Abrogation of *MMP-9* Gene Protects Against the Development of Retinopathy in Diabetic Mice by Preventing Mitochondrial Damage

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OBJECTIVE—In the development of diabetic retinopathy, mitochondrial dysfunction is considered to play an important role in the apoptosis of retinal capillary cells. Diabetes activates matrix metalloproteinase-9 (MMP-9) in the retina and its capillary cells, and activated MMP-9 becomes proapoptotic. The objective of this study is to elucidate the plausible mechanism by which active MMP-9 contributes to the mitochondrial dysfunction in the retina.

RESEARCH DESIGN AND METHODS—Using *MMP-9* gene knockout (MMP-KO) mice, we investigated the effect of MMP-9 regulation on diabetes-induced increased retinal capillary cell apoptosis, development of retinopathy, mitochondrial dysfunction and ultrastructure, and mitochondrial DNA (mtDNA) damage. To understand how diabetes increases mitochondrial accumulation of MMP-9, interactions between MMP-9 and chaperone proteins (heat shock protein [Hsp] 70 and Hsp60) were evaluated. The results were confirmed in the retinal mitochondria from human donors with diabetic retinopathy, and in isolated retinal endothelial cells transfected with *MMP-9* small interfering RNA (siRNA).

RESULTS—Retinal microvasculature of MMP-KO mice, diabetic for ~7 months, did not show increased apoptosis and pathology characteristic of retinopathy. In the same MMP-KO diabetic mice, activation of MMP-9 and dysfunction of the mitochondria were prevented, and electron microscopy of the retinal microvasculature region revealed normal mitochondrial matrix and packed lamellar cristae. Damage to mtDNA was protected, and the binding of MMP-9 with Hsp70 or Hsp60 was also normal. As in the retina from wild-type diabetic mice, activation of mitochondrial MMP-9 and alterations in the binding of MMP-9 with chaperone proteins were also observed in the retina from donors with diabetic retinopathy. In endothelial cells transfected with MMP-9 siRNA, high glucose–induced damage to the mitochondria and the chaperone machinery was ameliorated.

CONCLUSIONS—Regulation of activated MMP-9 prevents retinal capillary cells from undergoing apoptosis by protecting mitochondrial ultrastructure and function and preventing mtDNA damage. Thus, MMP-9 inhibitors could have potential therapeutic value in preventing the development of diabetic retinopathy by preventing the continuation of the vicious cycle of mitochondrial damage. ***Diabetes*** **60:3023–3033, 2011**

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Diabetic retinopathy is one of the major causes of acquired blindness in working adults, but despite extensive research in the field, the molecular mechanism of its development remains elusive. In the pathogenesis of this slow progressing disease, capillary cells and other retinal cells are lost by apoptosis before histopathology characteristic of diabetic retinopathy can be seen in the retina (1–4). However, how a diabetic environment accelerates retinal cell apoptosis is unclear.

Matrix metalloproteinases (MMPs), a class of approximately 25 zinc-dependent proteinases, regulate a variety of cellular functions, including apoptosis, proliferation, differentiation, and angiogenesis. In diabetes, MMPs are elevated in the retina and other tissues, and MMP-9, the largest member of the MMP family (5), is associated with many diabetes complications, including nephropathy, cardiomyopathy, and retinopathy (6). Our previous work has shown that in a diabetic environment, the activation of MMP-9 in the retina and its capillary cells is mediated by the Ras/Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase pathway, and activated MMP-9 induces the apoptosis of retinal capillary cells (7,8). The mechanism by which MMP-9 induces apoptosis in the pathogenesis of diabetic retinopathy remains to be explored.

Damage to the retinal mitochondria is considered to play a major role in the development of diabetic retinopathy. Retinal mitochondria become dysfunctional, superoxide levels are elevated, and mitochondrial permeability is significantly increased, which leads to leakage of cytochrome c into the cytosol and acceleration of the apoptosis of retinal capillary cells (9,10). Emerging work has shown that MMPs are not present in the matrix alone, they are also found in the mitochondria, and induction of MMP-9 in the myocyte mitochondria is considered to act as a negative regulator of mitochondrial function (11). Our recent work has shown that MMP-2, another important member of MMP family, is present in the retinal mitochondria, and in diabetes, MMP-2 damages retinal mitochondria by modulating connexin43 (12). As diabetes activates both MMP-2 and MMP-9 in the retina, accelerating apoptosis of retinal capillary cells (7,8,12), the role of MMP-9 in retinal mitochondrial dysfunction in diabetes, leading to retinal capillary cell loss, needs further investigation.

The aim of this study is to elucidate the plausible mechanism by which active MMP-9 contributes to the mitochondrial dysfunction in the retina, accelerating the apoptosis of capillary cells and ultimately resulting in retinopathy. Using *MMP-9* gene knockout (MMP-KO) mice, we have investigated the effect of *MMP-9* regulation on diabetes-induced increased retinal capillary cell apoptosis

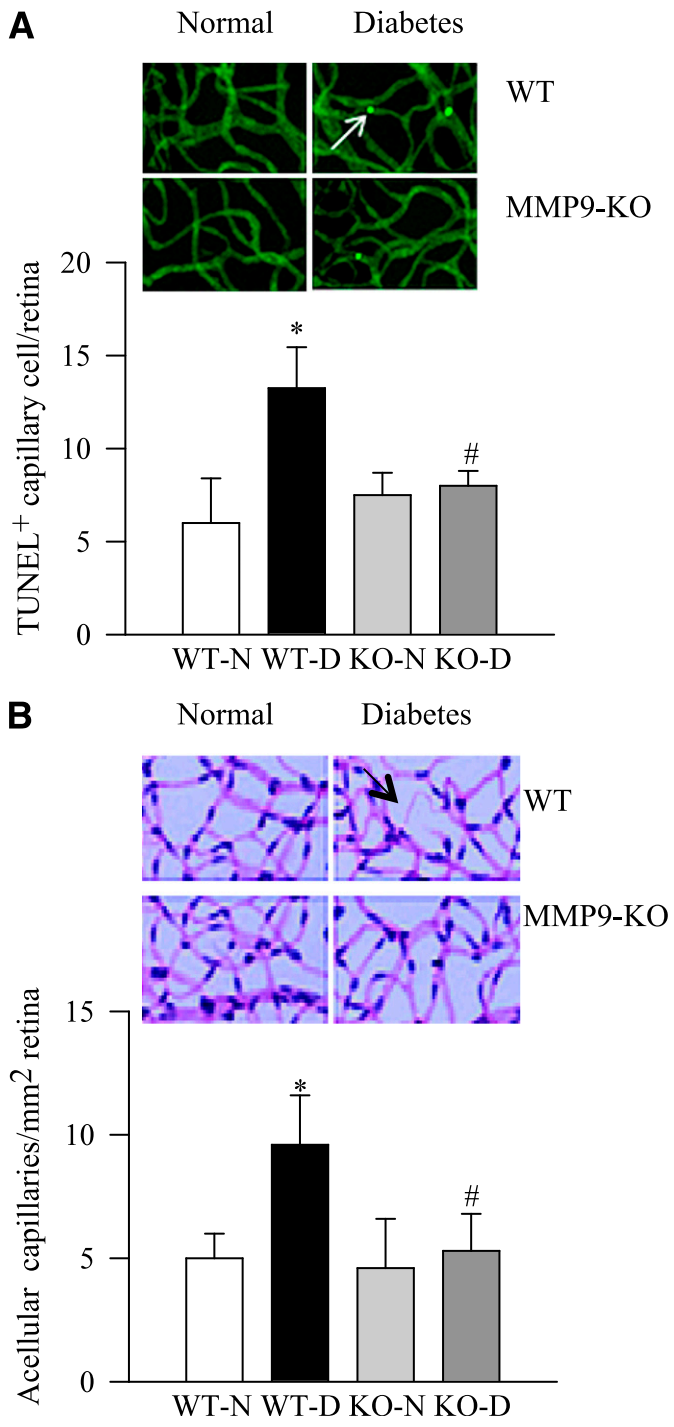


FIG. 1. Abrogation of *MMP-9* gene protects retinal microvasculature from accelerated apoptosis and the development of diabetic retinopathy. Trypsin-digested retinal microvasculature was prepared from MMP-KO and WT mice that were diabetic for ~7 months. *A*: Capillary cell apoptosis was detected by TUNEL staining of the microvasculature, and the arrow indicates TUNEL-positive capillary cell. *B*: After TUNEL staining, the microvessels were stained with periodic acid Schiff–hematoxylin and examined by light microscopy for the basement membrane tubes lacking cell nuclei and maintaining at least one-fourth the normal capillary caliber over their length. The arrow indicates acellular capillary. Results are expressed as mean ± SD using five to six mice in each group. *$P < 0.05$, compared with WT-N; #$P < 0.05$, compared with WT-D. (A high-quality digital representation of this figure is available in the online issue.)

and the development of retinopathy. To understand the mechanism, the effect of regulation of MMP-9 on retinal mitochondrial dysfunction, mitochondrial DNA (mtDNA) damage and ultrastructure was evaluated in MMP-KO mice. As mitochondrial function is largely controlled by their membrane structure, and translocases in the outer membrane (the TOM complex) and translocases in the mitochondrial inner membrane (the TIM complex) help facilitate import of proteins (13,14), we have also investigated the mechanism by which diabetes increases mitochondrial accumulation of MMP-9. The results are confirmed in the retina from human donors with diabetic retinopathy and also in isolated retinal endothelial cells exposed to high glucose with the MMP-9 gene silenced.

RESEARCH DESIGN AND METHODS

Mice. Male, wild-type (WT) C57BL/6 J or MMP-KO (B6.FVB [Cg]-MMP-9tm1Tvu/J) mice were obtained from Jackson Laboratory (Bar Harbor, ME). A group of WT and MMP-KO mice were made diabetic (WT-D and KO-D groups, respectively) by streptozotocin injection (55 mg/kg body wt) for five consecutive days. Mice with blood glucose 250 mg/dL or higher, 3 days after the last injection of streptozotocin, were considered diabetic (WT-N and KO-N groups). Age-matched normal WT and MMP-KO mice served as controls. MMP-KO mice have significantly decreased expression of MMP-9 in their retina, and these mice can be maintained in diabetic conditions (8). Seven months after induction of diabetes, mice were killed by carbon dioxide asphyxiation. One eye was used to remove the retina, and the other eye was incubated in 10% buffered formalin for histopathology. The treatment of the animals conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research and Institutional Guidelines (Wayne State University).

Human retina. Human postmortem eyes were obtained from Midwest Eye Banks (Ann Arbor, MI). Diabetic donors (10–12 years of diabetes) with retinopathy were aged 60–74 years, and nondiabetic donors aged 44–75 years. The eyes were enucleated between 6 and 9 h after death (8). A portion of the freshly isolated retina was processed for mitochondrial isolation.

Retinal endothelial cells. Bovine retinal endothelial cells (BRECs) were grown in Dulbecco's modified Eagle's medium containing 15% heat-inactivated FBS, 5% Nu-serum, 50 μg/mL heparin, and 50 μg/mL endothelial cell growth supplement. Cells from third to fourth passage were transfected with *MMP-9* small interfering RNA (siRNA) using transfection reagents and siRNA duplex from Santa Cruz Biotechnology (Santa Cruz, CA), as previously described (8,12). For control, the cells were transfected with nontargeting scramble RNA. At the end of the transfection, the medium was replaced with either 5 or 20 mmol/L glucose media, and the cells were incubated for four additional days. This method yields about 50% transfection efficiency, and the transfection process does not alter cell survival or proliferation (8). For control, parallel incubations were run using nontransfected cells, and for osmolarity control, cells were incubated in 20 mmol/L mannitol instead of 20 mmol/L glucose.

Apoptosis and histopathology in retinal microvessels. The retina from the formalin-fixed eyes was rinsed overnight with running water, and the microvasculature was isolated by incubating the retina at 37°C with 3% crude trypsin (Invitrogen-Gibco, Grand Island, NY) containing 200 mmol/L sodium fluoride for 45 to 70 min. The neuroretinal tissue was gently brushed away, and the apoptotic vascular cells (endothelial cells/pericytes) were detected by incubating the retinal microvasculature with terminal deoxyribonucleotide transferase-mediated dUTP nick-end labeling (TUNEL) stain (In Situ Cell Death kit; Roche Molecular Biochemicals, Indianapolis, IN). After TUNEL staining, the microvessels were stained with periodic acid Schiff–hematoxylin and examined by light microscopy (10,12).

Mitochondrial isolation. Mitochondria were isolated from the retina or BRECs using mitochondria isolation kit (Pierce, Rockford, IL) according to the manufactures' instructions, as routinely used in our laboratory (12). The mitochondrial pellet was washed with PBS and suspended in the mitochondria lysis buffer (2% CHAPS in 25 mmol/L Tris, 0.15 mol/L NaCl, pH 7.2). Protein concentration was quantified by the bicinchoninic acid assay (Sigma-Aldrich, St Louis, MO).

Activation of MMP-9. Activation of MMP-9 was quantified in the mitochondrial fraction by in situ zymography and confirmed by ELISA. For gelatinase activity, mitochondrial protein (10–20 μg) was separated on nonreducing 8% SDS-PAGE containing 0.1% gelatin. The gel was washed with 2.5% Triton X-100, and incubated for 18 h at 37°C in substrate buffer containing 50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L $CaCl_2$, 200 mmol/L $NaCl_2$, and 0.02% Brij-35. The gel was stained with 0.25% Brilliant Blue *R*-250 (Sigma-Aldrich) and then destained with

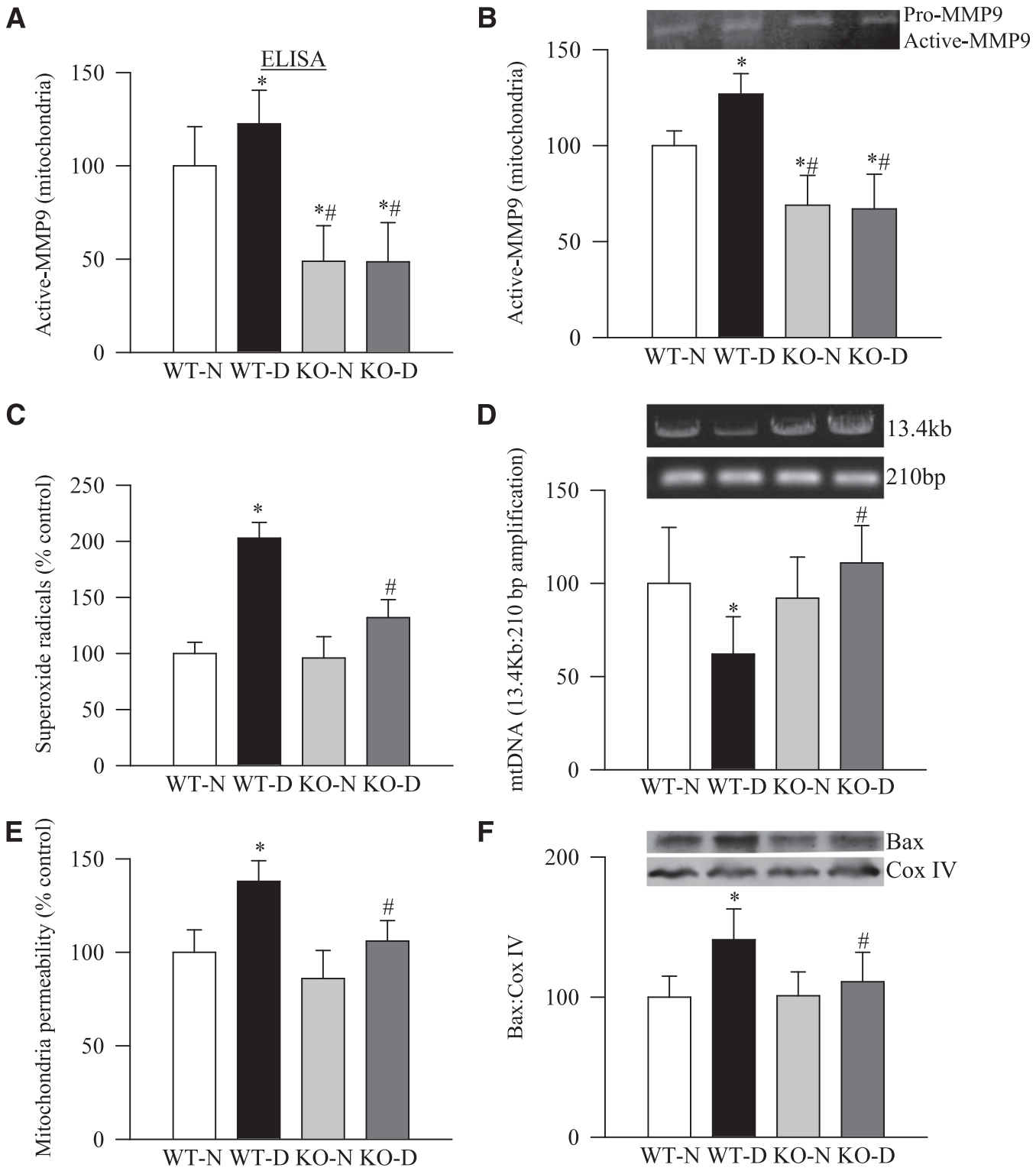


FIG. 2. Diabetes increases MMP-9 in retinal mitochondria, and MMP-9 regulates retinal mitochondrial dysfunction. The active MMP-9 was quantified in the retinal mitochondria by (*A*) ELISA and (*B*) in situ zymography. *C*: Mitochondrial superoxide levels were quantified fluorometrically using MitoTracker Red CM-H_2XRO. *D*: Damage of mtDNA was determined using mitochondrial genome–specific quantitative extended-length PCR with the GeneAmp XL PCR kit. *E*: Mitochondrial membrane potential was evaluated using a spectrophotometric method by measuring calcium chloride–induced decrease in absorbance. *F*: Translocation of Bax into the mitochondria was performed by quantifying the expression of Bax in the mitochondrial fraction. The values represented as mean ± SD are from five mice each in WT-N and KO-N groups, seven mice in WT-D, and six mice in KO-D. *$P < 0.05$, compared with WT-N; #$P < 0.05$, compared with WT-D.

10% acetic acid and 30% methanol (8). The intensity of the active band of MMP-9 (85–80 kD) was quantified using Un-Scan-It Gel digitizing software.

Activation of MMP-9 in the mitochondria was also performed using an ELISA kit (Amersham, Buckinghamshire, U.K.). The final color was developed using a specific peptide chromogen, and the resulting color was measured at 405 nm. Diabetes-induced activation of MMP-9 was calculated by quantifying the immunocaptured MMP-9 that was not treated with amino-phenyl mercuric acetate (7,8).

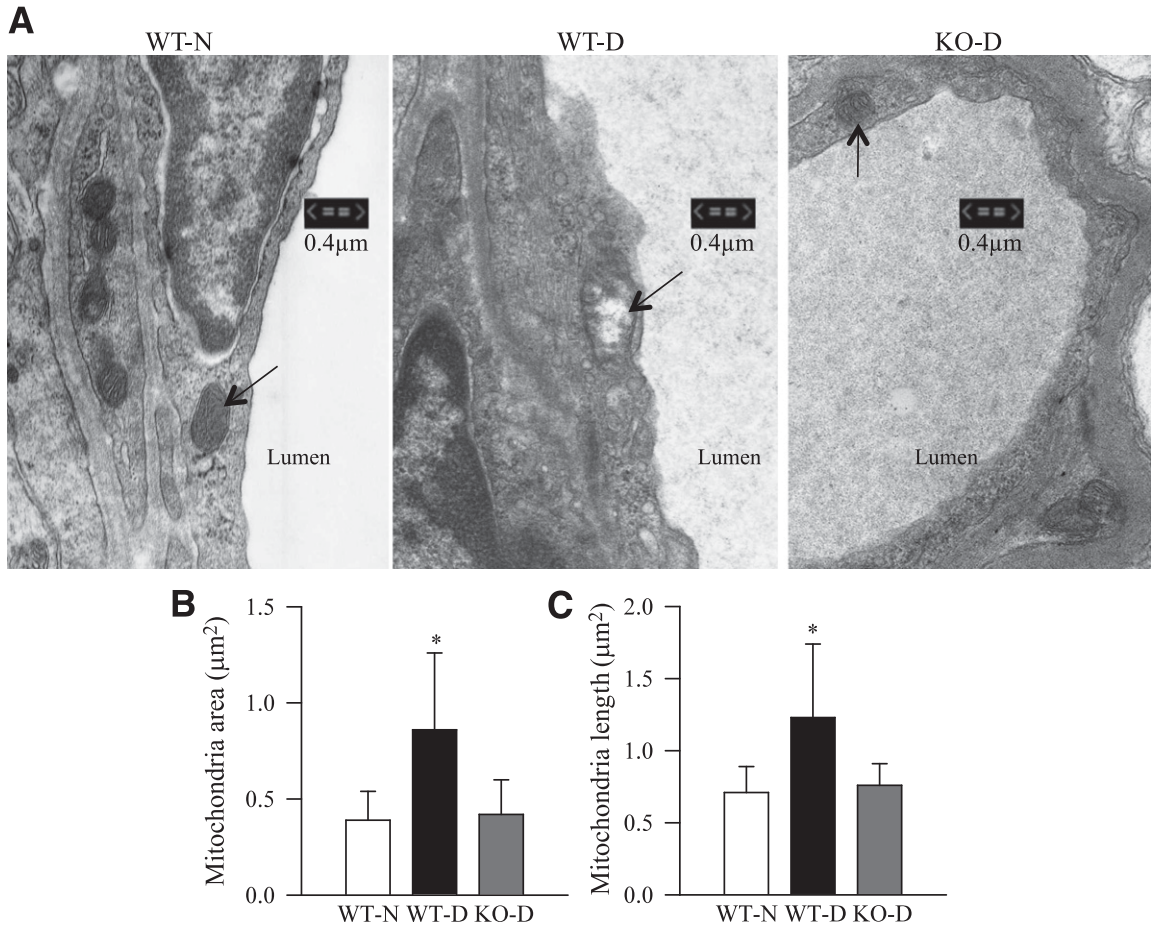


FIG. 3. Mitochondria in the retinal vasculature in MMP-KO mice are protected from diabetes-induced ultrastructure damage. Mitochondrial morphology was evaluated by TEM in the sections from the selected area near the microvasculature region. At least eight images were taken from each sample at 20,000× magnification, and the micrograph is representative of four to five mice in each group. Mitochondria in the endothelium region are marked with arrows. Average (*B*) area and (*C*) length (the longer diameter) of the mitochondria in the retinal endothelium was analyzed in a blind manner using ImageJ software. The histogram represents mean ± SD of 20 or more mitochondria per group. **P* < 0.05, compared with WT-N.

Apoptosis of retinal endothelial cells. A Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics, Indianapolis, IN) was used to detect cell apoptosis. Monoclonal antibodies directed against DNA and histones were used to quantify the relative amounts of mono- and oligonucleosomes generated from apoptotic cells (8).

Mitochondrial damage. Retinal mitochondrial superoxide levels were quantified using a cell-permeable probe, MitoTracker Red CM-H_2XROS (Molecular Probes, Eugene, OR). Freshly harvested retina was homogenized in mitochondria isolation buffer, as described previously (10). Mitochondrial protein (2–5 μg in PBS) was supplemented with 400 nmol/L Mitotracker Red CM-H_2XROS. After incubating for 30 min at 37°C, the resultant fluorescence was measured at 599-nm emission wavelength and 579-nm excitation wavelength using LS 55 fluorescence spectrometer (Perkin-Elmer, Waltham, MA).

The collapse of mitochondrial membrane potential was investigated by quantifying the swelling of the isolated mitochondria using a spectrophotometric method routinely used in our laboratory (10,12,15). Decrease in absorbance, induced by calcium chloride, was followed for 3–5 min at 540 nm.

Mitochondrial DNA damage was determined by mitochondrial genome–specific quantitative extended-length PCR with the GeneAmp XL PCR kit (Applied Biosystems, Foster City, CA). In brief, 15 ng DNA was amplified in a reaction mixture containing 1× XL PCR buffer II, 200 μmol/L dNTP, 1.1 mmol/L Mg $(OAc)_2$, 0.1 μmol/L genome-specific primers (mtDNA-long, forward AAA ATC CCC GCA AAC AAT GAC CAC CCC and reverse GGC AAT TAA GAG TGG GAT GGA GCC AA; mtDNA-short, forward CCT CCC ATT CAT TAT CGC CGC CCT TGC and reverse GTC TGG GTC TCC TAG TAG GTC TGG GAA) and 1 unit r*Tth* DNA polymerase. Mitochondrial DNA was amplified with 24 cycles, and PCR products were resolved on agarose gel. Relative amplification was quantified by normalizing the intensity of the long product to the short product (mtDNA = 13.4 kb/210 bp). These methods are routinely used in our laboratory (16).

Mitochondrial morphology. Ultrastructutre of the mitochondria in the retinal vasculature region was evaluated by transmission electron microscopy (TEM). The enucleated eyes were dissected along the equators, and the retina was incubated for 24 h in 2.5% glutaraldehyde (in 100 mmol/L phosphate buffer, pH 7.4). The retina was postfixed in 1% buffered osmium tetroxide and dehydrated using graded ethanol solutions. After embedding the samples in 812 resin (Electron Microscope Science, Hatfield, PA), ultrathin transverse sections (70–80 nm) of the areas near the microvasculature region were prepared. The sections were stained with uranyl acetate and lead citrate and viewed by TEM (Zeiss EM 900, Oberkochen, Germany). At least eight random images were recorded from each independent preparation and enlarged to 20,000× magnification. Mitochondria in the endothelial cells were analyzed in a blind manner by ImageJ software to calculate their area (μm^2) and average length (μm) as previously described (17).

Localization of MMP-9 in retinal endothelial cells. BRECs grown on 12-mm diameter cover slips coated with 0.1% gelatin were incubated with 5 mmol/L or 20 mmol/L glucose for 4 days. At the end of the incubation, the cells were incubated with 500 nmol/L MitoTracker (green; Molecular Probes) for 30 min (12,16). The cells were washed with PBS, fixed in cold methanol for 20 min, and permeabilized in 0.25% Triton X-100 for 15 min. They were blocked in 5% BSA for 1 h and incubated with anti–MMP-9 antibody (Santa Cruz Biotechnology). After washing the cells with PBS, they were incubated with anti-rabbit secondary antibody (Taxes red conjugated; Molecular Probes), washed with PBS, and mounted on slides using Vecta Shield containing DAPI (Vector Laboratories, Burlingame, CA). The slides were examined under a Zeiss ApoTome using 40× magnification (Carl Zeiss Inc., Chicago, IL).

Western blot analysis. Retinal mitochondrial protein (25–30 μg) was separated on a 4–20% SDS-PAGE and transferred to nitrocellulose membranes using a semidry transfer. The membranes were incubated with the antibody against the target protein (Bax, TOM34, and TIM44 from Santa Cruz

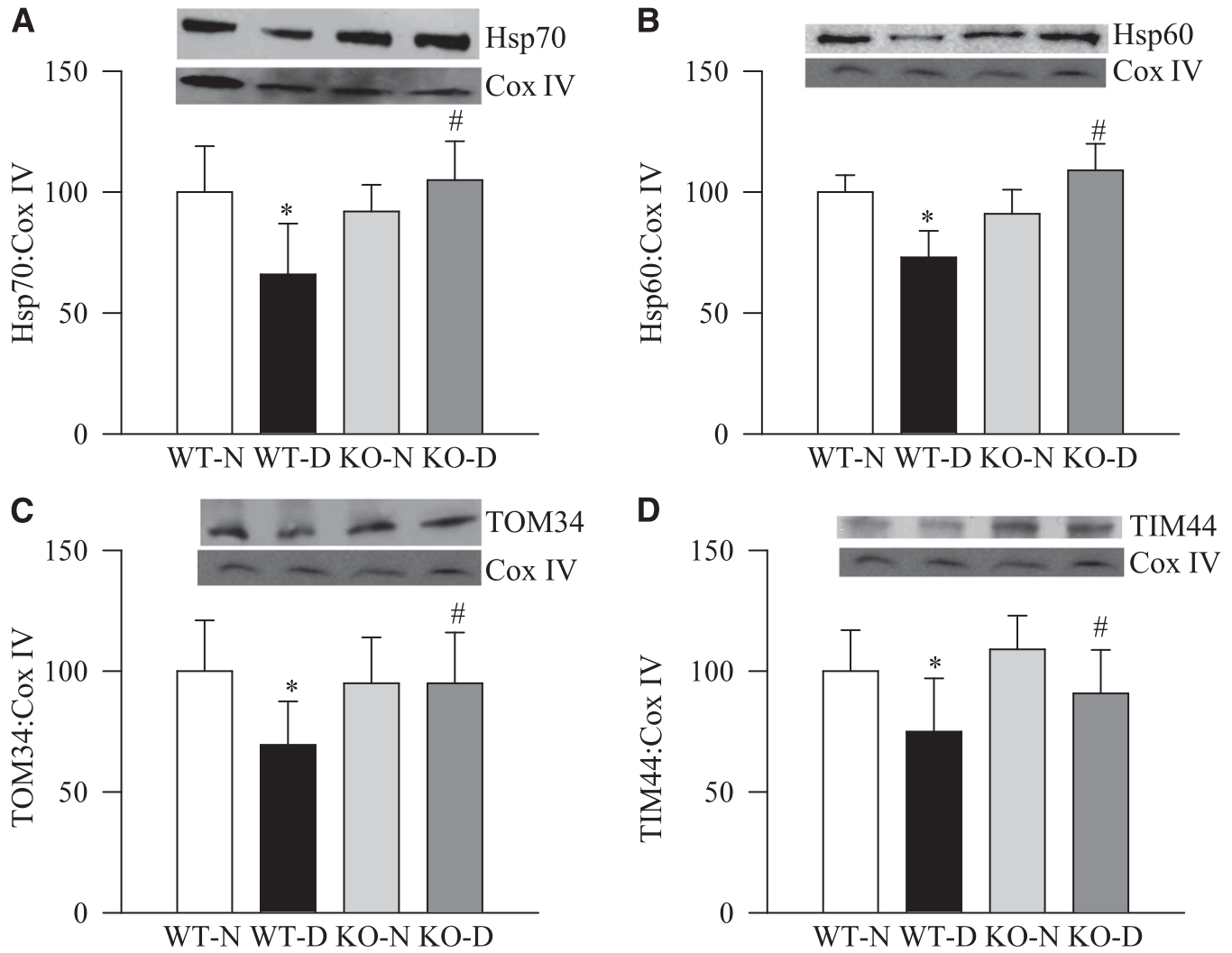


FIG. 4. Diabetes-induced decreases in chaperone proteins and translocases in the retinal mitochondria are ameliorated in MMP-KO mice. The levels of (*A*) Hsp70, (*B*) Hsp60, (*C*) TOM34, and (*D*) TIM44 were quantified in retinal mitochondria by Western blot technique using their respective antibodies. Cox IV was used as a loading protein. Each measurement was performed in six mice each in WT-N and KO-D groups, five in WT-D, and four in KO-N, and the values are expressed as mean ± SD. **P* < 0.05, compared with WT-N; #*P* < 0.05, compared with WT-D.

Biotechnology; heat shock protein 70 [Hsp70] and Hsp60 from Abcam, Cambridge, MA). Membranes were reprobed with Cox IV to evaluate the lane-loading control. The band intensities were quantified by Un-Scan-It software.

Binding of MMP-9 with chaperones and TIM44. Retinal or cell homogenate (150–200 μg) was incubated overnight at 4°C with 2 μg of anti–MMP-9 antibody. Agarose beads A/G (Santa Cruz Biotechnology) were mixed with protein antibody for 1 h at 4°C. The immune precipitate was collected and washed three times with the lysis buffer without EDTA or EGTA. Samples were boiled in 2× Laemmli buffer and subjected to electrophoresis on 10% SDS-PAGE (12,15). Western blot analysis was performed using antibodies against Hsp70, Hsp60, or TIM44.

Statistical analysis. Each measurement was made in duplicate, and the assay was repeated three or more times. Data are expressed as mean ± SD. Statistical analysis was performed using the nonparametric Kruskal-Wallis test followed by Mann-Whitney test, and $P < 0.05$ was considered statistically significant.

RESULTS

Mouse retina. Diabetes of ~7 months in WT mice, as expected, significantly increased the apoptosis of capillary cells. The number of TUNEL-positive cells in the trypsin-digested retinal microvasculature was about twofold higher compared with the WT-nondiabetic (WT-N) mice (Fig. 1*A*). In the same retinal vasculature, the number of degenerative capillaries was also significantly increased (Fig. 1*B*). Consistent with the increase in active MMP-9 in the retina in diabetes (7,8), retinal mitochondria also had an ~25% increase in active MMP-9, as confirmed by both ELISA and in situ zymography assays (Fig. 2*A* and *B*). In the same WT-diabetic (WT-D) mice, mitochondrial superoxide levels were elevated by >100%, mtDNA was damaged (40% reduction in the amplification of the long fragment of mtDNA), mitochondrial permeability was increased, and the translocation of Bax into the mitochondria was increased by ~50% compared with the values from age-matched WT-N mice (Fig. 2*C*–*F*). A significant number of mitochondria in WT-D mice in the endothelium region of the microvasculature were elongated and swollen with electron-lucent matrix and partial cristolysis compared with the intact and tightly packed lamellar cristae in WT-N mice (Fig. 3*A*). The average area and length of the mitochondria in the endothelial region were ~40% larger in WT-D mice compared with WT-N mice (Fig. 3*B* and *C*). The chaperone proteins (Hsp70 and Hsp60) and the translocators (TOM34 and TIM44) were significantly decreased in the retinal mitochondria (Fig. 4*A*–*D*), but the binding of MMP-9 with Hsp70, Hsp60, or TIM44 was increased by 40–80% (Fig. 5).

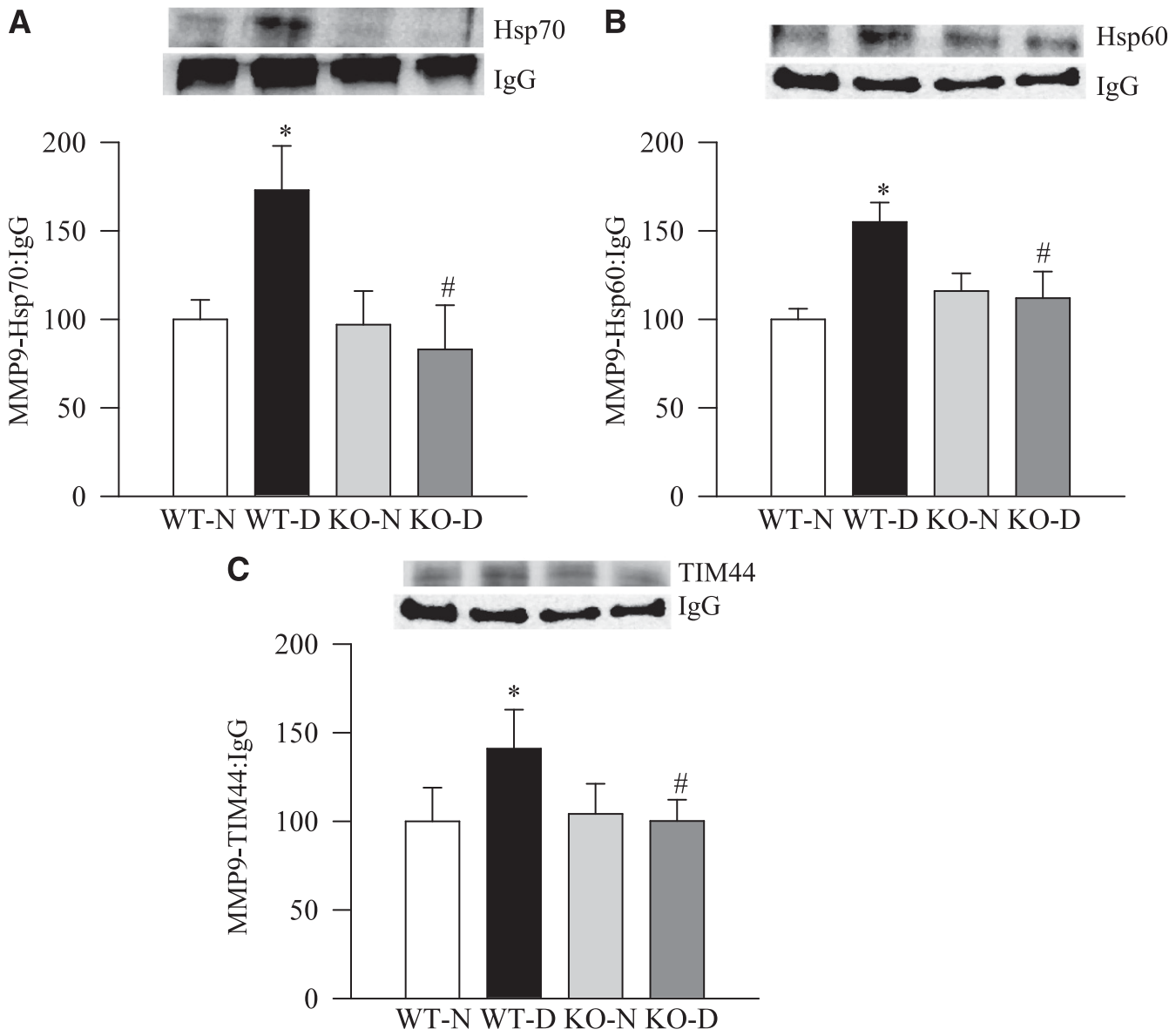


FIG. 5. Binding of MMP-9 with the chaperones and TIM44. Retinal homogenate was immunoprecipitated with anti–MMP-9. Relative abundance of Hsp70, Hsp60, or TIM44 was determined by Western blotting using their respective antibodies. IgG was used as the loading control. Results are expressed as mean ± SD of five to seven mice in each group. The values obtained from mice in the WT-N group are considered as 100%. *$P < 0.05$ and #$P < 0.05$, compared with WT-N and WT-D, respectively.

Retinal microvasculature of MMP-KO mice was protected from diabetes-induced accelerated apoptosis, and the number of degenerative capillaries was also significantly lower compared with the values obtained from WT-D mice (Fig. 1). In the same MMP-KO mice, diabetes had no effect on active MMP-9 in the retinal mitochondria; this was significantly lower compared with WT-D mice (Fig. 2). In addition, mitochondrial dysfunction was protected; the levels of mitochondrial superoxide, damage to the mtDNA, and swelling were significantly lower in MMP-KO diabetic mice compared with WT-D mice (Fig. 2*C–F*). TEM of the endothelium region of the retinal microvasculature from MMP-KO diabetic mice showed mitochondria with normal ultrastructure; their average area and length were significantly lower compared with the WT-D group, and the cristae were more tightly packed and lamellar compared with the WT-D mice (Fig. 3). Chaperone proteins and the translocase system remained normal in the mitochondria; expressions of Hsp70, Hsp60, TOM34, and TIM44 were significantly higher in the retinal mitochondria from MMP-KO diabetic mice compared with WT diabetic mice, and these values were similar to those observed in MMP-KO mice that are nondiabetic (KO-N) and WT-N mice (Fig. 4). Similarly, an increase in the binding of retinal MMP-9 with Hsp70, Hsp60, or TIM44, as observed in WT diabetic mice, was also prevented in the MMP-KO diabetic mice (Fig. 5).

Human retina. To confirm our results obtained from the rodent model, major parameters were quantified in the retinal mitochondria prepared from human donors with diabetic retinopathy. Mitochondria from donors with diabetic retinopathy had 60–70% increase in active MMP-9 compared with age-matched, nondiabetic donors, and significantly decreased expression of Hsp70 and Hsp60. The binding of MMP-9 with Hsp70 or with Hsp60 was also significantly increased (Fig. 6).

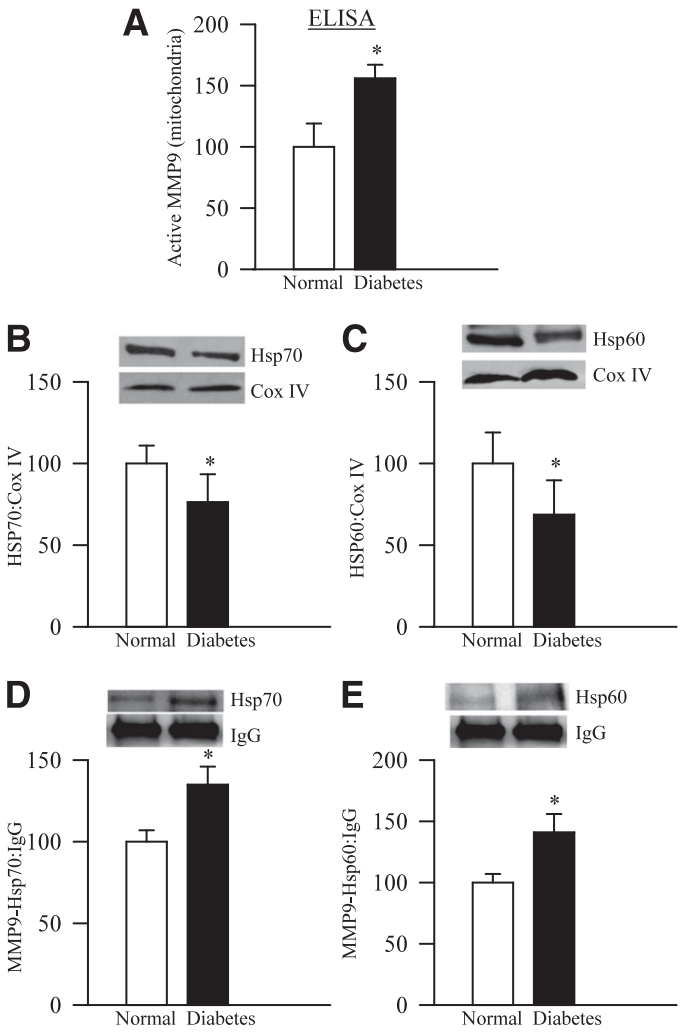


FIG. 6. MMP-9 is activated in retinal mitochondria from patients with diabetic retinopathy, and binding of MMP-9 with chaperones is increased. Mitochondria were prepared from the retina of donors with diabetic retinopathy and age-matched nondiabetic subjects by differential centrifugation. *A*: Active MMP-9 was quantified by ELISA technique. Expression of (*B*) Hsp70 and (*C*) Hsp60 was quantified in the mitochondria by Western blot technique, and binding of MMP-9 with (*D*) Hsp70 or (*E*) Hsp60 by immunoprecipitating MMP-9. Each measurement was performed in duplicate in the samples obtained from four nondiabetic donors and five donors with diabetic retinopathy. The values are represented as mean ± SD. *$P < 0.05$, compared with the values from nondiabetic donors.

Endothelial cells. Exposure of retinal endothelial cells, the site of histopathology characteristic of diabetic retinopathy, to high glucose increased the accumulation of MMP-9 in the mitochondria; cells incubated in high glucose had increased staining of MMP-9 (red) in the mitochondria compared with the cells exposed to normal glucose (Fig. 7*A*). In the same cell preparations, the active MMP-9 was ~40% higher in the mitochondria from the cells exposed to high glucose as confirmed by both in situ zymography (Fig. 7*B*) and ELISA (Fig. 7*C*), and cell apoptosis was elevated by >75% (Fig. 7*D*). Consistent with the retina, high glucose decreased mitochondrial Hsp70 and Hsp60 expressions and increased the binding of MMP-9 with Hsp70 or Hsp60 as evidenced by their increased expression in the cells immunoprecipitated for MMP-9 (Fig. 8).

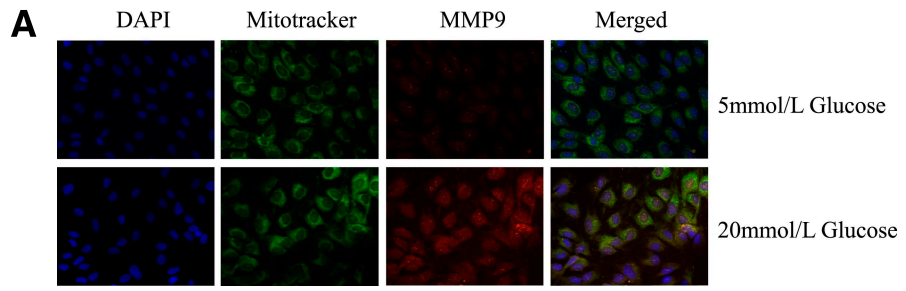
Regulation of MMP-9 by siRNA prevented a glucose-induced increase in mitochondrial MMP-9 (Fig. 7*B* and *C*) and cell apoptosis (Fig. 7*D*). Furthermore, a decrease in mitochondrial Hsp70 and Hsp60, and increase in the binding of MMP-9 with these chaperone proteins, experienced by BRECs in high-glucose conditions, was also ameliorated in the cells transfected with *MMP-9* siRNA. The values obtained in high-glucose conditions from the cells transfected with MMP-9 siRNA were significantly different from the cells untransfected or transfected with scramble RNA (Fig. 8).

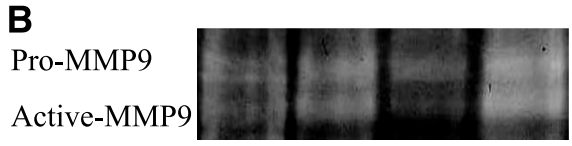
DISCUSSION

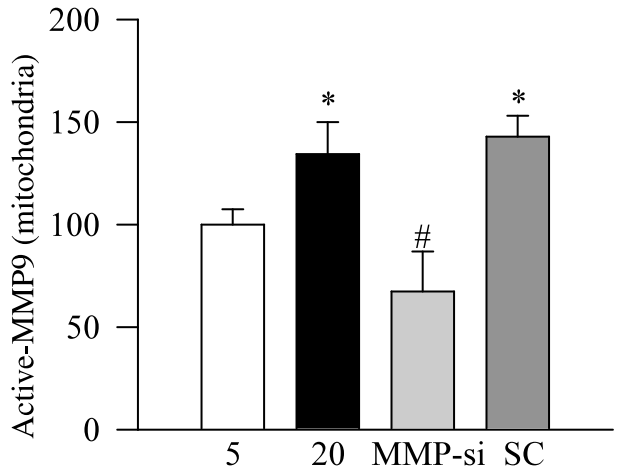
This is the first report demonstrating a direct role of MMP-9 in the development of diabetic retinopathy; here we show that the genetic ablation of MMP-9 ameliorates diabetic mice from the development of retinopathy. MMP-KO mice, diabetic for ~7 months, are protected from histopathology characteristic of early signs of retinopathy, and retinal capillary cell apoptosis is not accelerated. Their retinal mitochondria have normal levels of active MMP-9, function, and ultrastructutre, and mtDNA is not damaged. Furthermore, abrogation of *MMP-9* gene also protects retinal mitochondrial transport machinery and chaperone proteins from diabetes-induced abnormalities. Retinal mitochondria from donors with diabetic retinopathy have similar alterations in active MMP-9 and chaperone proteins. Silencing of *MMP-9* gene using *MMP-9* siRNA in isolated retinal endothelial cells, the site of histopathology associated with diabetic retinopathy, prevents damage to the mitochondrial chaperone machinery, experienced by cells in high glucose. Together, these results strongly imply a significant role for mitochondrial MMP-9 in the development of diabetic retinopathy, and the mechanism appears to be through damage of retinal mitochondria.

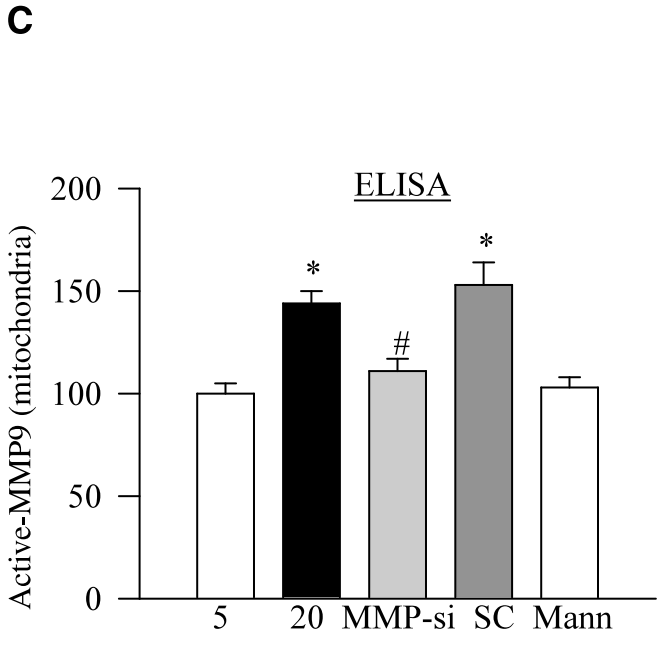
Matrix metalloproteinases can cleave extracellular matrix constituents and nonmatrix proteins (18). These enzymes exert various roles at different stages of the disease. In diabetes, retinal MMPs are postulated to facilitate an increase in vascular permeability through proteolytic degradation of the tight junction proteins and disruption of the overall tight junction complex (19–21). We have shown that the activation of MMP-9, the most complex member of the MMP family (5,22), is modulated by a small molecular weight G protein, H-Ras, and triggers apoptosis of retinal capillary cells (7). Now we provide convincing data showing a direct role of MMP-9 in the development of diabetic retinopathy. The retinal vasculature of diabetic mice with the *MMP-9* gene abrogated is protected from accelerated apoptosis and from the histopathology characteristic of diabetic retinopathy. This implies that the inhibitors of MMP-9 have potential to impede the development of retinopathy in diabetic patients. Optimistically, these inhibitors are being used in clinical trials for other diseases, including chronic obstructive pulmonary disease and multiple sclerosis (23).

The retina and its capillary cells experience increased oxidative stress in diabetes, and increased oxidative stress is considered to play a significant role in the development of diabetic retinopathy (4,24–26). Dysfunctional retinal mitochondria become swollen and their permeability is increased. Cytochrome c leaks into the cytosol, and Bax translocates into the mitochondria (9,10). Data presented here show increased active MMP-9 in the mitochondria and its capillary cells in hyperglycemic conditions, and these results are in agreement with the results from others showing association of active mitochondrial MMP-9 with mechanical dysfunction of cardiac myocytes (27).









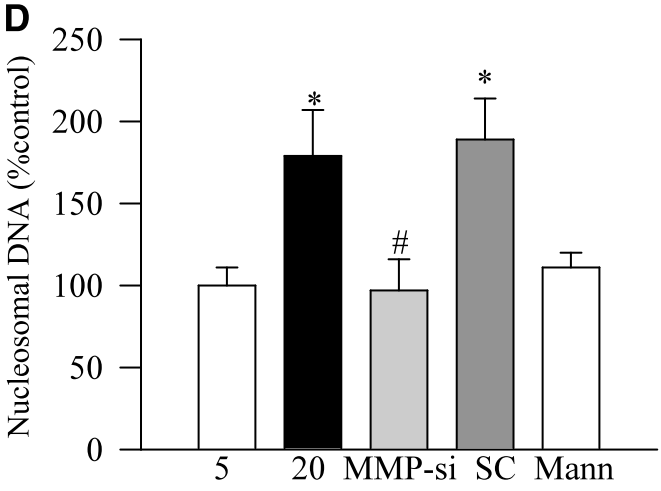


FIG. 7. Effect of high glucose on mitochondrial MMP-9 in retinal endothelial cells. *A*: Localization of MMP-9 in retinal endothelial cells was performed immunohistochemically. For mitochondria, MitoTracker (green) was used, and for MMP-9, Texas Red–conjugated antibody was used. The coverslips were mounted using Vecta Shield containing DAPI (blue, nucleus). The slides were examined under a Zeiss ApoTome using 40× magnification. The picture is representative of three to four different preparations. *B*: Gelatinase activity of MMP-9 was measured in the incubation medium by in situ zymography. The active form of MMP-9 (~80 kD) was quantified and presented in the accompanying histogram. *C*: Active MMP-9 in the mitochondrial fraction was quantified by performing ELISA. *D*: Cell apoptosis was quantified by performing ELISA for cytoplasmic histone–associated DNA fragments using a commercially available kit from Roche Diagnostics. Each measurement was performed in three to four different cell preparations. The values obtained from the untransfected cells incubated in 5 mmol/L glucose are considered as 100% (control). 5 and 20, untransfected cells incubated in 5 or 20 mmol/L glucose for 4 days; MMP-si and SC, cells transfected with *MMP-9* siRNA or with scramble RNA, respectively; Mann, cells incubated in 20 mmol/L mannitol for 4 days. *$P < 0.05$, compared with the values obtained from cells incubated in 5 mmol/L glucose; #$P < 0.05$, compared with values from cells in 20 mmol/L glucose for 4 days. (A high-quality digital representation of this figure is available in the online issue.)

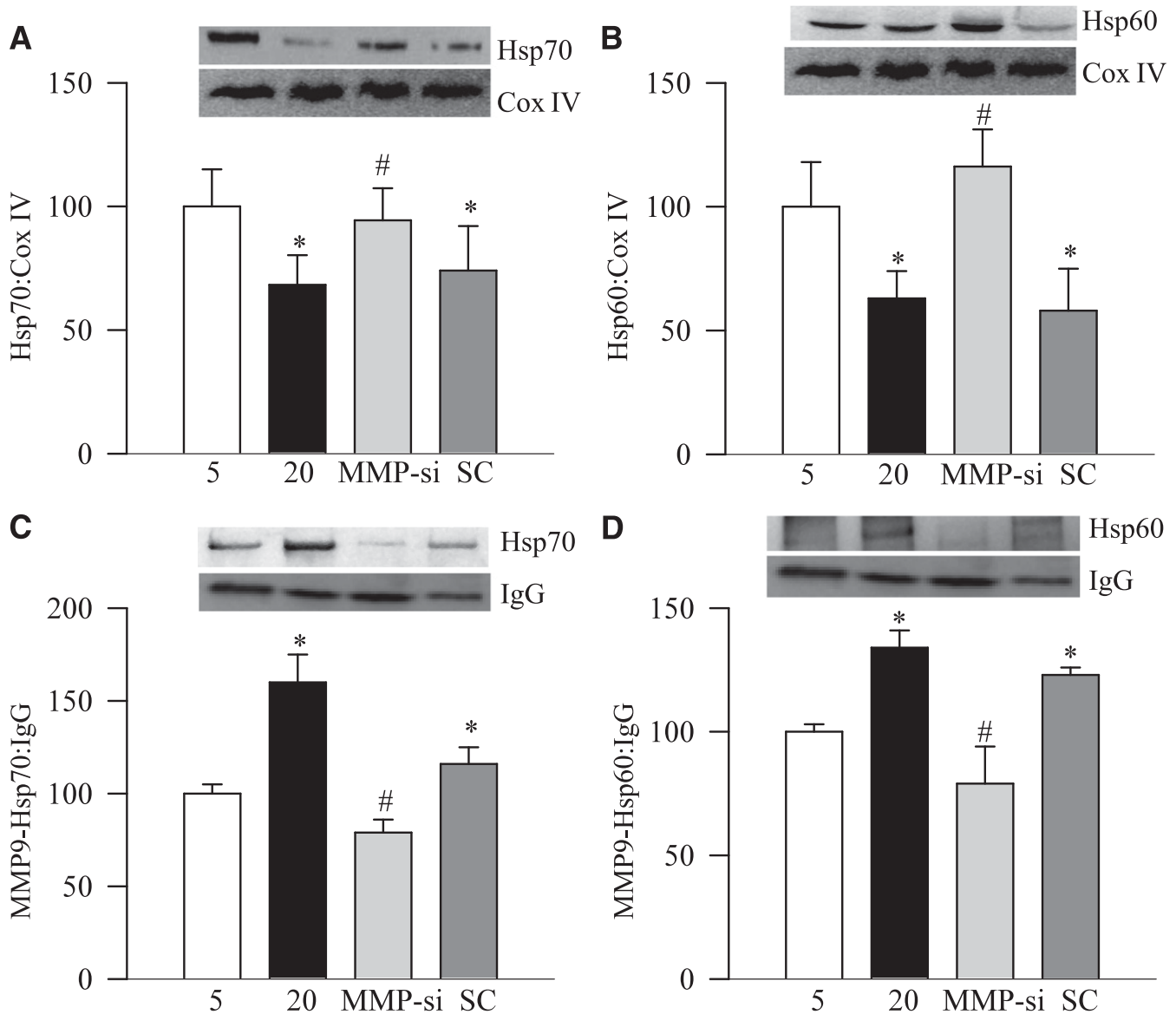


FIG. 8. ***MMP-9*** **siRNA regulates glucose-induced decrease in the mitochondrial Hsp70 and Hsp60, and binding of MMP-9 with these chaperone proteins. Expressions of (*A*) Hsp70 and (*B*) Hsp60 were quantified in mitochondria prepared from BRECs transfected with** ***MMP-9*** **siRNA (MMP-si) or scramble RNA (SC), and incubated in 20 mmol/L glucose for 4 days. For binding, MMP-9 was immunoprecipitated from cell homogenate using anti–MMP-9, and the relative abundance of (*C*) Hsp70 and (*D*) Hsp60 was determined by Western blotting using IgG as the loading control. Results are expressed as mean ± SD of three or more experiments. The values obtained from the cells incubated in 5 mmol/L glucose are considered as 100%. *$P < 0.05$, compared with 5 mmol/L glucose; #$P < 0.05$, compared with 20 mmol/L glucose.**

The retinae of *MMP*-KO diabetic mice are protected from increased superoxide radicals and mtDNA damage, and their mitochondrial permeability remains normal. This suggests that the retinal-activated MMP-9 in diabetes damages mitochondria and increases their permeability, Bax moves into the mitochondria, and apoptotic machinery is activated. There remains a possibility that the TUNEL staining used to detect apoptosis of retinal capillary cells is also detecting late necrosis. However, the beneficial effect of *MMP-9* gene knockout on diabetes-induced increased translocation of proapoptotic Bax into the mitochondria and on the swelling of the mitochondria further confirms the role of MMP-9 in mitochondrial damage resulting in apoptosis. Furthermore, *MMP-9* gene ablation also prevents diabetes-induced ultrastructural changes in the mitochondria in the retinal microvasculature, suggesting a major role of MMP-9 in diabetes-induced mitochondrial dysfunction and disruption. Consistent with these, MMP-9 is considered as a negative regulator of mitochondrial function (11,28), and active MMP in cardiac myocytes is postulated to initiate a negative feedback cycle that degrades mitochondrial membrane potential and impairs function (29). Mitochondrial ultrastructural alterations are observed in cells undergoing apoptosis, and the swollen state is considered to coincide with the beginning stages of cell death (30). In the pathogenesis of diabetic neuropathy, elongated and swollen mitochondria with disrupted cristate are observed in ganglion cells (31,32). Our results demonstrating the prevention of diabetes-induced elongation and partial cristolysis of the retinal endothelium mitochondria by ablation of *MMP-9* genes strongly imply that the damage to the mitochondria could be one of the major mechanisms by which active MMP-9 contributes to the development of diabetic retinopathy. The loss of pericyte is also considered as one of the earliest morphological changes

in the development of diabetic retinopathy (33), and the possibility that similar MMP-9–mediated mitochondrial damage, as seen in retinal endothelial cells, is also operating in the retinal pericytes cannot be ruled out.

To understand the molecular mechanism by which MMP-9 is increased in the retinal mitochondria in diabetes, we investigated the chaperone and transport system. Mitochondrial protein import and sorting are mediated by the translocase complexes in the membranes and chaperones in the aqueous compartments operating along the import pathways (13,14). The Hsps play an important role in protein–protein interactions and in prevention of unwanted protein aggregation. These molecular chaperones bind and stabilize proteins at intermediate stages of folding, assembly, and aid in the transport of proteins across subcellular membranes to the appropriate cellular compartments. The Hsp60 chaperone system is a prominent component of the mitochondrial protein quality control system, and it helps in the folding of mitochondrial proteins after import has been completed (34). The other chaperone, Hsp70, is mostly antiapoptotic and is shown to inhibit translocation of Bax into mitochondria and release of cytochrome c from mitochondria. Mitochondrial Hsp70 acts as the first chaperone that a newly imported polypeptide chain encounters in the mitochondria (34,35). Our results show that the ablation of *MMP-9* gene in mice prevents a diabetes-induced decrease in mitochondrial Hsp70 and Hsp60, suggesting that MMP-9 has a critical role in regulating mitochondrial Hsp70 and Hsp60.

The Hsp70 helps maintain cellular homeostasis, and acts as a carrier of peptides or proteins. Although Hsp60 is mainly a mitochondrial protein, cytosolic Hsp60 prevents translocation of the proapoptotic protein Bax into mitochondria (36,37). Increased binding of retinal MMP-9 with Hsp60 and Hsp70 in diabetes, and regulation of these bindings by inhibition of MMP-9 activation (MMP-KO mice and *MMP-9* siRNA–transfected cells) clearly suggests that in diabetic conditions, Hsp70 and Hsp60 are important in chaperoning MMP-9 to the mitochondria.

Mitochondria also have a well-defined translocase system to import nuclear-encoded proteins into mitochondrial membranes (13,14). This system functions as a receptor for recognition of the targeting and/or intramitochondrial sorting signals, and also a driving force for translocating the polypeptide chain. The TOM and TIM complexes translocate proteins across the mitochondrial outer and inner membranes, and both the inner membrane potential and matrix Hsp70 are essential for releasing the preprotein from the TOM complex. The precursor proteins are bound to the molecular chaperones in the cytosol and are delivered to the TOM import machinery of the mitochondrial outer membrane (13,38). TIM44 serves as an adaptor protein for the binding of mitochondrial Hsp70 to the membrane (35). Crossing of proteins into the mitochondria and transporting into the inner membrane are also regulated by the membrane potential (39). Protection of decreased levels of TOM34, TIM44, Hsp70, and Hsp60 in the retinal mitochondria and inhibition of increased mitochondrial permeability, experienced by retina and its capillary cells in diabetes, by abrogation of *MMP-9* strongly suggests the role of MMP-9 in modulating mitochondrial transport system.

Recent work from our laboratory has shown that human donors with diabetic retinopathy have increased MMP-9 in their retinal microvessels, and the activation is achieved through the Ras/Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase pathway signaling cascade (8). The results presented here clearly show that the retinal mitochondria of the same donors also have activated MMP-9 and decreased mitochondrial Hsp70 and Hsp60, but the binding of MMP-9 with Hsp70 and with Hsp60 is increased, further strengthening the role of mitochondrial MMP-9 in the development of diabetic retinopathy.

In summary, the activation of MMP-9 in the retinal mitochondria is one of the key events in diabetes that initiates mitochondrial dysfunction, damages mitochondrial structure, and activates apoptotic machinery. MMP-9 is transported to the mitochondria by Hsp70/Hsp60, and activated MMP-9 damages mitochondrial integrity and its DNA. Thus, inhibition of MMP-9 could have potential therapeutic value in preventing the continuation of the vicious cycle of mitochondrial damage that the retina experiences in diabetes (16), ultimately inhibiting the development and continued progression of diabetic retinopathy.

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R.A.K. developed the experimental plan, performed the literature search, and wrote and edited the manuscript. G.M., J.M.d.S., and Q.Z. researched data.

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