INTRODUCTION

Dopamine, an important neurotransmitter in the nervous system, is synthesized from tyrosine by tyrosine hydroxylase in dopaminergic neurons and released in accordance with a circadian clock. Dopamine affects several neuronal cells in the retina, including photoreceptors and horizontal and bipolar cells, through five G-protein-coupled receptors named D1 to D5. Dopamine levels are reduced in the retinas of diabetic rodent models, and supplementation of dopamine in these animals prevents visual defects. In type 1 diabetic mice,
Dopamine deficiency contributes to early visual dysfunction, assessed by changes in spatial frequency threshold and contrast sensitivity, which is improved by intraperitoneal treatment with dopamine receptor agonists or the dopamine precursor levodopa (L-dopa). A preclinical study in patients with diabetes revealed that early retinal dysfunction assessed by electroretinography is detectable prior to clinically recognized retinopathy and can be reversed by L-dopa treatment. Dopamine is therefore likely to be beneficial for hyperglycemia-induced early visual dysfunction in diabetes; however, its effect on retinal vascular dysfunction and protective mechanism remain unknown.

Diabetic retinopathy (DR) is the most common microvascular complication of diabetes and the leading cause of blindness in working-age populations. DR, a progressive metabolic disease from a non-proliferative stage to a proliferative stage, is caused by several risk factors including poor glycemic control, duration of diabetes, and hypertension; however, the treatment of DR remains challenging. Clinical trials showed that blood glucose normalization in patients with type 1 or 2 diabetes does not arrest DR progression caused by persistent hyperglycemic stress, which is commonly referred to as hyperglycemic memory (HGM) or metabolic memory. The Diabetes Control and Complications Trial (DCCT) and the following up Epidemiology of Diabetes Interventions and Complications (EDIC) studies demonstrated that poor glycemic control leads to the development of diabetic complications, including DR, long after intensive glycemic control is achieved in patients with type 1 diabetes. The United Kingdom Prospective Diabetes Study (USPDS) showed the long-term effects of intensive glycemic control on vitreous hemorrhage and retinal photocoagulation in patients with type 2 diabetes. The role of HGM in the development and progression of DR was also demonstrated in diabetic animal models, including diabetic dogs and rats. It is now clear that HGM is the pivotal phenomenon in the development of DR, so exploration of the pathological mechanisms of HGM is needed to facilitate the development of new therapies for DR.

The underlying mechanisms of HGM have been investigated to uncover the pathophysiology of diabetic microvascular and macrovascular complications. Reactive oxygen species (ROS) generation is critically involved in the persistent hyperglycemic stress caused by HGM in endothelial cells and experimental animals. Hyperglycemia-induced upregulation of the pro-oxidant enzymes protein kinase C (PKC) β and NADPH oxidase subunit p47phox that persisted after restoration of normoglycemia in the retinas of diabetic rats. In the aortas of insulin-supplemented diabetic mice, activation of the mitochondrial adaptor protein p66Shc by PKC βII persisted after return to normoglycemia, and the persistent p66Shc activation was associated with continued ROS production and PKC βII upregulation, leading to a vicious cycle. Sustained ROS generation upregulates expression of NFκB subunit p65 and monomethylation of histone H3 at lysine 4 by the methyltransferase Set7/9, leading to overexpression of inflammatory genes including vascular cell adhesion molecule-1 and monocyte chemoattractant protein 1. Hence, an understanding of the persistent ROS generating machinery and its role in HGM-induced retinopathy is the real challenge in the development of effective therapies.

We hypothesized that dopamine ameliorates HGM-induced retinal microvascular dysfunction in DR by inhibiting persistent oxidative stress and mitochondrial dysfunction. We tested this in an HGM mouse model and found that intravitreal injection of L-dopa alleviated HGM-induced persistent oxidative stress, mitochondrial membrane potential (ΔΨm) collapse and fission, and adherens junction disassembly and subsequent vascular leakage in the retina. Furthermore, we found that L-dopa supplementation mitigated HGM-induced pericyte degeneration, acellular capillary and pericyte ghost generation, and endothelial apoptosis. Our findings provide a useful window into the mechanism of HGM-induced retinal microvascular dysfunction and suggest that dopamine might be a therapeutic agent for the treatment of DR.

2 MATERIALS AND METHODS

2.1 Cell culture

Human retinal endothelial cells (HRECs) were purchased from the Applied Cell Biology Research Institute (Cell Systems, Kirkland, WA, USA) and grown, as previously described. For experiments, endothelial cells from passages 4 to 7 were incubated for 12 h in low-serum medium supplemented with 2% fetal bovine serum, 0.1 ng/ml basic fibroblast growth factor, and antibiotics, and then subjected to treatments with 5.5 mM D-glucose for 6 days (normal glucose), 30 mM glucose for 6 days (high glucose), or 30 mM glucose for 3 days followed by 5.5 mM glucose for 3 days (HGM). Retinal Müller cells (rMC-1), purchased from Kerafast (Boston, MA, USA), were maintained at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and antibiotics (culture medium).
2.2 Measurement of ROS levels in endothelial cells

Intracellular and mitochondrial ROS levels were measured using CellROX™ deep red and MitoSOX™ red mitochondrial superoxide indicator (Thermo Fisher Scientific, Waltham, MA, USA), respectively, as previously described. Fluorescence intensities of single stained cells were determined by confocal microscopy (K1-Fluo; Nanoscope Systems, Daejeon, Korea). The ROS levels were determined by comparing the average fluorescence intensities of treated cells with those of control cells (fold change).

2.3 Analysis of mitochondrial fission and ∆Ψm in endothelial cells

Mitochondrial fission was analyzed using MitoTracker Red CMXRos, as previously described. Briefly, endothelial cells were incubated at 37°C with 100 nmol/L MitoTracker Red CMXRos (Thermo Fisher Scientific) and 0.5 μg/ml Hoechst 33342 (MilliporeSigma) for 30 min. Cells with disrupted and predominantly spherical mitochondria were identified as having mitochondrial fission. Thirty cells per experiment were used to calculate the percentage of cells undergoing mitochondrial fission. ∆Ψm was determined using 2 μmol/L JC-1 (Thermo Fisher Scientific) and confocal microscopy, as previously described. Data are expressed as the J-aggregate to monomer fluorescence intensity ratio.

2.4 Visualization and internalization of VE-cadherin in endothelial cells

Endothelial cells were incubated with 10 μM dopamine for 30 min and stimulated with 10 ng/ml vascular endothelial growth factor (VEGF) for 90 min. Vascular endothelial (VE)-cadherin was visualized by confocal microscopy as previously described. Briefly, following fixation and permeabilization, endothelial cells were incubated overnight with a monoclonal VE-cadherin antibody (1:200; Santa Cruz Biotechnology, Dallas, TX, USA) at 4°C. The cells were then probed with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (1:200; MilliporeSigma) for 2 h and stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; MilliporeSigma) for 10 min. VE-cadherin levels were represented by histograms and quantitatively analyzed using the peak fluorescence intensities of the histograms at the single-cell level.

Internalization of VE-cadherin was visualized by immunofluorescence as previously described. Briefly, the cells were incubated for 30 min at 4°C with a monoclonal antibody against the cell-surface VE-cadherin extracellular domain (BV6; MilliporeSigma) and then acid washed for 10 min with a low-pH buffer containing 100 mM glycine, 20 mM magnesium acetate, and 50 mM potassium chloride (pH 2.2). Following fixation and permeabilization, the cells were probed with an FITC-conjugated goat anti-mouse antibody for 2 h and stained with 1 μg/ml DAPI for 10 min.

2.5 In vitro endothelial cell monolayer permeability assay

In vitro endothelial cell monolayer permeability assays were performed as previously described. Briefly, endothelial cells were grown to confluence on gelatin-coated inserts (0.4-μm polycarbonate membranes) of Transwell Permeable Supports (CoStar Group, Washington DC, USA). After culturing for 5 days, cells on the inserts were incubated with 10μM dopamine for 30 min, treated with 10 ng/ml VEGF for 90 min, and probed with 1 mg/ml 40-kDa FITC-dextran (MilliporeSigma) for the last 60 min. The amount of FITC-dextran in the lower chamber was measured with a microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA).

2.6 Measurement of endothelial cell apoptosis

Apoptosis of endothelial cells was assessed by TUNEL assay as previously described. Briefly, cells were fixed with 1% (w/v) paraformaldehyde and 70% (v/v) ethanol on ice and stained with an APO-BrdU TUNEL assay kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol and with 1 μg/ml DAPI for 10 min. Apoptotic cells were visualized by confocal microscopy and expressed as the percentage of TUNEL-positive cells.

2.7 Western blot analysis

Lysates from HRECs and mouse retinas were separated by 12% SDS–PAGE and transferred to polyvinylidene fluoride membranes. The membranes were then incubated with antibodies against tyrosine hydroxylase (1:1000, MilliporeSigma), phospho-Drp1 (Ser616) (1:1000, Cell Signaling Technology, Danvers, MA, USA), mitochondrial fission factor (MFF) (1:1000, Cell Signaling Technology), Bcl-2-associated X protein (BAX) (1:1000, Cell Signaling Technology), cytochrome c (1:500, Cell Signaling Technology), or β-actin (1:2000, Cell Signaling Technology),
followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized using a ChemiDoc (Bio-Rad, Hercules, CA, USA).

2.8 | Generation of diabetic mice and treatment of mice with insulin and L-dopa

Six-week-old male C57BL/6 mice were obtained from DBL (EumSeong, Korea). The mice were maintained under pathogen-free conditions in a temperature-controlled room with a 12-h light/dark cycle. Diabetic mice were generated by single daily intraperitoneal injections over 5 consecutive days of streptozotocin (50 mg/kg body weight; MilliporeSigma) freshly prepared in 100 mM citrate buffer (pH 4.5), as previously described.28 Mice with fasting blood glucose concentrations ≥19 mM and polyuria were considered diabetic. For insulin supplementation, 12 weeks after the first streptozotocin injection, diabetic mice were anesthetized with 3% isoflurane and implanted with an Alzet Mini-Osmotic Pump 2004 (Durect, Cupertino, CA, USA), which delivered human recombinant insulin (MilliporeSigma) at a rate of 58.4 pmol/min/kg. At 18 weeks, a new insulin pump was implanted to maintain normal blood glucose levels for 12 weeks. For L-dopa supplementation, mice were anesthetized with 3% isoflurane and injected intravitreally with 2 μl of 10 mmol/L L-dopa every two days for 12 weeks. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA) and were approved by the Institutional Animal Care and Use Ethics Committee of Kangwon National University.

2.9 | Measurement of VEGF levels in mouse retinas

VEGF levels in mouse retinas were determined using the QuantiKine mouse VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA) as previously described.27 Lysates were extracted from the retinas of normal, diabetic, and insulin-supplemented diabetic (HGM) mice, and VEGF levels were determined using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) according to the manufacturer’s protocol.

2.10 | Measurement of in vivo TGase activity and vascular leakage in mouse retinas

In vivo TGase activity in the mouse retinas was determined by confocal microscopy as previously described.23 Briefly, mice were anesthetized with 3% isoflurane, and 48 μl of 100 mM 5-(biotinamido)pentylamine was injected into the left ventricle. Enucleated eyes were fixed with 4% paraformaldehyde for 45 min. The retinas were then whole-mounted on glass slides in the Maltese cross configuration and incubated with FITC-conjugated streptavidin (1:200, v/v) for 1 h. In vivo TGase activity was visualized by confocal microscopy and quantified by measuring the fluorescence intensities.

Microvascular leakage in the retinas was investigated using fluorescein angiography.23 Briefly, mice were anesthetized, and 1.25 mg 500 kDa FITC-dextran (MilliporeSigma) was injected into the left ventricle. Enucleated eyes were fixed with 4% paraformaldehyde, and whole-mounted retinas were visualized by confocal microscopy (Nanoscope Systems). Vascular leakage was quantitatively analyzed by measuring the fluorescence intensities.

2.11 | Miles vascular permeability assay in mouse ears and retinas

Miles vascular permeability assay in mouse ears was performed as previously described.29 Briefly, Evans blue solution (45 mg/kg, MilliporeSigma) was intravenously injected into the tail vein and allowed to circulate for 30 min. The mice were then anesthetized and injected intradermally in the middle part of the ears with 10 μl of either 3 ng/μl VEGF or a combination of 3 ng/μl VEGF and 1.9 ng/μl dopamine in PBS. PBS alone was injected into ears as a control. After 30 min, the mice were killed by cervical dislocation and dissected ears were incubated overnight in formamide at 65°C. For the Miles assay in mouse eyes, 4 h after Evans blue injection, retinas were isolated and incubated overnight in formamide at 65°C. The amount of extravasated Evans blue was measured by spectrophotometry (Molecular Device).

2.12 | Measurement of ROS generation and ΔΨm in mouse retinas

ROS levels in mouse retinas were determined using dihydroethidium (Thermo Fisher Scientific).23 Mouse eyes were enucleated and quickly frozen in OCT compound (Sakura Finetek USA, Torrance, CA, USA). Unfixed retinal tissues were cut with a microtome-cryostat (Leica Biosystems, Wetzlar, Germany) at 10 μm thickness, and the sections were stained at 37°C with 5 μmol/L dihydroethidium in serum-free M199 medium for 30 min. The sections were also incubated with 5 μmol/L MitoSOX™ red (Thermo Fisher Scientific) to measure
mitochondrial ROS levels in the retina. The ROS levels were visualized by confocal microscopy (Nanoscope Systems) and quantified by measuring the fluorescence intensities.

To measure $\Delta \Psi_m$ in the retinas, the retinal sections were stained at 37°C with 2 $\mu$mol/L JC-1 in serum-free M199 medium for 30 min. The $\Delta \Psi_m$ was visualized by confocal microscopy and quantified by measuring the fluorescence intensities.

2.13 | **Visualization of tyrosine hydroxylase expression in mouse retinas**

Expression of tyrosine hydroxylase was visualized in sections of mouse retinas by immunohistochemistry. Mouse eyes were fixed with 4% paraformaldehyde, transferred to 30% sucrose, incubated at 4°C overnight, and frozen in OCT compound. The eyes were then cut with a microtome-cryostat (Leica Biosystems) at 10 $\mu$m thickness. The sections were permeabilized with 1% Triton X-100 and stained overnight with anti-tyrosine hydroxylase polyclonal antibody (1:200, MilliporeSigma) at 4°C. The sections were probed with an FITC-conjugated goat anti-rabbit antibody (1:300, MilliporeSigma) for 2 h and stained with 1 $\mu$g/ml DAPI for 10 min at room temperature.

2.14 | **Visualization of VE-cadherin and pericytes in mouse retinas**

VE-cadherin in mouse retinas was visualized by confocal microscopy as previously described. Following whole-mounting in the Maltese cross configuration, the retinas were delipidated with ice-cold acetone for 3 min at −20°C and permeabilized with 1% Triton X-100 for 4 h. The retinas were then incubated overnight at 4°C with a monoclonal VE-cadherin antibody (1:100, BD Biosciences) and probed for 2 h with an FITC-conjugated goat anti-rat antibody (1:300, Thermo Fisher Scientific). VE-cadherin in the superficial vascular plexus and deep capillary plexus was visualized in the retinas by confocal microscopy as illustrated in Figure 6A.

To visualize pericytes, whole-mounted retinas were incubated overnight at 4°C with a monoclonal neuron-glial antigen 2 (NG2) antibody (1:200, MilliporeSigma) and probed for 2 h with an FITC-conjugated goat anti-rabbit antibody (1:300) and the endothelial cell-specific marker Alexa 647-Isoclectin B4 (1:1000; Thermo Fisher Scientific). The number of NG2-positive pericytes was counted in the superficial vascular plexus of the retina per mm² vascular area.

2.15 | **Retinal trypsin digestion assay and apoptosis of retinal vasculature**

Retinal vasculature was isolated by trypsin digestion as previously described. Briefly, mouse retinas were isolated from eyes fixed in 10% neutral buffered formalin (MilliporeSigma) and incubated for 3 h with 3% trypsin in 0.1 M Tris buffer (pH 7.8) at 37°C. The digested retinas were flat-mounted on glass slides, stained with Periodic Acid Schiff (MilliporeSigma), and observed under a light microscope. The numbers of acellular capillaries and pericyte ghosts were counted per mm² vascular area.

To assess apoptosis in the retinal vasculature, flat-mounted digested retinas were stained with the APO-BrdU TUNEL assay kit (BD Biosciences) and incubated for 1 h with Alexa Fluor 647-Isoclectin B4 (Thermo Fisher Scientific). TUNEL-positive cells in the stained retinas were visualized using confocal microscopy.

2.16 | **Statistical analysis**

Data were analyzed using OriginPro 2015 software (OriginLab, Northampton, MA, USA). Data are expressed as means ± standard deviation (SD) of at least three independent in vitro experiments or six independent in vivo experiments. Statistical significance was determined using one-way ANOVA with Holm–Sidak’s multiple comparisons test. $p$ Values of <.05 were considered statistically significant.

3 | **RESULTS**

3.1 | **Persistent hyperglycemic stress in the retinas of HGM mice**

To investigate whether hyperglycemic stress persists in the retinas of HGM mice after blood glucose normalization, diabetic mice were exposed to hyperglycemic conditions for 12 weeks and then supplemented for 12 weeks with human recombinant insulin using osmotic pumps (Figure 1A). Compared with non-diabetic controls, diabetic mice showed elevated water consumption, decreased body weight, and hyperglycemia, all of which were normalized by insulin supplementation (Figure 1B,C). Hyperglycemia stimulated ROS generation in the retinas of diabetic mice; however, glucose normalization by insulin supplementation had no effect on the ROS generation in HGM mice (Figure 1D,E). In vivo TGase activity was also elevated by hyperglycemia in the retinas of the diabetic mice, but the TGase activation was not affected by glucose normalization (Figure 1F). Because ROS-mediated TGase activation
is involved in vascular leakage in diabetic retinas, we studied vascular leakage in whole-mounted retinas. Hyperglycemia induced vascular leakage in the retinas of diabetic mice, and blood glucose normalization had no effect on the vascular leakage (Figure 1G,H). These results suggest that oxidative stress, TGase activation, and vascular leakage are involved in persistent hyperglycemic stress in the retinas of HGM mice.

We next investigated whether tyrosine hydroxylase, the dopaminergic neuronal marker in retinal amacrine cells, is associated with persistent hyperglycemic stress in the retinal sections of HGM mice. Hyperglycemia reduced the expression of tyrosine hydroxylase in the retinas of diabetic mice, and the reduced expression was not recovered by glucose normalization in HGM mice (Figure 1I).

3.2 Inhibitory effects of dopamine on HGM-induced persistent oxidative stress and mitochondrial dysfunction in HRECs and mouse retinas

To study the effect of dopamine on persistent hyperglycemic stress, we first tested whether dopamine can inhibit sustained oxidative stress by measuring intracellular and
mitochondrial ROS levels in HRECs treated with HGM conditions (Figure 2A). High glucose conditions led to elevated intracellular ROS levels, which were sustained after glucose normalization but were inhibited by dopamine treatment (Figure 2B,C). Similarly, elevated mitochondrial ROS levels were maintained in HRECs under HGM conditions but were suppressed by dopamine treatment (Figure 2D,E). Dopamine also suppressed intracellular and mitochondrial ROS generation in HRECs treated with high glucose conditions. To validate our in vitro studies, we studied the role of dopamine in retinal sections of HGM mice supplemented with L-dopa for 12 weeks. Hyperglycemia led to elevated mitochondrial ROS levels in the retinas of diabetic mice, and the elevated mitochondrial ROS levels were sustained after blood glucose normalization but attenuated by L-dopa supplementation (Figure 2F,G). These results indicate that dopamine inhibits HGM-induced persistent intracellular and mitochondrial ROS generation in HRECs and mouse retinas.

Because mitochondrial dysfunction is important in the development of DR, we investigated the inhibitory effect of dopamine on mitochondrial dysfunction by visualizing mitochondrial fission and $\Delta \Psi_m$ collapse in HRECs. Exposure of HRECs to high glucose conditions induced mitochondrial fission and subsequent increase in the number of mitochondria per cell, which persisted after glucose normalization. These effects were inhibited by dopamine or mdivi-1, a potent inhibitor of Drp1-mediated mitochondrial fission (Figure 3A–C). The $\Delta \Psi_m$ collapse induced by the high-glucose treatment was sustained after glucose

![Figure 2](image-url)
normalization and this sustained $\Delta \Psi_m$ collapse was inhibited by dopamine or mdivi-1 treatment (Figure 3D,E). We further explored the inhibitory effect of dopamine on HGM-induced mitochondrial dysfunction by Western blot (Figure 3F–H). High-glucose-induced phosphorylation of Drp1 was sustained after glucose normalization, but the sustained phosphorylation was inhibited by dopamine. Dopamine also inhibited HGM-induced sustained expression of MFF, a key molecule that recruits Drp1 as a part of the mitochondrial fission machinery. Mdivi-1 had the same qualitative effects as dopamine on HGM-induced phosphorylation of Drp1 and expression of MFF. These results suggest that dopamine inhibits HGM-induced mitochondrial dysfunction by inhibiting sustained mitochondrial fission and $\Delta \Psi_m$ collapse in HRECs.

To support our in vitro findings, we investigated the preventive effect of L-dopa supplementation on $\Delta \Psi_m$ collapse, Drp1 phosphorylation, and MFF expression in the retinas of HGM mice. Hyperglycemia induced $\Delta \Psi_m$ collapse in retinal sections from diabetic mice, and the $\Delta \Psi_m$ collapse persisted after normoglycemia was improved by L-dopa treatment (Figure 4A,B). Hyperglycemia-induced increases in Drp1 phosphorylation and MFF expression also persisted in the retinas of HGM mice after normoglycemia.
but they were suppressed by L-dopa (Figure 4C–E). Taken together, our results demonstrate that dopamine inhibits HGM-induced oxidative stress and mitochondrial dysfunction in HRECs and mouse retinas.

3.3 Inhibitory effects of dopamine on HGM-induced adherens junction disassembly and vascular leakage in HRECs and mouse retinas

To understand the role of dopamine in vascular leakage, we investigated the inhibitory effects of dopamine on VEGF-induced adherens junction disassembly and vascular permeability in HRECs. Hyperglycemia elevated the levels of VEGF in the retinas of diabetic mice, and the elevated levels were sustained after blood glucose normalization in HGM mice (Figure 5A), highlighting the important role of VEGF in diabetes-associated retinopathy. In HRECs, VEGF induced VE-cadherin disassembly and subsequent internalization of VE-cadherin, which were suppressed by dopamine (Figure 5B–E). We investigated the role of dopamine in VEGF-induced vascular leakage by in vitro endothelial cell monolayer permeability assay in HRECs and in vivo Miles vascular permeability assay in mouse ears. In HRECs, VEGF caused an increase in endothelial cell permeability, which was reversed by dopamine (Figure 5F). In vivo vascular leakage was elevated by intradermal injection of VEGF into mouse ears compared with that in PBS-injected ears, and the elevated vascular leakage was prevented by dopamine (Figure 5G,H). These results indicate that dopamine prevents VEGF-induced adherens junction disassembly and subsequent increase in vascular permeability in HRECs and mouse ears.
To support our findings in HRECs and mouse ears, we further studied the effect of dopamine on HGM-induced adherens junction disassembly and vascular leakage in mouse retinas. Compared with non-diabetic controls, the retinas of diabetic mice displayed hyperglycemia-induced VE-cadherin disassembly in the superficial and deep vascular layers, which persisted after return to normoglycemia (Figure 6A,B). The HGM-induced disassembly of VE-cadherin was alleviated by L-dopa supplementation. High levels of FITC-dextran extravasation were sustained in the retinas of HGM mice after blood glucose normalization, but the HGM-induced vascular leakage was inhibited by L-dopa supplementation (Figure 6C,D). We further studied the inhibitory effect of dopamine against HGM-induced retinal vascular leakage by Evans blue assay. Evans blue extravasation persisted after glucose normalization in the retinas of HGM mice but was suppressed by L-dopa (Figure 6E). Together, our findings demonstrate that L-dopa ameliorates HGM-induced adherens junction disassembly and subsequent microvascular leakage in mouse retinas.

3.4 Inhibitory effects of L-dopa on microvascular abnormalities and endothelial apoptosis in the retinas of HGM mice

To investigate whether microvascular abnormalities that represent retinopathy persist after normoglycemia, we analyzed pericyte degeneration and the numbers of acellular capillaries and pericyte ghosts in the retinas of HGM mice. Hyperglycemia led to a reduction in the numbers of NG2-positive pericytes in the retinas of diabetic
mice, which persisted after blood glucose normalization in HGM mice but was reversed by L-dopa (Figure 7A,B). The numbers of acellular capillaries and pericyte ghosts in trypsin-digested retinas of diabetic mice increased by hyperglycemia, and the increased numbers were maintained after blood glucose normalization but were reduced by L-dopa treatment (Figure 7C–E). These results show that hyperglycemia-induced microvascular abnormalities in the mouse retina are sustained after blood glucose normalization but are ameliorated by L-dopa supplementation.

We next studied the role of L-dopa in endothelial cell apoptosis by Western blot and TUNEL staining of the retinas of HGM mice. The expression of BAX and cytochrome c was maintained in the retinas of HGM mice after return to normoglycemia, but L-dopa treatment attenuated the HGM-induced expression of these proteins (Figure 8A–C). Hyperglycemia increased the number of TUNEL-positive cells in diabetic retinas, and the HGM-induced increase in the number of apoptotic cells was normalized by L-dopa (Figure 8D,E). These results suggest that L-dopa alleviates HGM-induced endothelial apoptosis in the mouse retina. Taken together, our results suggest that HGM causes microvascular leakage and abnormalities through persistent oxidative stress and mitochondrial dysfunction after blood glucose normalization in the mouse retina, and that dopamine ameliorates the HGM-induced retinal microvascular dysfunction by reversing the persistent hyperglycemic stress (Figure 8F).
DISCUSSION

Dopamine is synthesized in the retina by tyrosine hydroxylase in dopaminergic amacrine neurons and is involved in visual signaling. Tyrosine hydroxylase, the rate-limiting enzyme of catecholamine synthesis, catalyzes the hydroxylation of tyrosine to L-dopa, which is converted into dopamine by aromatic amino acid decarboxylase. Hyperglycemia decreases tyrosine hydroxylase-positive neurons and the dopamine levels in retina of diabetic mice. Dopamine is beneficial for hyperglycemia-induced visual dysfunction in the retinas of diabetic mice. Dopamine is beneficial for hyperglycemia-induced visual dysfunction in the retinas of diabetic mice. HGM, or persistent hyperglycemic stress after glucose normalization, is a pivotal phenomenon in the development of DR. HGM-induced retinopathy was initially observed by Engerman and Kern, who found that DR is not improved by good glycemic control in diabetic dogs. The DCCT-EDIC studies demonstrated that episodes of poor glycemic control contribute to the development of DR long after improved glucose control is achieved in patients with type 1 diabetes. This long-lasting effect is also reported in patients with type 2 diabetes in the UKPDS study. In the retinas of diabetic rats, oxidative stress and hypermethylation of mitochondrial proteins are not inhibited by reversal of hyperglycemia after poor glycemic control. Therefore, understanding the underlying mechanism of HGM is essential for the development of new therapies for HGM-induced retinopathy. In this study, we demonstrated that dopamine ameliorated HGM-induced retinal vascular dysfunction by normalizing persistent oxidative stress and mitochondrial dysfunction. HGM induced persistent oxidative stress and hypermethylation of mitochondrial proteins.
stress, mitochondrial fission and $\Delta \Psi_m$ collapse, and subsequent adherens junction disassembly and microvascular leakage leading to microvascular abnormalities, as shown by increases in endothelial apoptosis and the numbers of acellular capillaries and pericyte ghosts. L-dopa ameliorated HGM-induced vascular dysfunction by inhibiting the hyperglycemic stress in the retina, suggesting dopamine as a possible therapeutic agent for HGM-induced retinopathy.

Mitochondria play a central role in the pathogenesis of DR. Oxidative stress induces mitochondrial dysfunction by impairing mitochondrial DNA stability and mitochondrial dynamics, causing subsequent mitochondrial fission. Mitochondrial fission through cytochrome c release promotes retinal vascular apoptosis; however, the role of mitochondrial dysfunction in HGM-induced retinopathy is unknown. We showed that in HRECs, HGM induced persistent mitochondrial ROS generation and subsequent mitochondrial dysfunction through sustained $\Delta \Psi_m$ collapse, mitochondrial fission, and subsequent increase in the number of mitochondria after glucose normalization. In the retinas of diabetic mice, HGM consistently induced persistent mitochondrial ROS generation, $\Delta \Psi_m$ collapse, and increases in Drp1 phosphorylation and MFF expression after return to normoglycemia. These results indicate that mitochondrial dysfunction contributes to the pathogenesis of HGM-induced microvascular dysfunction and therefore might be an effective target for the treatment of DR.

Our results show that in addition to its role in hyperglycemia-induced neuronal dysfunction, dopamine ameliorates HGM-induced microvascular damage. Although DR is considered a diabetic microvascular complication, it is reported that neurodegeneration is also involved in DR pathogenesis, and thus DR is a neurovascular complication caused by microvascular dysfunction and neurodegeneration. Although dopamine deficiency contributes to early visual dysfunction in rodent models of DR, dopamine ameliorates HGM-induced microvascular damage. These results suggest dopamine as a possible therapeutic agent for HGM-induced retinopathy.
diabetes, and this hyperglycemia-induced visual dysfunction is improved by dopamine treatment.\textsuperscript{3,5} the role of dopamine in diabetes-associated retinal vascular damage has been unclear. Our results demonstrate that dopamine has a protective effect against persistent microvascular leakage and abnormalities induced by HGM. In HRECs, dopamine prevented VEGF-induced adherens junction disassembly and subsequent endothelial cell permeability. In the retinas of HGM mice, L-dopa supplementation suppressed HGM-induced adherens junction disassembly and subsequent microvascular leakage, and it ameliorated microvascular abnormalities including pericyte degeneration, acellular capillary and pericyte ghost generation, and endothelial apoptosis. Furthermore, L-dopa suppressed HGM-induced persistent mitochondrial ROS generation and $\Delta \Psi_m$ collapse in retinal neuronal cells. Together, these results suggest that dopamine might ameliorate HGM-induced retinopathy by normalizing microvascular abnormality and neuronal dysfunction.

It is likely that dopamine ameliorates HGM-induced microvascular dysfunction in the retina by inhibiting VEGF-induced oxidative stress leading to vascular leakage. VEGF, a vascular permeability factor, plays a key role in DR.\textsuperscript{25} Previously, it was reported that VEGF activates TGase2 by intracellular Ca\textsuperscript{2+} elevation and ROS generation and subsequently induces microvascular leakage through adherens junction disassembly in the retinas of diabetic mice.\textsuperscript{23,26} We found that VEGF levels were persistently elevated in the retinas of HGM mice and that dopamine prevented vascular leakage induced by intradermal injection of VEGF into mouse ears. Consistent with our in vivo results, dopamine treatment of HRECs inhibited VEGF-induced intracellular events including intracellular and mitochondrial ROS generation, adherens junction disassembly, and subsequent endothelial cell permeability. However, in retinal Müller cells, high glucose conditions induced sustained ROS generation, which was not inhibited by dopamine treatment (data not shown). Dopamine also had no effect on HGM-induced VEGF expression in retinal Müller cells, indicating that dopamine normalizes endothelial dysfunction rather than VEGF expression in the retina. It is reported that dopamine inhibits vascular permeability induced by VEGF in mice.\textsuperscript{38} In human umbilical vein endothelial cells, dopamine dephosphorylates VEGF receptor 2 by activating the Src-homology-2-domain-containing protein tyrosine phosphatase 2 through D2 dopamine receptors.\textsuperscript{39} These findings suggest that VEGF-induced molecular events play a key role in the pathogenesis of HGM-induced retinopathy, and that dopamine ameliorates these events by regulating VEGF 2 receptors. It is still necessary, however, to elucidate the molecular mechanism of dopamine action in the retina.

In conclusion, HGM induces DR through persistent oxidative stress, mitochondrial dysfunction, and microvascular abnormalities in the retina after blood-glucose normalization. Dopamine alleviates HGM-induced microvascular leakage and abnormalities by inhibiting the persistent oxidative stress and mitochondrial dysfunction. Our results provide a possible mechanism of HGM-induced vascular dysfunction and suggest dopamine as a possible therapeutic agent for the treatment of DR.

AUTHOR CONTRIBUTIONS
Yeon-Ju Lee designed and performed experiments, analyzed data, and wrote the manuscript. Hye-Yoon Jeon, Ah-Jun Lee, and Minsoo Kim designed experiments and analyzed data. Kwon-Soo Ha conceptualized the study, designed experiments, analyzed and interpreted data, and wrote the manuscript. All authors approved the final version of the manuscript.

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DISCLOSURES
The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT
The datasets generated during the current study are available from the corresponding author on reasonable request.

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REFERENCES
1. Popova E. Role of dopamine in distal retina. J Comp Physiol A Neuroethol Sens Neural Behav Physiol. 2014;200:333-358.
2. Mohana Devi S, Mahalaxmi I, Aswathy NP, Dhivya V, Balachandar V. Does retina play a role in Parkinson’s disease? Acta Neurol Belg. 2020;120:257-265.
3. Aung MH, Park HN, Han MK, et al. Dopamine deficiency contributes to early visual dysfunction in a rodent model of type 1 diabetes. J Neurosci. 2014;34:726-736.
4. Chesler K, Motz C, Vo H, et al. Initiation of L-DOPA treatment after detection of diabetes-induced retinal dysfunction reverses retinopathy and provides neuroprotection in rats. Transl Vis Sci Technol. 2021;10:8.
5. Kim MK, Aung MH, Mees L, et al. Dopamine deficiency mediates early rod-driven inner retinal dysfunction in diabetic mice. Invest Ophthalmol Vis Sci. 2018;59:572-581.
6. Motz CT, Chesler KC, Allen RS, et al. Novel detection and restorative levodopa treatment for preclinical diabetic retinopathy. Diabetes. 2020;69:1518-1527.
7. Wang W, Lo ACY. Diabetic retinopathy: pathophysiology and treatments. Int J Mol Sci. 2018;19:1816.
8. Simo-Servat O, Hernandez C, Simo R. Diabetic retinopathy in the context of patients with diabetes. *Ophthalmic Res*. 2019;62:211-217.

9. Kowluru RA. Diabetic retinopathy, metabolic memory and epigenetic modifications. *Vision Res*. 2017;139:30-38.

10. Semeraro F, Morescalchi F, Cancarini A, Russo A, Rezzola S, Costagliola C. Diabetic retinopathy, a vascular and inflammatory disease: therapeutic implications. *Diabetes Metab*. 2019;45:517-527.

11. Pirola L, Balcerzcyk A, Okabe J, El-Osta A. Epigenetic phenomena linked to diabetic complications. *Nat Rev Endocrinol*. 2010;6:665-675.

12. Holman RR, Paul SK, Bethel MA, Matthews DR, Neil HA. 10-year follow-up of intensive glucose control in type 2 diabetes. *N Engl J Med*. 2008;359:1577-1589.

13. Zhang L, Chen B, Tang L. Metabolic memory: mechanisms and implications for diabetic retinopathy. *Diabetes Res Clin Pract*. 2012;96:286-293.

14. Engerman RL, Kern TS. Progression of incipient diabetic retinopathy during good glycemic control. *Diabetes*. 1987;36:808-812.

15. Kowluru RA, Mohammad G. Epigenetics and mitochondrial stability in the metabolic memory phenomenon associated with continued progression of diabetic retinopathy. *Sci Rep*. 2020;10:6655.

16. Jeon HY, Lee A-J, Ha K-S. Polymer-based delivery of peptide drugs to treat diabetes: normalizing hyperglycemia and preventing diabetic complications. *Biochit J*. 2022;16:111-127.

17. Paneni F, Volpe M, Luscher TF, Cosentino F. SIRT1, p66(Shc), and Set7/9 in vascular hyperglycemic memory: bringing all the strands together. *Diabetes*. 2013;62:1800-1807.

18. Lee YJ, Lee YJ, Jeon HY, et al. The vicious cycle between transglutaminase 2 and reactive oxygen species in hyperglycemic memory-induced endothelial dysfunction. *FASEB J*. 2019;33:12655-12667.

19. Ihnat MA, Thorpe JE, Kamat CD, et al. Reactive oxygen species mediate a cellular ‘memory’ of high glucose stress signalling. *Diabetología*. 2007;50:1523-1531.

20. Paneni F, Mochlaria F, Akhmedov A, et al. Gene silencing of the mitochondrial adaptor p66(Shc) suppresses vascular hyperglycemic memory in diabetes. *Circ Res*. 2012;111:278-289.

21. Di Lisa F, Giorgio M, Ferdinandy P, Schulz R. New aspects of p66Shc in ischaemia reperfusion injury and other cardiovascular diseases. *Br J Pharmacol*. 2017;174:1690-1703.

22. Okabe J, Orlowsky C, Balcerzcyk A, et al. Distinguishing hyperglycemic changes by Set7 in vascular endothelial cells. *Circ Res*. 2012;110:1067-1076.

23. Lee YJ, Kim M, Lee JY, et al. The benzodiazepine anesthetic midazolam prevents hyperglycemia-induced microvascular leakage in the retinas of diabetic mice. *FASEB J*. 2018;32:6089-6099.

24. Bhatt MP, Lim YC, Kim YM, Ha KS. C-peptide activates AMPKalpha and prevents ROS-mediated mitochondrial fission and endothelial apoptosis in diabetes. *Diabetes*. 2013;62:3851-3862.

25. Lee YJ, Jung SH, Kim SH, et al. Essential role of transglutaminase 2 in vascular endothelial growth factor-induced vascular leakage in the retina of diabetic mice. *Diabetes*. 2016;65:2414-2428.

26. Lee YJ, Jung SH, Hwang J, et al. Cysteamine prevents vascular leakage through inhibiting transglutaminase in diabetic retina. *J Endocrinol*. 2017;235:39-48.

27. Jeon HY, Lee YJ, Kim YS, et al. Proinsulin C-peptide prevents hyperglycemia-induced vascular leakage and metastasis of melanoma cells in the lungs of diabetic mice. *FASEB J*. 2019;33:750-762.

28. Seo JA, Jeon HY, Kim M, et al. Anti-metastatic effect of midazolam on melanoma B16F10 cells in the lungs of diabetic mice. *Biochem Pharmacol*. 2020;178:114052.

29. Lim YC, Bhatt MP, Kwon MH, et al. Prevention of VEGF-mediated microvascular permeability by C-peptide in diabetic mice. *Cardiovasc Res*. 2014;101:155-164.

30. Chou JC, Rollins SD, Fawzi AA. Tryptsin digest protocol to analyze the retinal vasculature of a mouse model. *J Vis Exp*. 2013;13:e50489.

31. Kowluru RA. Mitochondrial stability in diabetic retinopathy: lessons learned from epigenetics. *Diabetes*. 2019;68:241-247.

32. Daubner SC, Le T, Wang S. Tyrosine hydroxylase and regulation of dopamine synthesis. *Arch Biochem Biophys*. 2011;508:1-12.

33. Lahouaoui H, Coutanson C, Cooper HM, Bennis M, Dkhissi-Benyahya O. Diabetic retinopathy alters light-induced clock gene expression and dopamine levels in the mouse retina. *Mol Vis*. 2016;22:959-969.

34. Kowluru RA. Effect of reinstitution of good glycemic control on retinal oxidative stress and nitrative stress in diabetic rats. *Diabetes*. 2003;52:818-823.

35. Kim D, Sankaramoorthy A, Roy S. Downregulation of Drp1 and Fis1 inhibits mitochondrial fission and prevents high glucose-induced apoptosis in retinal endothelial cells. *Cell*. 2020;9:1662.

36. Wu S, Zhou F, Zhang Z, Xing D. Mitochondrial oxidative stress causes mitochondrial fragmentation via differential modulation of mitochondrial fission-fusion proteins. *FEBS J*. 2011;278:941-954.

37. Simo R, Stitt AW, Gardner TW. Neurodegeneration in diabetic retinopathy: does it really matter? *Diabetologia*. 2018;61:1902-1912.

38. Basu S, Nagy JA, Pal S, et al. The neurotransmitter dopamine inhibits angiogenesis induced by vascular permeability factor/vascular endothelial growth factor. *Nat Med*. 2001;7:569-574.

39. Sinha S, Vohra PK, Bhattacharya R, Dutta S, Sinha S, Mukhopadhyay D. Dopamine regulates phosphorylation of VEGF receptor 2 by engaging Src-homology-2-domain-containing protein tyrosine phosphatase 2. *J Cell Sci*. 2009;122:3385-3392.

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