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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. 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.

1. Introduction

Diabetic retinopathy is a common and serious microvascular complication of diabetes [1]. The normal function of retinal vessels requires interactions between the inner endothelial cells and surrounding pericytes [2]. Pericyte loss is one of the earliest detectable lesions in the diabetic retina [3] and may be responsible for the retinal vascular leakage and sight-threatening neovascularization [4]. Pericytes express several angiogenic growth factors, which are critical for determining whether vessels are stable or will undergo angiogenesis [5]. 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 [6,7]. 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 [7]. Indeed, although many classes of glucose-lowering oral agents have been available for clinical use, the prevalence of diabetic retinopathy is increasing [8].

Abbreviations: ROS, reactive oxygen species; DPP-4, dipeptidyl peptidase-4; GLP-1, glucagon-like peptide-1; VEGF, vascular endothelial growth factor; ICAM-1, intercellular adhesion molecule-1; PAI-1, plasminogen activator inhibitor-1; PEDF, pigment epithelium-derived factor; OIR, oxygen-induced retinopathy; db/db, C57BL/KsJ-Lepr$^{db/db}$ diabetic mice; db/+, nondiabetic heterozygous littermates; P, postnatal day; PBS, phosphate-buffered saline; E/P, endothelial cell/pericytes; DAPI, diamidinophenylindole; FITC, fluorescein isothiocyanate; PI, propidium iodide; SEM, standard error of the mean; SDF, stromal cell-derived factor-1; MCP-1, monocyte chemoattractant protein-1; PGF-2, placental growth factor-2; IGFBP, insulin-like growth factor binding protein; FGF-2, fibroblast growth factor-2; PF-4, platelet factor-4.

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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 [9]. Recently, Blaslov et al. reported that circulating DPP-4 activity is associated with the progression and severity of diabetic retinopathy [10]. 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 [11,12]. 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 [13]. Saxagliptin improved retinal capillary flow in diabetic patients without the clinical signs of microvascular alteration [14]. 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 [15]. 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 [16–19]. 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-Lepr$^{db/db}$ 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 [20]. 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 [21]. To determine the E/P ratio, the total numbers of pericytes and endothelial cells were counted in 10 randomly selected areas (magnification 400×) 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) × 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 10,000 ×*g* for 5 min, 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.

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

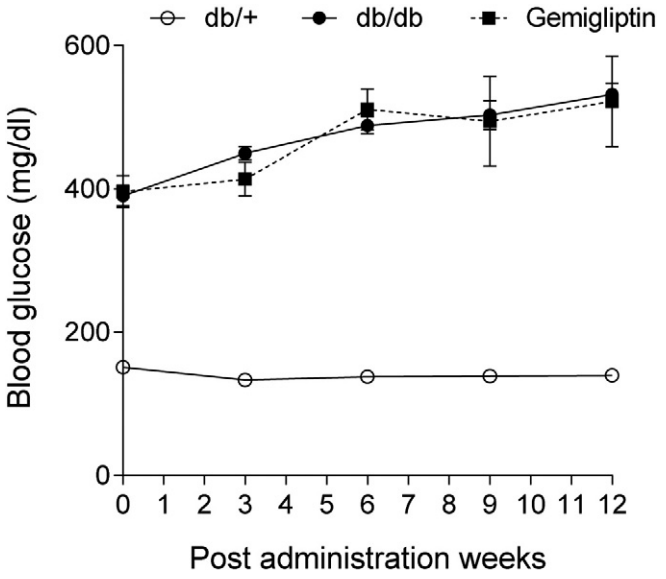

Fig. 1. Blood glucose levels. Values are expressed as the mean ± SEM, n = 10.

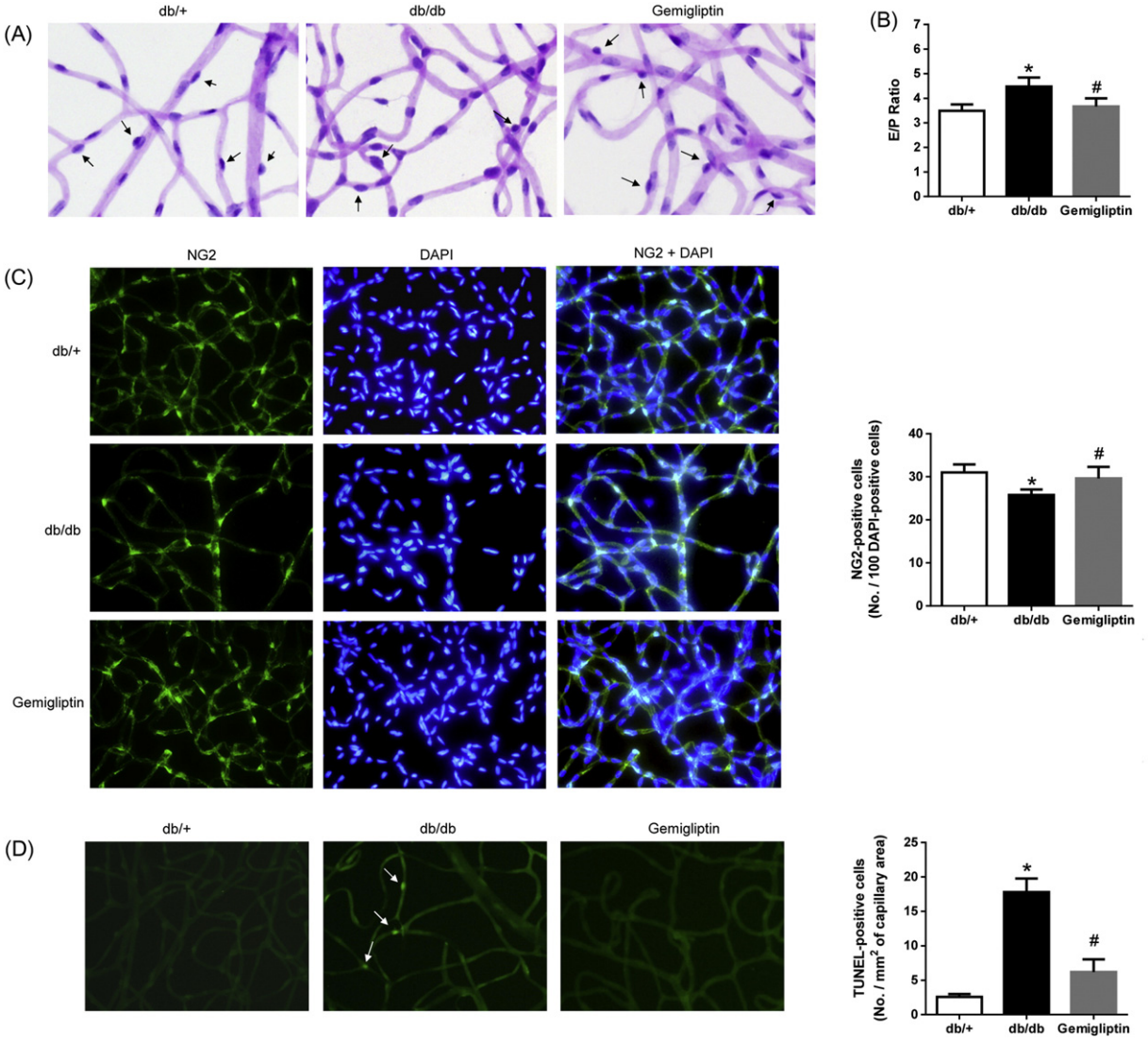

Fig. 2. Inhibitory effects of gemigliptin on diabetes-induced pericyte loss. (A) Periodic acid Schiff and hematoxylin-stained retinal digested microvessels. Pericytes were identified in retinal digest preparations based on morphologic criteria (shape, staining intensity, and relative position in the 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.

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).

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).

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

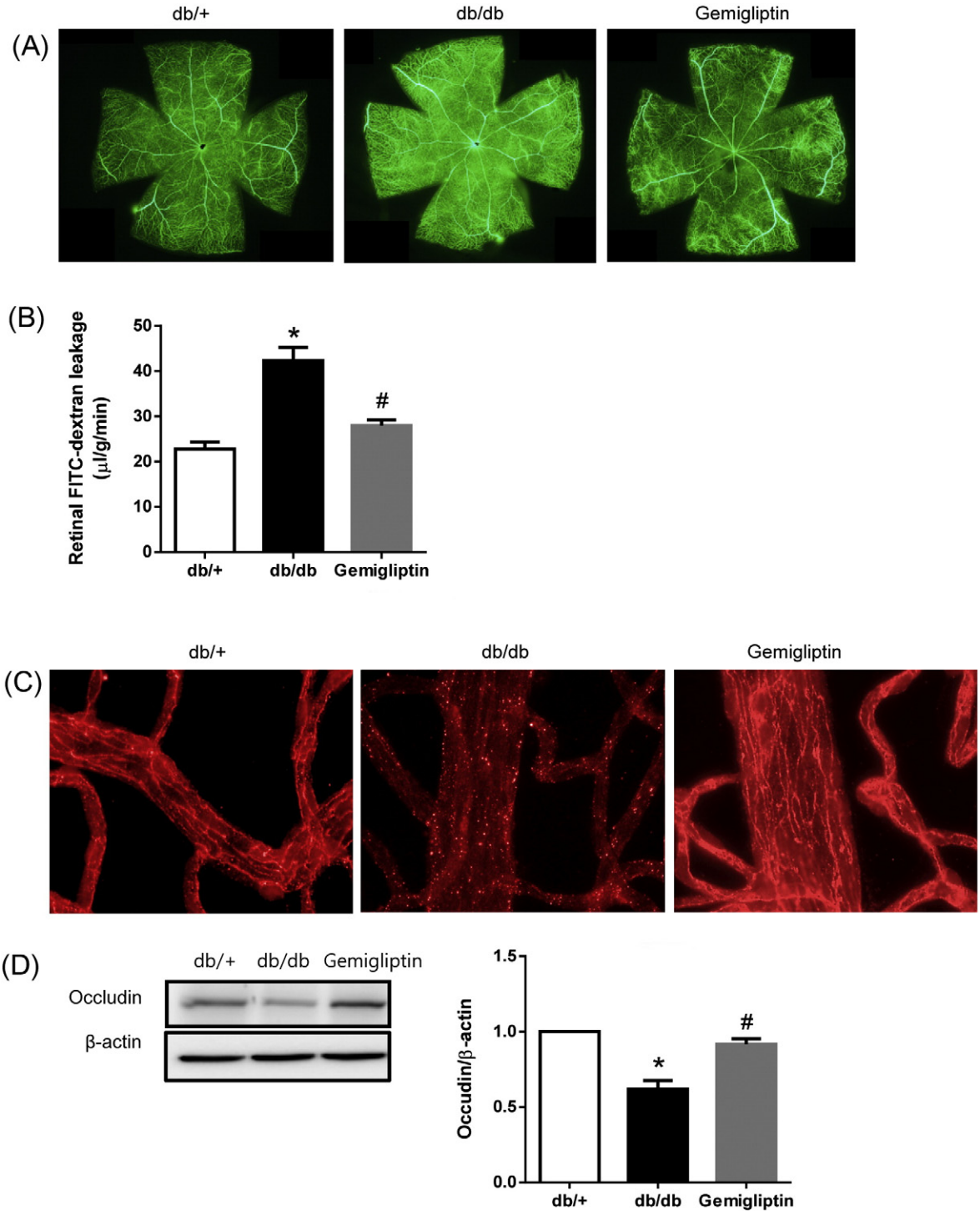

Fig. 3. Inhibitory effects of gemigliptin on diabetes-induced blood–retinal barrier breakdown. (A) FITC–dextran angiography on retinal flat mounts. (B) Quantitative analysis of retinal vascular permeability. (C) Immunofluorescence staining for occludin protein in retinal whole mounts. In normal db/+ 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.

(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.

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

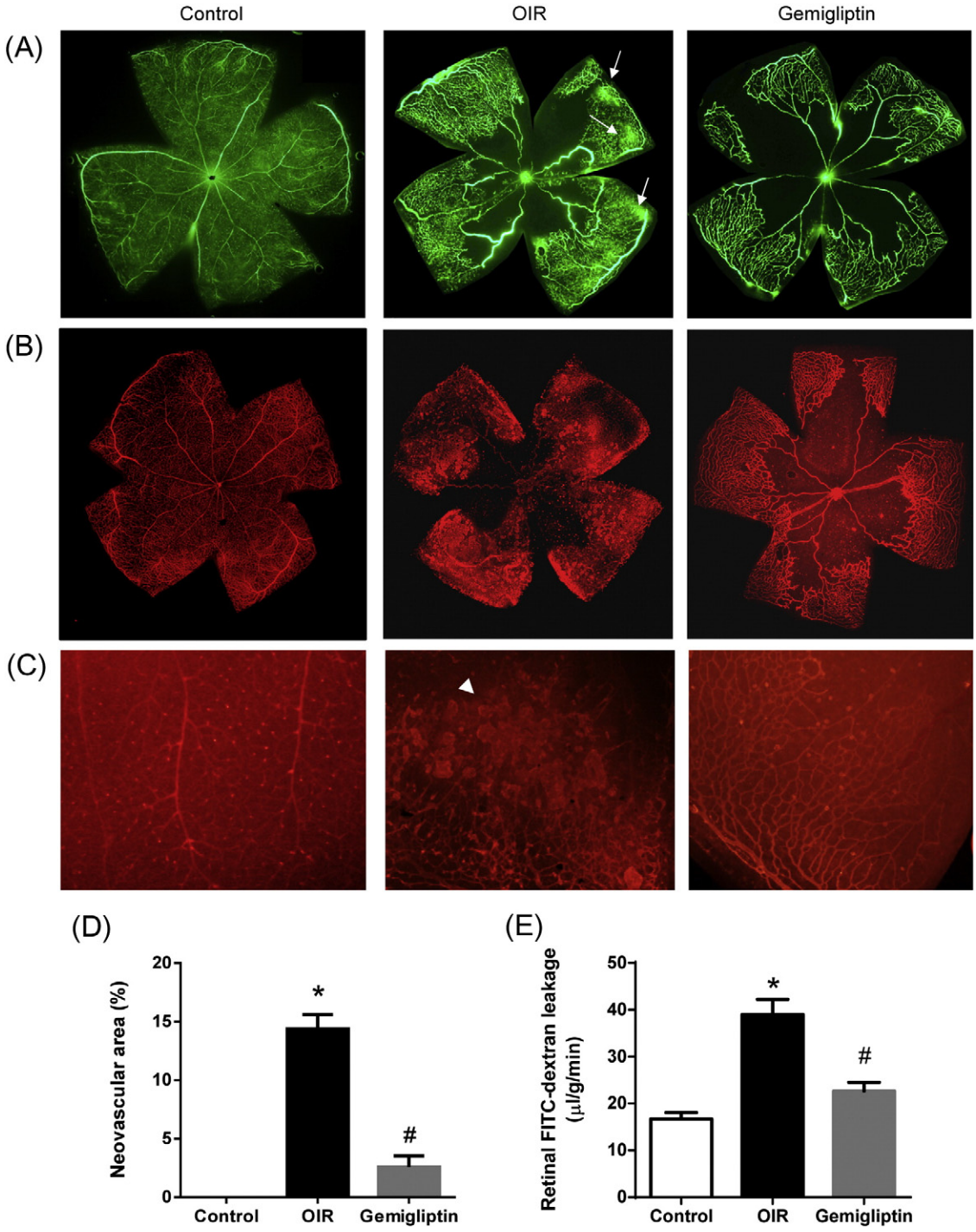

Fig. 4. Effect of gemigliptin on ischemia-induced retinal neovascularization. Mice were exposed to 75% oxygen from P7 to P12. The mice were returned to room air and received an intraperitoneal injection of 50 mg/kg/day of gemigliptin for 5 days. (A) Retinal fluorescein angiography at P17. The arrow indicates the area of retinal vascular leakage. (B) Isolectin B4-stained retinal whole-mounts. (C) High-magnification images for isolectin B4-stained retinas (×100). 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.

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.

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

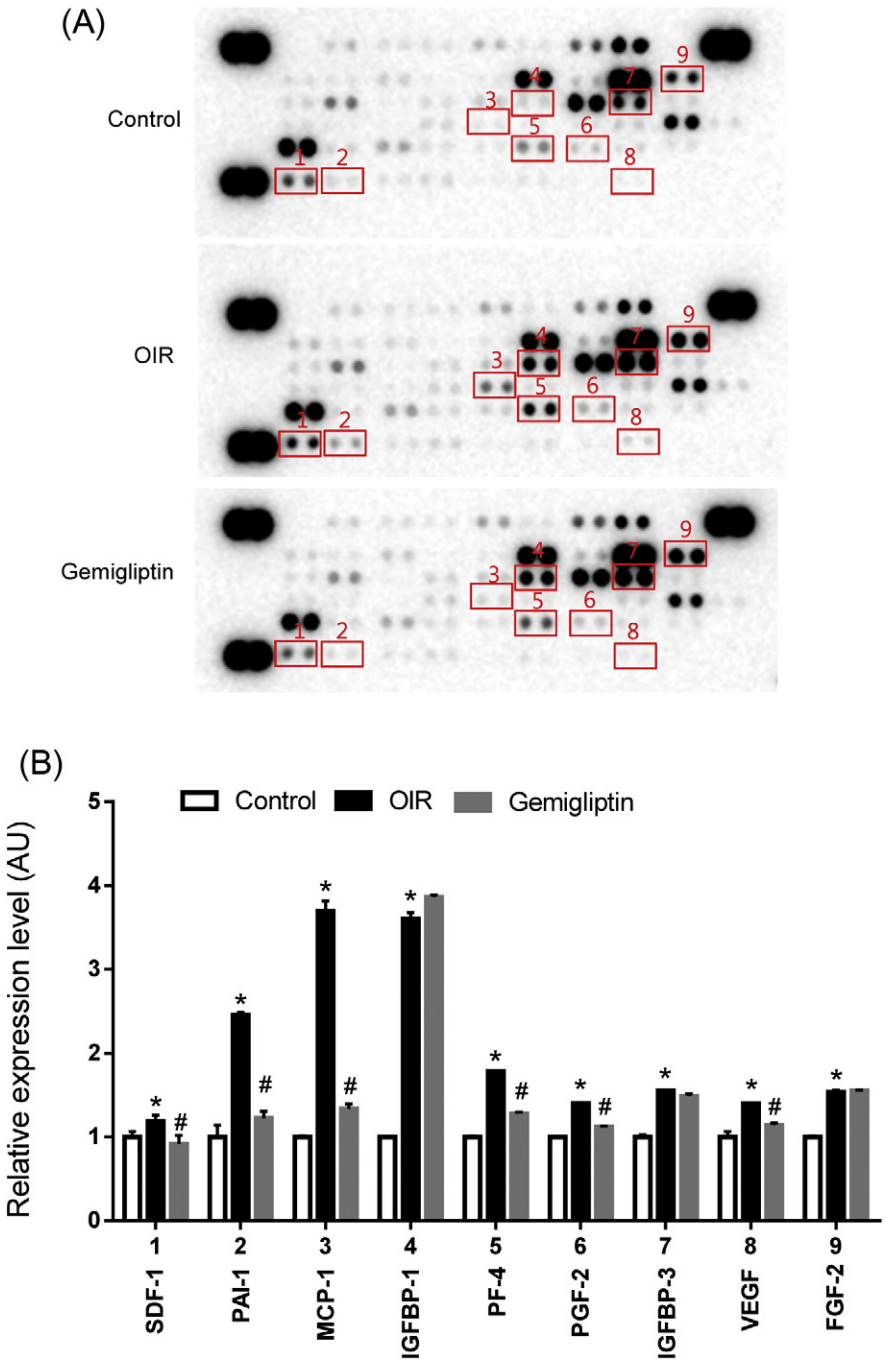

Fig. 5. Effect of gemigliptin on the expression levels of 55 angiogenesis-related proteins. The positive controls are located in three corners of the arrays, and the negative control is located in the lower right corner of the arrays. Modulated proteins in retinas treated with gemigliptin are highlighted 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.

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 DPP-4 protein more than that of siRNA treatment alone (Fig. 7C and D). These results suggest that the inhibitory effect

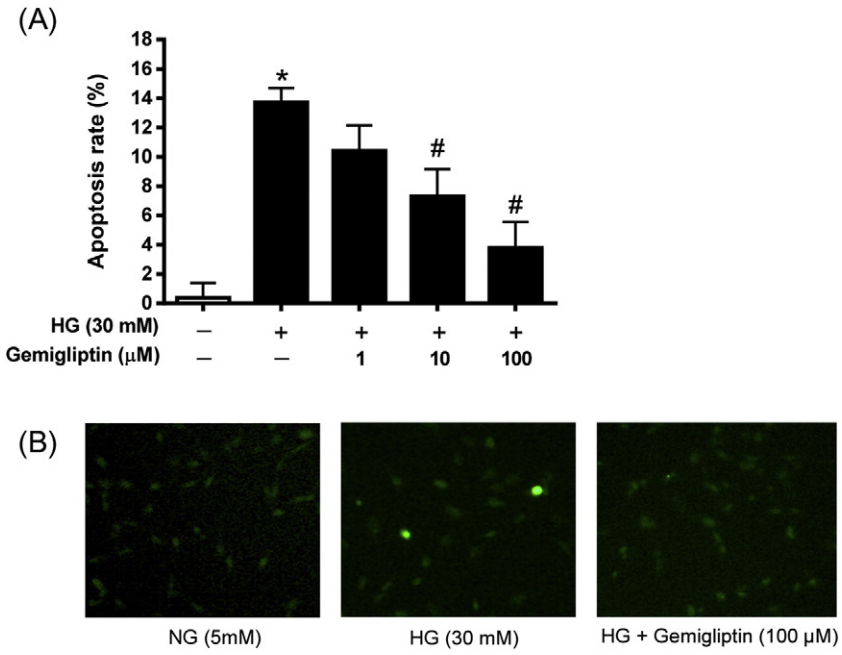

Fig. 6. Gemigliptin inhibits high glucose-induced pericyte apoptosis. (A) Primary human retinal pericytes were exposed to high glucose (HG, 30 mmol/L) or normal glucose (NG, 5 mmol/L) for 1 week with or without gemigliptin (0, 1, 10 and 100 μmol/L). Apoptotic cells were detected using an FITC-labeled annexin V protein and flow cytometry. (B) TUNEL staining at 100× 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.

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.

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 [22]. MCP-1 has been shown to contribute to the recruitment of inflammatory cells into the diabetic retina [23] and indirectly induces apoptosis in retinal pigment epithelial cells by infiltrating inflammatory cells [24]. 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.

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 [14]. 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 [25]. 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 [26], heart [27] and brain [28]. Recently, Goncalves et al. reported that DPP-4 inhibition also has beneficial effects in retinal vessels and neurons [11]. DPP-4 is widely expressed in various cells types [29] and in vascular endothelial cells [30] and pericytes [31]. 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 [11]. Avolio et al. reported that DPP-4 mRNA was expressed at relatively low levels in pericytes under normal conditions [31]. 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 DPP-4 inhibitors in the retina is mainly due to the inhibition of DPP-4.

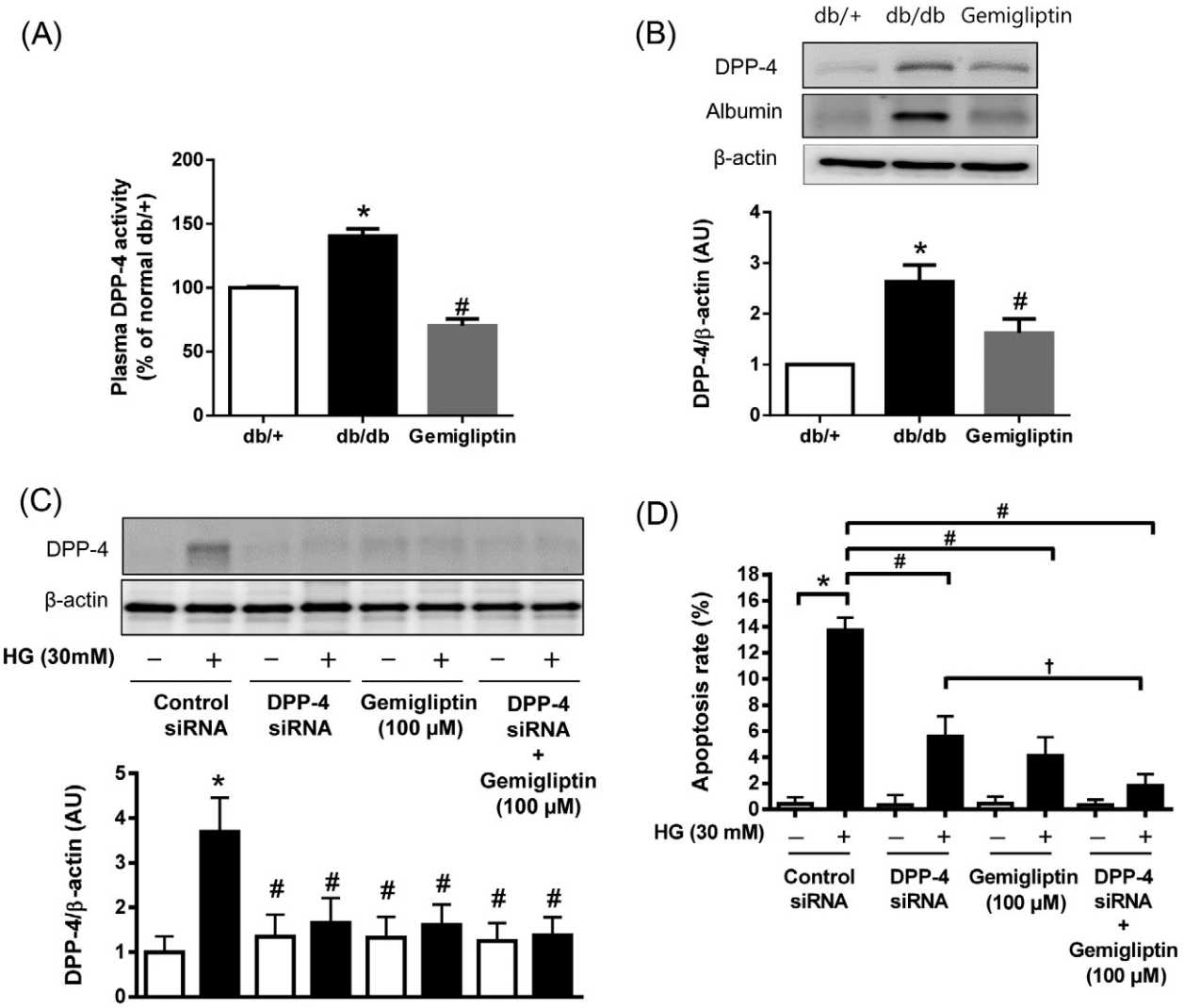

Fig. 7. High glucose-induced pericyte apoptosis is synergistically suppressed by a combination treatment using gemigliptin and DPP-4 siRNA. (A) DPP-4 activity was measured in the plasma. (B) Retinal protein levels of DPP-4 were analyzed by Western blotting. Values in the bar graphs represent the mean ± 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 DPP-4 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.

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 DPP-4 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 [32]. Vasoregression in diabetic retinopathy starts with pericyte loss [33]. Moreover, the loss of pericytes leads to increased vascular permeability resulting in vascular leakage and macular edema [34]. 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 [4]. 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 [21], and a more than 25% decrease in pericyte density was observed after 15 months of diabetes in db/db mice [35]. TUNEL-positive apoptotic pericytes were found in 18-week-old db/db mice [36]. 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,

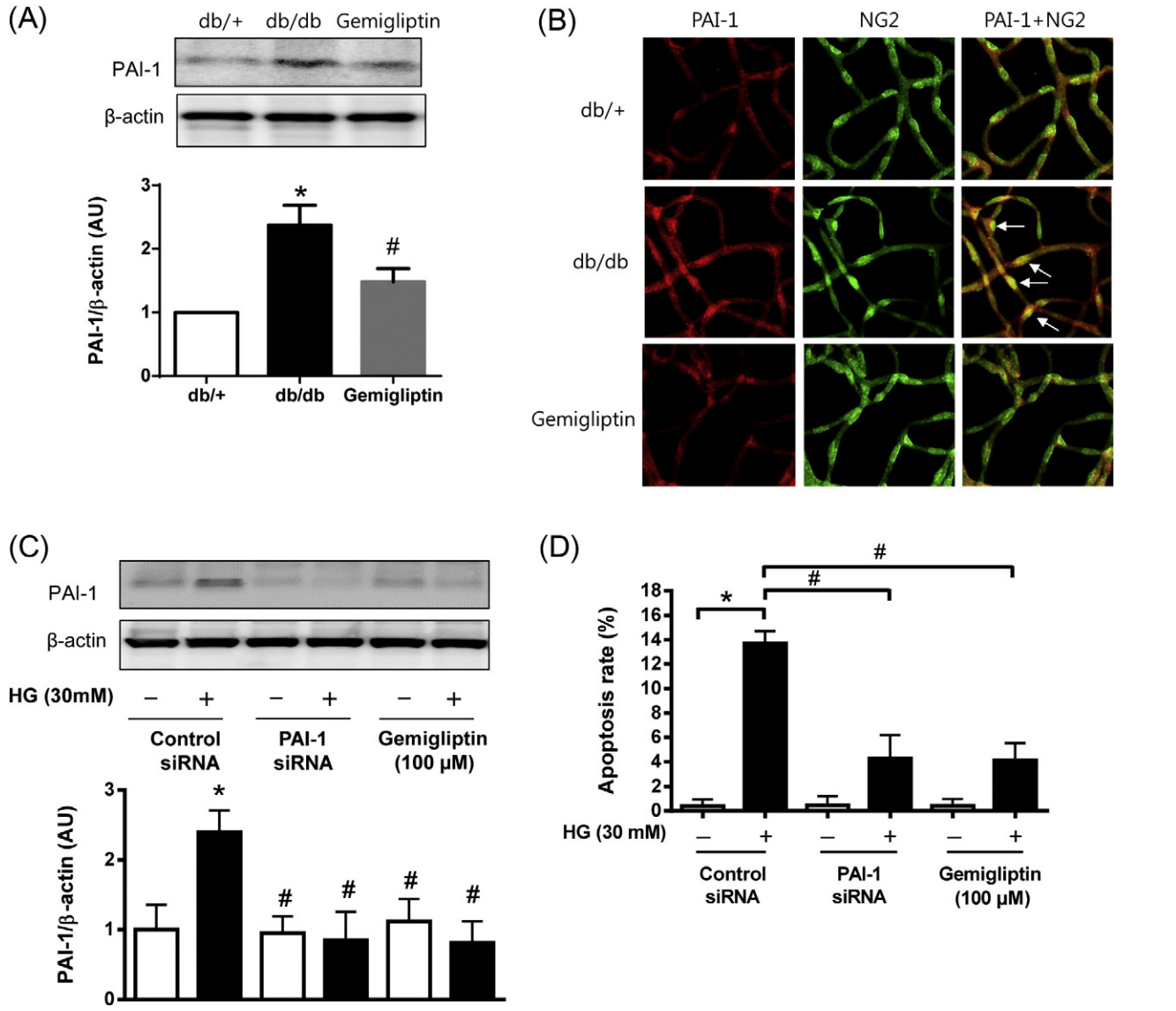

Fig. 8. Gemigliptin suppressed PAI-1 expression, which inhibited high glucose-induced pericyte apoptosis.(A) Retinal protein levels of PAI-1 were analyzed by Western blotting. Values in the bar graphs represent the mean ± SEM, n = 4. *P<0.05 vs. db/+ mice, #P<0.05 vs. db/db mice. (B) Immunofluorescence 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.

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 [37] and pericytes [38]. PAI-1 plays an important role in the development of diabetic retinopathy. High levels of PAI-1 have been observed in serum [39], vitreous [40] and retinal microvasculature [41] 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 [42]. 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 [43]. 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 [44]. In contrast, the intravitreal injection of exogenous human PAI-1 protein in rats with retinopathy of prematurity inhibited retinal neovascularization [45]. This paradoxical role of PAI-1 in angiogenesis is dependent on the cell type and PAI-1 concentration [46]. High levels (micromolar) of PAI-1 prevented angiogenesis, whereas low levels (physiological levels; nanomolar) of PAI-1 conversely facilitated angiogenesis [47]. 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 [48], whereas anti-apoptotic effects also have been observed in these cells [49]. 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 [50], 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 [51]. 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 [52]. 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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Conflict of interest

Eunsoo Jung and Sung-Ho Kim are employees of LG Life Sciences, R&D Center. No other author has a conflict of interest to disclose.

Author contribution statement

E.J. researched data and wrote the manuscript. J.K. researched data and reviewed the manuscript. C.S.K researched data. S.H.K. contributed to the discussion and reviewed the manuscript. M.H.C. wrote the manuscript.

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