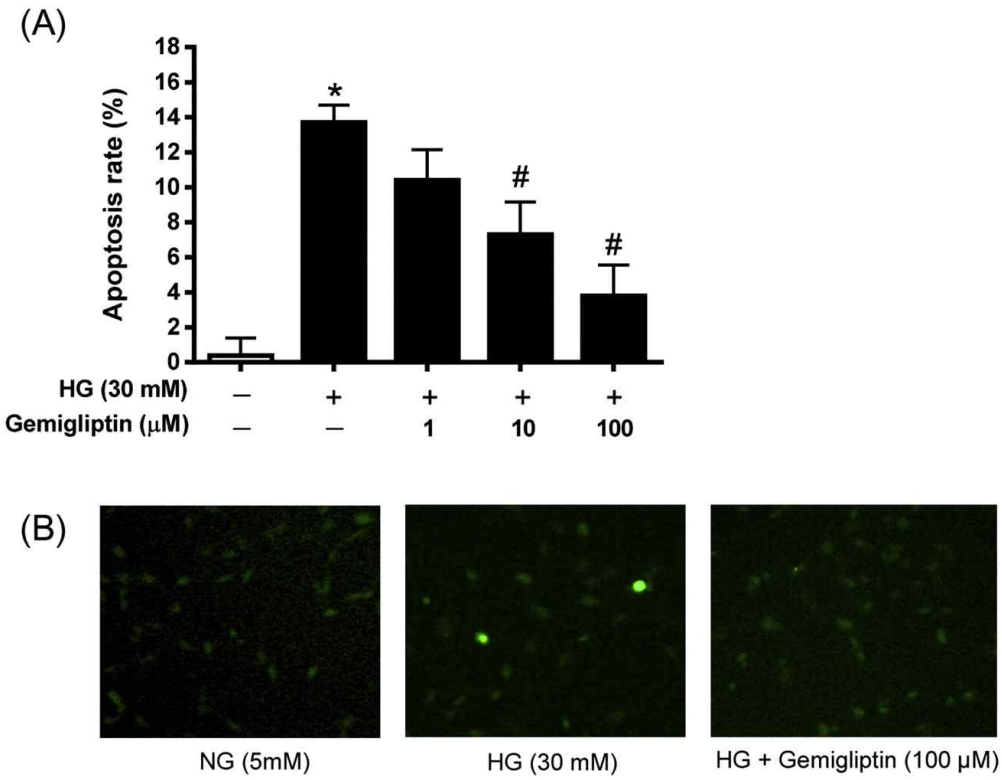


Fig. 6 Gemigliptin inhibits high glucose-induced pericyte apoptosis (A) Primary human retinal pericytes were exposed to high glucose (HG, 30 mmol/L) or normal glucose (NG, 5 mmol/L) for 1 week with or without gemigliptin (0, 1, 10 and 100 μmol/L). Apoptotic cells were detected using an FITC-labeled annexin V protein and flow cytometry. (B) TUNEL staining at 100x magnification. Each bar represents the mean ± SEM from three independent experiments. *P<0.05 vs. normal glucose group, #P<0.05 vs. high glucose group.

3.6 High glucose-induced pericyte apoptosis is synergistically suppressed by a combination treatment using gemigliptin and DPP-4 siRNA

To determine the effect of gemigliptin on DPP-4, plasma DPP-4 activity and retinal DPP-4 protein levels were examined in *db/db* mice. Gemigliptin decreased the activity of plasma DPP-4 to 40% of normal *db/+* mice, corresponding to a 50% decrease compared to *db/db* mice (Fig. 7A). Retinal DPP-4 protein levels were also reduced by gemigliptin treatment compared to vehicle treatment in *db/db* mice (Fig. 7B). However, the retinal DPP-4 protein levels in *db/db* mice might be affected by the extravasation of the soluble form of the protein because retinal vascular permeability was significantly increased in the *db/db* mice and was suppressed by gemigliptin. Moreover, the increased presence of extravasated serum albumin into retinal parenchyma was also observed in *db/db* mice (Fig. 7B).

We next examined DPP-4 protein levels in primary human retinal pericytes under high glucose conditions with or without gemigliptin treatment. DPP-4 is expressed at low levels under normal conditions. However, high glucose induced pericyte apoptosis along with elevated DPP-4 protein levels (Fig. 7C and D). When pericytes were treated with control siRNA or DPP-4 siRNAs, the DPP-4 siRNAs markedly down-regulated DPP-4 expression (Fig. 7C) and prevented high glucose-induced pericyte apoptosis (a 59% reduction compared to the high glucose group, Fig. 7D). Interestingly, a combination of DPP-4 siRNA with gemigliptin induced the enhanced apoptosis inhibition (an 87% reduction compared with the high glucose group) more than either DPP-4 siRNA or gemigliptin alone (a 71% reduction), although

treatment with gemigliptin alone or a combination of siRNA and gemigliptin did not reduce the expression of DPP4 protein more than that of siRNA treatment alone (Fig. 7C and D). These results suggest that the inhibitory effect of gemigliptin on high glucose-induced pericyte apoptosis might be caused not only by its ability to inactivate DPP-4 but also by its off-target effects.

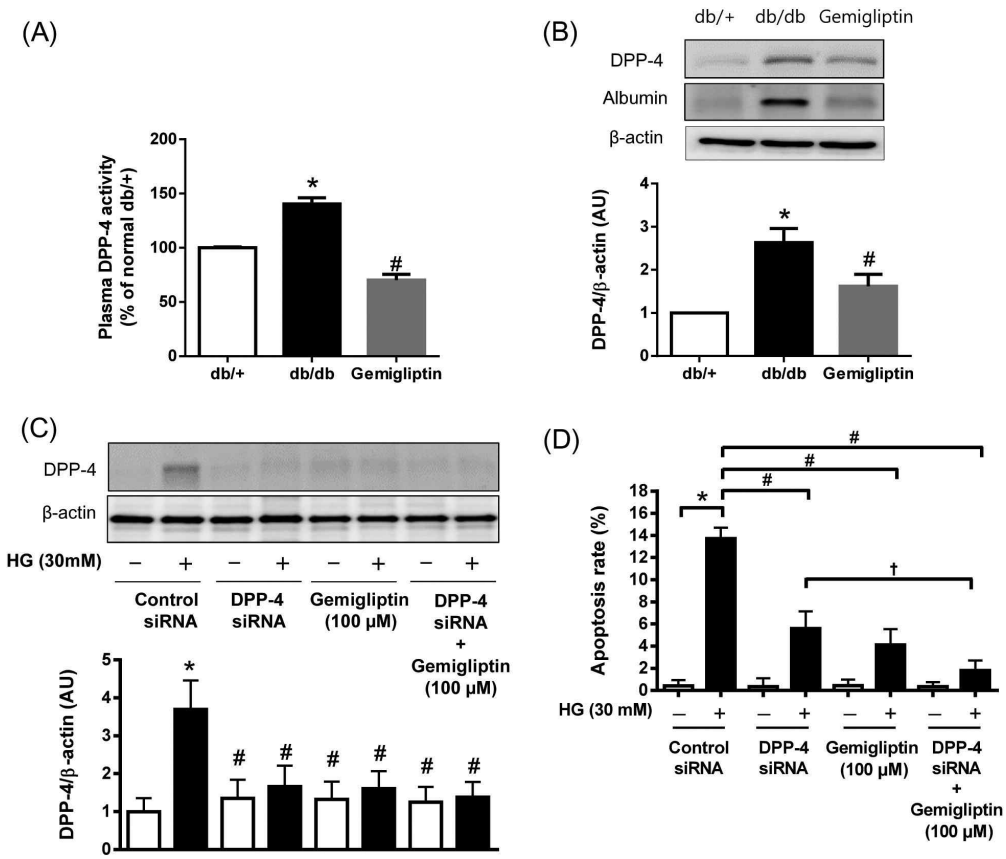


Fig. 7 High glucose-induced pericyte apoptosis is synergistically suppressed by a combination treatment using gemigliptin and DPP-4 siRNA. (A) DPP-4 activity was measured in the plasma. (B) Retinal protein levels of DPP-4 were analyzed by western blotting. Values in the bar graphs represent the mean ± SEM, n = 4. *P<0.05 vs. *db/+* mice, #P<0.05 vs. *db/db* mice. (C) After the pericyte transfection with control siRNA or DPP4 siRNA, Western blot analysis for DPP-4 was performed in pericytes under high glucose (30 mmol/L) with or without gemigliptin (100 μmol/L). (D) Apoptotic cell counts were assessed using FACS analysis. Data represent three

independent experiments. Each bar represents the mean ± SEM from three independent experiments. *P<0.05 vs. normal glucose group, #P<0.05 vs. high glucose group, † P<0.05 vs. DPP-4 siRNA group

3.7 Gemigliptin suppressed PAI-1 expression, which inhibited high glucose-induced pericyte apoptosis

A protein array indicated that gemigliptin markedly suppressed the expression of PAI-1 and MCP-1. MCP-1 expression is increased in both diabetic and ischemic retinopathy. However, despite the pro-angiogenic properties of MCP-1, MCP-1 deficiency did not prevent retinal neovascularization in MCP-/- mice with ischemic retinopathy (Davies et al., 2008). MCP-1 has been shown to contribute to the recruitment of inflammatory cells into the diabetic retina (Funatsu et al., 2005) and indirectly induces apoptosis in retinal pigment epithelial cells by infiltrating inflammatory cells (Yang et al., 2011). Based on these findings, we can exclude the notion of MCP-1 directly promoting retina neovascularization and diabetes-induced pericyte apoptosis. Thus, we examined whether PAI-1 is involved in the inhibitory role of gemigliptin in high glucose-induced pericyte apoptosis to determine the underlying mechanisms by which gemigliptin suppresses diabetic retinopathy. In *db/db* mice, PAI-1 protein was increased 2.3-fold in retinal tissues compared with normal *db/+* mice retinal tissues. Gemigliptin treatment significantly decreased retinal PAI-1 levels (Fig. 8A). Additionally, we examined the localization of PAI-1 in the retinal vasculature using immunostaining to determine whether pericytes are the cellular source of PAI-1. As expected, PAI-1 and NG-2 double-positive pericytes were observed in *db/db* mice (Fig. 8B).

Based on the *in vivo* experiments, we also observed high glucose-induced PAI-1 expression in primary human retinal pericytes (Fig. 8C). When pericytes were transfected with control siRNA or PAI-1 siRNA, the PAI-1 siRNA effectively down-regulated PAI-1

expression (Fig. 8C) and also attenuated high glucose-induced pericyte apoptosis (Fig. 8D). This PAI-1 overexpression and pericyte apoptosis induced by high glucose was also significantly inhibited by gemigliptin (Fig. 8C and D). These data suggest that the effect of gemigliptin on high glucose-induced pericyte apoptosis may be mediated at least in part through the inhibition of PAI-1.

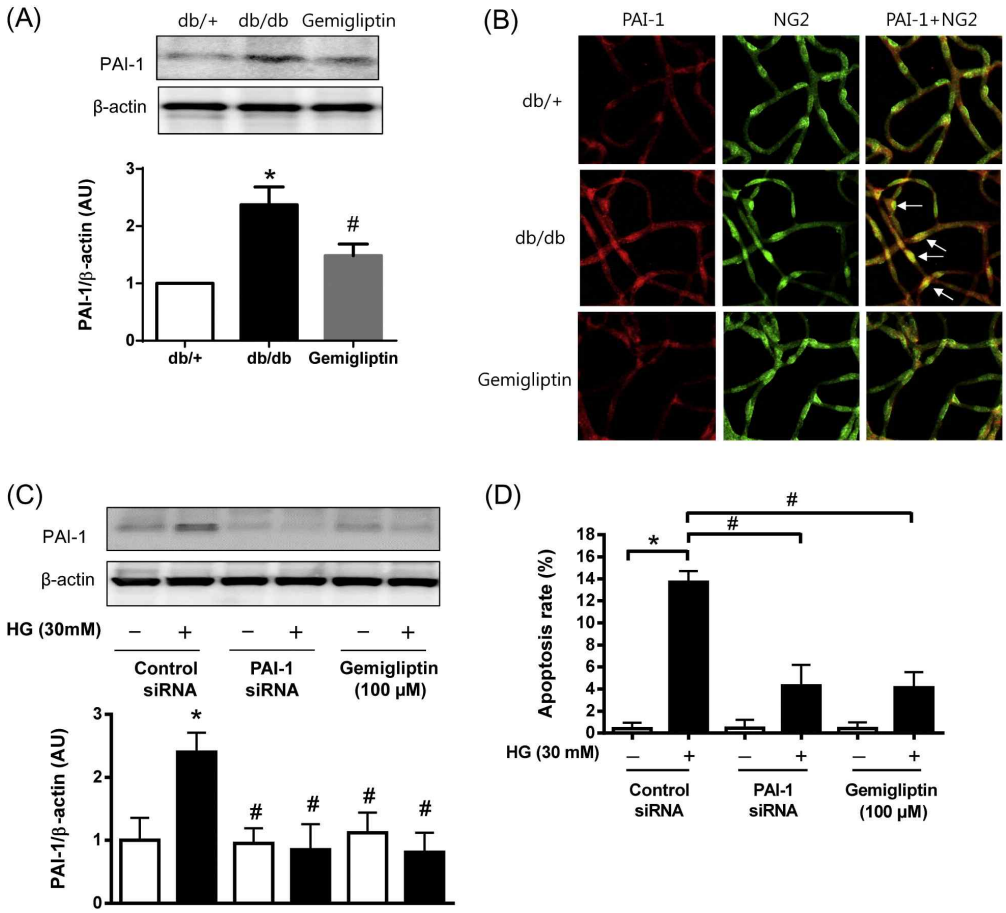


Fig. 8 Gemigliptin suppressed PAI-1 expression, which inhibited high glucose-induced pericyte apoptosis. (A) Retinal protein levels of PAI-1 were analyzed by Western blotting. Values in the bar graphs represent the mean ± SEM, n = 4. *P<0.05 vs. *db/+* mice, #P<0.05 vs. *db/db* mice. (B) Immunofluorescence staining for PAI-1 (red) and NG2 (green) in retinal vessels. White arrows indicate PAI-1-expressed pericytes. (C) After the pericyte transfection with control siRNA or PAI-1 siRNA, Western blot analysis for PAI-1 was performed in pericytes under high glucose (30 mmol/L) with or

without gemigliptin (100 μmol/L). (D) Apoptotic cell counts were assessed by FACS analysis. Data represent three independent experiments. Each bar represents the mean ± SEM from three independent experiments. *P<0.05 vs. normal glucose group, #P<0.05 vs. high glucose group.

4. Discussion

Few experimental studies have demonstrated the effect of DPP-4 inhibitors on diabetic retinopathy. One small clinical study in type 2 diabetic patients without retinopathy demonstrated that a DPP-4 inhibitor improved retinal capillary blood flow and vasodilation (Ott et al., 2014). Here, we provide the first evidence that gemigliptin has preventive effects on diabetes-induced pericyte injury and ischemia-induced retinal neovascularization. Furthermore, we demonstrated that the effects of gemigliptin on retinal vascular alterations can be achieved without a reduction of hyperglycemia in *db/db* mice or nondiabetic OIR mice, suggesting that gemigliptin's protective effects are independent of its effects on glucose homeostasis. More importantly, our results suggest that the preventive effects of gemigliptin on diabetic retinopathy occur through a PAI-1 dependent mechanism.

In the present study, gemigliptin did not reduce blood glucose levels in *db/db* mice. Similarly, a previous report showed that alogliptin did not exhibit beneficial effects on blood glucose in *db/db* mice aged between 8 and 16 weeks. This ineffectiveness of DPP-4 inhibitors on hyperglycemia in this animal model can be explained by the reduction in GLP-1 receptor expression in pancreas (Kawashima et al., 2011). Therefore, we investigated the retinoprotective possibility of gemigliptin in this animal model of diabetes independent of glucose levels.

DPP-4 inhibition has beneficial effects on the vasculature (Shah et al., 2011), heart (Bostick et al., 2014) and brain (Kosaraju et al., 2013). Recently, Goncalves et al. reported that DPP-4 inhibition also has beneficial effects in retinal vessels and neurons (Goncalves et al., 2014). DPP-4 is widely expressed in various cells types (Barnett,

2006) and in vascular endothelial cells (Ludwig et al., 2002) and pericytes (Avolio et al., 2015). Regarding the retina, the expression of DPP-4 protein was enhanced in the retinal tissues of STZ-induced diabetic rats, and immunofluorescence staining showed that it was mainly expressed in ganglion cells (Goncalves et al., 2014). Avolio et al. reported that DPP-4 mRNA was expressed at relatively low levels in pericytes under normal conditions (Avolio et al., 2015). Consistent with this report, our results showed that retinal pericytes have relatively low expression levels of DPP-4 under normal conditions, but high glucose induces a significant increase in DPP-4. Gemigliptin prevented the increase in DPP-4 protein in pericytes with high glucose. However, it has been claimed that the beneficial effect of DPP4 inhibitors in the retina is mainly due to the inhibition of DPP-4. Our study clearly showed that high glucose-induced pericyte apoptosis was prevented by siRNA-mediated DPP-4 reduction. In particular, a combination of DPP-4 siRNA with gemigliptin is more effective than either DPP-4 siRNA or gemigliptin alone, although gemigliptin alone or a combination of siRNA and gemigliptin did not reduce the expression of DPP4 proteins more than that of siRNA treatment alone. Although we could not measure DPP-4 enzyme activity in the pericytes due to the technical limitations of the assay sensitivity (data not shown), our results provide the initial evidence that gemigliptin exerts a DPP-4 inhibitory action as well as additional off-target activities in retinas.

The retina has the highest pericyte density among all vascular beds (Sims, 2000). Vasoregression in diabetic retinopathy starts with pericyte loss (Hammes et al., 2011). Moreover, the loss of pericytes leads to increased vascular permeability resulting in vascular leakage and macular edema (Shin et al., 2014). A loss of pericytes in diabetic

retinopathy also triggers the development of acellular capillaries and capillary nonperfusion, which leads to retinal ischemia and sight-threatening neovascularization (Arboleda-Velasquez et al., 2015). Pericyte loss also occurs in diabetic rodent models. Although it has been known that pericytes begin to die relatively early in the course of diabetic retinopathy, the time of onset of pericyte loss in *db/db* mice is a controversial issue. Twenty three-week-old *db/db* mice have been shown to exhibit an increase in E/P ratio and acellular capillaries (Midena et al., 1989), and a more than 25% decrease in pericyte density was observed after 15 months of diabetes in *db/db* mice (Cheung et al., 2005). TUNEL-positive apoptotic pericytes were found in 18-week-old *db/db* mice (Zhang et al., 2013). In this study, we terminated gemigliptin treatment in *db/db* mice at 20 weeks of age. Similar to previous studies, pericyte loss was already visible at this early time point.

Pericytes may be a valid target for the treatment of diabetic retinopathy. Here, we hypothesized that the reduction in PAI-1 by gemigliptin may contribute to the inhibition of pericyte loss and retinal neovascularization. To test the hypothesis, we examined the pathogenic role of PAI-1 in human primary retinal pericytes under high glucose conditions in the presence or absence of gemigliptin. The current study demonstrates that both diabetic retinas in *db/db* mice and ischemic retinas in OIR mice exhibited the PAI-1 overexpression. Gemigliptin treatment restored its expression to near-normal levels in these animals, in parallel with a marked inhibition in pericyte injury, vascular leakage and retinal neovascularization. In addition, the down-regulation of PAI-1 with siRNA ameliorated high glucose-induced pericyte apoptosis in vitro. Consistent with these results, the reduction of PAI-1 by gemigliptin

treatment also attenuated pericyte injury under high glucose conditions. These findings provide evidence that overexpressed PAI-1 in retinal tissues confers its pro-apoptotic and angiogenic effects in the retina, and gemigliptin inhibits the up-regulation of PAI-1, which may account for its beneficial effect in diabetic retinopathy and ischemia-induced retinopathy.

PAI-1 is a primary regulator of fibrinolysis and is a biosynthetic product of retinal endothelial cells (Munjal et al., 1994) and pericytes (Canfield et al., 1989). PAI-1 plays an important role in the development of diabetic retinopathy. High levels of PAI-1 have been observed in serum (Mansouritorghabe et al., 2013), vitreous (Hattenbach et al., 1999) and retinal microvasculature (Grant et al., 1996) of patients with diabetes. Furthermore, the retinal vasculature of transgenic mice that overexpress PAI-1 exhibited an increase in the basal membranes and E/P ratio, similar to diabetic retinopathy (Grant et al., 2000). In an animal model of laser-induced choroidal neovascularization, PAI-1 deficiency inhibited subretinal neovascularization in PAI-$1^{-/-}$ mice, and the restoration of PAI-1 expression by an adenoviral vector expressing human PAI-1 cDNA induced subretinal neovascularization (Lambert et al., 2001). This finding is similar to the results of ischemia-induced retinal neovascularization. The loss of PAI-1 reduced retinal neovascularization in PAI-$1^{-/-}$ mice with OIR (Basu et al., 2009). In contrast, the intravitreal injection of exogenous human PAI-1 protein in rats with retinopathy of prematurity inhibited retinal neovascularization (Penn and Rajaratnam, 2003). This paradoxical role of PAI-1 in angiogenesis is dependent on the cell type and PAI-1 concentration (Balsara and Ploplis, 2008). High levels (micromolar) of PAI-1 prevented angiogenesis, whereas low levels (physiological

levels; nanomolar) of PAI-1 conversely facilitated angiogenesis (Devy et al., 2002). Taken together with results observed previously in proliferative retinopathy models, our results confirm that PAI-1 has pro-angiogenic activity in retinal tissues under pathological conditions, such as diabetes and hypoxia.

Additionally, apart from modulating angiogenesis, experimental studies have shown that PAI-1 has both deleterious and beneficial effects on apoptosis. PAI-1 induces apoptosis in vascular endothelial cells (Al-Fakhri et al., 2003), whereas anti-apoptotic effects also have been observed in these cells (Chen et al., 2004). However, to date, there have been no reports on the exact role of PAI-1 in retinal pericytes. We demonstrated that pericyte loss occurred with PAI-1 increases in diabetic mice retinas, indicating that pericyte loss may be partially correlated with PAI-1. Consistent with a previous report (Kane et al., 2005), we also showed that high glucose induced an up-regulation of PAI-1 in retinal pericytes. The down-regulation of PAI-1 with siRNA suppressed high glucose-induced pericyte apoptosis. These results suggest that PAI-1 down-regulation might be helpful for the prevention of diabetic retinopathy. Although we did not provide concrete evidence of whether PAI-1 deficiency actually ameliorates diabetic retinopathy in animal models, the down-regulation of PAI-1 by gemigliptin reduced high-glucose-induced pericyte apoptosis both in vitro and in vivo.

Gemigliptin has been used clinically to treat type 2 diabetes. Our surprising finding of direct ocular effects of gemigliptin on diabetic retinopathy and ischemia-induced retinopathy provides evidence that the oral administration of gemigliptin may be a promising therapeutic approach to diabetic retinopathy. An oral dose of 50 mg/day gemigliptin is recommended for glycemic control in diabetic patients.

Although we used a relatively high dose of gemigliptin in animal models, a dose of 50 mg/day gemigliptin may be sufficient to obtain the same relative beneficial effects on diabetic retinopathy in human subjects. The elimination half-life of gemigliptin was shown to be 17.1 h (Lim et al., 2008). The elimination half-life of gemigliptin in *db/db* mice was 1.5 h (our unpublished data). Due to its short half-life in *db/db* mice, we selected an oral dose of 100 mg/kg gemigliptin for this animal model. Moreover, *db/db* mice are obese, and their body surface area is approximately 2 times larger than that of normal C57BL/6 mice (Cheung et al., 2009). Thus, we also selected an oral dose of 50 mg/kg gemigliptin for the OIR mice.

In summary, our study demonstrated that gemigliptin has both anti-apoptotic and anti-angiogenic effects in the retinas of diabetic mice and ischemia-induced retinopathy mice through the down-regulation of PAI-1. These novel findings provide insight into the retinoprotective effects of gemigliptin that are independent of glycemic control.

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국문 초록

DPP-4 억제제의 당뇨 합병증에서 다양한 혈당 비의존성 기전 연구

정 은 수
서울대학교 수의과대학
수의학과 수의병인생물학 및 예방수의학(독성학) 전공

2006년 Januvia의 미국 허가를 시작으로, incretin 기반의 2형 당뇨 치료제는 매해 큰 성장을 거듭하며 당뇨 치료를 위한 처방 2차 약제로 널리 사용되고 있다. DPP-4 (dipeptidyl peptidase-4) 억제제는 해당 효소를 억제하여, DPP-4 에 의해 분해되는 GLP-1 의 기능 연장을 이용한 혈당강하 효과를 기반으로 하는데, DPP-4 억제제들은 혈당강하 효능 외에도 당뇨 합병증에 대한 긍적적인 효능이 일부 보고되었다. 따라서 본 연구에서는 DPP-4 억제제중 하나인 gemigliptin을 이용하여 혈당 강하 약효와 무관한 직접적인 당뇨병성 신증 및 망막증의 예방 효능을 확인하였으며, 나아가 gemigliptin의 혈당강하 외 신규 작용 기전을 규명하였다.

첫 번째 보호 기전은, 고혈당 상태에서 형성되는 advanced glycation end product 형성을 gemigliptin이 직접적으로 억제하였다. Methyglyoxal modified AGE-bovine serum albumin formation, AGE-collagen cross-linking assay 에서 기존 알려진 AGE 형성 억제제 (aminoguanidine)보다 강력한 효과가 확인되었다. 제2형 당뇨모델 (*db/db* 마우스)에서도 gemigliptin 12주 투약 후 AGE serum level은 줄어들었다.

두 번째로는 제2형 당뇨모델(*db/db*마우스)에서 gemigliptin 12주 투약 후 혈당 강하와 상관없이 당뇨병성 신증을 보호하는 효과를 확인하였다. 당뇨병성 신증에서 확인되는 albuminuria 억제, DPP-4 효소 활성 및 발현 억제, podocyte apoptosis (8-hydroxydeoxyguanosine 감소)가 억제되었고, 보호 기전으로 oxidative damage 억제(advanced oxidation protein products 감소), RAGE 와 ILK 발현 억제 (receptor for advanced glycation end products, integrin-linked kinase)가 확인되었다.

세 번째로는 당뇨병성 망막증의 여러 요인 중, 2형 당뇨 모델 *db/db* 마우스에서 retinal vascular leakage를 억제하는 것과 OIR (oxygen induced retinopathy) 모델에서 망막 신생혈관생성을 억제하는 기전을 밝혔다. 고혈당 상태인데도 gemigliptin을 투약한 그룹에서는 retinal pericyte apoptosis 와 vascular leakage 가 감소되었고, OIR 모델에서는 여러angiogenesis related protein의 발현이 줄어드는 것을 확인하였는데, 특히 PAI-1 (plasminogen activator inhibitor-1)의 발현이 gemigliptin에 의해 현저히 억제되는 것을 확인하였다. 또한 *in vitro* cultured pericyte 에서 high-glucose에 의한 pericyte의 apoptosis가 PAI-1 siRNA에 의해 억제되는 것을 확인하였으며, 이러한 현상이 gemigliptin에 의해서도 동일하게 관찰되었다. 따라서 본 연구를 통해 gemigliptin의 anti-angiogenic, anti-apoptotic activities를 처음 규명하였다.

Gemigliptin의 이러한 microvascular 에서의 anti-glycation, anti-oxidant, anti-apoptotic, anti-angiogenic 기능은 당뇨환자에서의 고혈당으로 인한 vascular damage의 많은 부분을 보호할 수 있는 기전으로 생각되고, 혈당을 줄이는 1차 endpoints 외에 추가적인 장점이 될 수 있다. 따라서, 본 연구 결과를 바탕으로 많은 임상 시험들을 통해, 당뇨 환자에서 실질적인 benefits으로 구현될 수 있기를 기대한다.

핵심 단어: 2형 당뇨, DPP-4 억제제, 당뇨 합병증, 당뇨병성 신증, 당뇨병성 망막증
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