

Therapeutic Effects of PPAR α Agonists on Diabetic Retinopathy in Type 1 Diabetes Models

Ying Chen,^{1,2} Yang Hu,^{1,2} Mingkai Lin,^{1,2,3} Alicia J. Jenkins,^{2,4} Anthony C. Keech,⁵ Robert Mott,¹ Timothy J. Lyons,² and Jian-xing Ma^{1,2}

Retinal vascular leakage, inflammation, and neovascularization (NV) are features of diabetic retinopathy (DR). Fenofibrate, a peroxisome proliferator-activated receptor α (PPAR α) agonist, has shown robust protective effects against DR in type 2 diabetic patients, but its effects on DR in type 1 diabetes have not been reported. This study evaluated the efficacy of fenofibrate on DR in type 1 diabetes models and determined if the effect is PPAR α dependent. Oral administration of fenofibrate significantly ameliorated retinal vascular leakage and leukostasis in streptozotocin-induced diabetic rats and in Akita mice. Favorable effects on DR were also achieved by intravitreal injection of fenofibrate or another specific PPAR α agonist. Fenofibrate also ameliorated retinal NV in the oxygen-induced retinopathy (OIR) model and inhibited tube formation and migration in cultured endothelial cells. Fenofibrate also attenuated overexpression of intercellular adhesion molecule-1, monocyte chemoattractant protein-1, and vascular endothelial growth factor (VEGF) and blocked activation of hypoxia-inducible factor-1 and nuclear factor- κ B in the retinas of OIR and diabetic models. Fenofibrate's beneficial effects were blocked by a specific PPAR α antagonist. Furthermore, *Ppara* knockout abolished the fenofibrate-induced downregulation of VEGF and reduction of retinal vascular leakage in DR models. These results demonstrate therapeutic effects of fenofibrate on DR in type 1 diabetes and support the existence of the drug target in ocular tissues and via a PPAR α -dependent mechanism. *Diabetes* 62:261–272, 2013

With the rising incidence of diabetes, the prevalence of the vascular complications of diabetes are increasing, in spite of recent advances in therapies targeting hyperglycemia, hypertension, and dyslipidemia (1,2). Diabetic retinopathy (DR) is a feared and common microvascular complication of diabetes and one of the most common sight-threatening conditions in developed countries (3). DR is a chronic, progressive, and multifactorial disorder, primarily affecting retinal capillaries (4,5). Diabetes induces retinal inflammation, blood-retinal barrier breakdown,

and increased retinal vascular permeability, leading to diabetic macular edema (DME) (6). In proliferative DR, overproliferation of capillary endothelial cells results in retinal neovascularization (NV), which can cause severe vitreous cavity bleeding, retinal detachment, and vision loss (7,8).

Unlike type 2 diabetes, in type 1 diabetes, obesity, the metabolic syndrome, and dyslipidemia are less common, although when present in people with type 1 diabetes, they are risk factors for micro- and macrovascular complications (9,10). Retinopathy in both type 1 and type 2 diabetes develops retinal vascular leakage, inflammation, NV, and fibrosis (11). Even though it is well established that vascular endothelial growth factor (VEGF) mediates the pathologic processes of vascular leakage and angiogenesis in DR, anti-VEGF compounds are not always effective in all patients with DR (12). This may be ascribed to the fact that DR is mediated by multiple angiogenic, inflammatory, and fibrogenic factors such as VEGF, tumor necrosis factor- α (13), intercellular adhesion molecule-1 (ICAM-1) (14), and connective tissue growth factor (15), and thus, blockade of VEGF alone is not sufficient to ameliorate all of the perturbed signaling.

Fenofibrate, a peroxisome proliferator-activated receptor α (PPAR α) agonist, available clinically for >30 years for the treatment of dyslipidemia (16,17), is particularly effective in improving the lipid profile in hypertriglyceridemia and low HDL syndromes (18), and for reducing some cardiovascular events (19). Recent studies reported that activation of PPAR α suppresses transforming growth factor- α -induced matrix metalloproteinase-9 expression in human keratinocytes (20), blocks tumor angiogenesis via vascular NADPH oxidase (21), modulates endothelial production of inflammatory factors (22), and improves wound healing in pediatric burn patients (23). In the retinal pigment epithelium, fenofibrate modulates cell survival signaling (24) and reduces diabetic stress-induced fibronectin and type IV collagen overexpression (25). Moreover, fenofibrate also prevents interleukin-1 β -induced retinal pigment epithelium disruption through inhibition of the activation of AMP-activated protein kinase (26).

Recent studies suggest that PPAR α is an emerging therapeutic target in diabetic microvascular complications (27–29). Two recent, large, prospective, placebo-controlled clinical trials have demonstrated protective effects of fenofibrate against DR in type 2 diabetic patients. The Fenofibrate Intervention in Event Lowering in Diabetes (FIELD) Study reported that fenofibrate monotherapy significantly reduced the cumulative need for laser therapy for DR by 37% (30), nephropathy progression by 14% (31), and amputations (23), including microvascular amputations, by 37% (32) in type 2 diabetic patients. The Action to Control Cardiovascular Risk in Diabetes (ACCORD) Lipid Study of combination simvastatin and fenofibrate

From the ¹Department of Physiology, The University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma; the ²Harold Hamm Diabetes Center, The University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma; the ³Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China; the ⁴Department of Medicine, The University of Melbourne, Melbourne, Australia; and the ⁵National Health and Medical Research Council Clinical Trials Centre, University of Sydney, Sydney, Australia.

Corresponding author: Jian-xing Ma, jian-xing-ma@ouhsc.edu.

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Y.C. and Y.H. contributed equally to this study.

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See accompanying commentary, p. 36.

demonstrated a 40% reduction in progression of proliferative DR in type 2 diabetic patients over simvastatin only (33). Despite these exciting clinical findings, several unanswered questions remain. Is fenofibrate effective against DR in type 1 diabetes? Is the fenofibrate effect on DR a direct action on retinal vasculature or through the systemic lipid-lowering effect? Are the ocular fenofibrate effects PPAR α dependent? This study was designed to address these important questions.

In the current study, we explore whether fenofibrate has therapeutic effects on DR in type 1 diabetes animal models, and on ischemia-induced retinal NV, and whether such effects are dependent on PPAR α .

RESEARCH DESIGN AND METHODS

Animals. Brown Norway (BN) rats, male Akita mice and their age-matched wild-type (Wt) littermates (C57BL/6 mice), and *Ppara*^{-/-} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Rodents were kept in a 12-h light-dark cycle with an ambient light intensity of 85 \pm 18 lux. Care, use, and treatment of the animals were in strict agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and local ethics committee approval was obtained.

Oral fenofibrate administration. Fenofibrate (Sigma-Aldrich, St. Louis, MO) was given as a 0.25 or 0.15% admixture with rodent chow (5001; LabDiet/TestDiet, Ft. Worth, TX). The diabetic or nondiabetic Wt and male, heterozygous Akita mice at 1 week after diabetes onset and male streptozotocin (STZ)-induced diabetic rats at 1 week after diabetes onset were fed chow with fenofibrate for 3 and 7 weeks, respectively.

Western blot analysis. For in vivo studies, two eyecups from each rodent were combined and homogenized, and protein concentration was measured by the Bradford method (34). The same amount (50 μ g) of total protein from each rodent was used for Western blot analysis using the enhanced chemiluminescence system, as described previously (35).

Immunohistochemistry. Frozen retinal sections (4 μ m) were incubated overnight (4°C) with primary antibodies followed by several PBS washes. The sections were incubated (30 min) with secondary antibodies, and the nuclei were counterstained with DAPI (Sigma-Aldrich). The sections were then mounted in antifade medium and viewed on a laser-scanning confocal microscope (model LSM 510; Carl Zeiss Meditec, Jena, Germany).

Primary antibody dilutions were 1:200 for the anti-nuclear factor- κ B (anti-NF- κ B; p65) antibody (Abcam, Cambridge, MA), 1:300 for anti-CD31 antibody (BD Pharmingen, San Jose, CA), 1:1,000 for anti-nonphosphorylated β -catenin antibody (Cell Signaling Technology, Danvers, MA), and 1:100 for rabbit anti-PPAR α antibody (Abcam, Cambridge, MA).

The secondary antibodies were fluorescein isothiocyanate (FITC) (or Texas Red)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA), fluorescent anti-rat IgG with mouse adsorbed (Vector Laboratories, Burlingame, CA) (or Texas Red-conjugated goat anti-rat IgG; Invitrogen, Carlsbad, CA), and Texas Red-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratory, Inc.) at a dilution of 1:200.

The oxygen-induced retinopathy model and analysis of retinal NV. The oxygen-induced retinopathy (OIR) model was induced in BN rats as described previously (36). BN rats at postnatal day 7 (P7) were placed in a 75% oxygen chamber until P12. Fluorescein retinal angiography and quantification of preretinal vascular cells were performed at P18 as previously described (37).

STZ-induced diabetic rats. Experimental diabetes was induced by an intraperitoneal injection of STZ (50 mg/kg) into anesthetized BN rats (8 weeks old) after an overnight fast. To induce diabetes in mice, the mice received five daily injections of STZ. Blood glucose levels were measured 48 h after the STZ injection and monitored weekly thereafter. Only animals with consistently elevated glucose levels >350 mg/dL were considered diabetic. No exogenous insulin treatment was given.

Intravitreal injection. In brief, animals were anesthetized with a 50:50 mix of ketamine (100 mg/mL) and xylazine (20 mg/mL), and pupils were dilated with topical phenylephrine (2.5%) and tropicamide (1%). A sclerotomy was created ~0.5 cm posterior to the limbus, and a glass injector (~33 gauge) connected to a syringe filled with fenofibrate in 10% rat serum, 0.1% DMSO, and 0.9% NaCl, and the same volumes of the vehicle as control, into the contralateral eye. Fenofibrate and GW7647 (Sigma-Aldrich) were dissolved in DMSO and diluted with 10% rat or mouse serum before injection.

Retinal angiography. Rats were anesthetized and perfused with 50 mg/mL 2 \times 10⁶-molecular-weight FITC-dextran (Sigma-Aldrich) as described by Smith et al. (37). The animals were immediately killed. The eyes were enucleated and fixed with 4% paraformaldehyde in PBS for 10 min. The retina was separated

and flat mounted, and vasculature was then examined under a fluorescence microscope (Axioplan2 Imaging; Carl Zeiss) by an operator masked to treatment allocation.

For quantification of preretinal vascular cells, eyes were fixed, sectioned, and stained as described previously (38). The preretinal nuclei were counted by an operator masked to therapy, averaged, and compared.

Retinal vascular permeability assay. Retinal vascular permeability was measured according to a documented method (39) with minor modifications. Evans blue (Sigma-Aldrich) was injected through the femoral vein (10 mg/kg body weight) under microscopic inspection. Two hours after the injection, the mice were perfused via the left ventricle with PBS (pH 7.4). Evans blue dye in the retina was measured and normalized by total retinal protein concentrations.

Retinal vascular leukostasis assay. The assay followed a documented protocol (40). In brief, anesthetized rats were perfused with PBS to remove nonadherent leukocytes in vessels. The adherent leukocytes in the vasculature and vascular endothelial cells were stained with FITC-conjugated concanavalin-A (40 μ g/mL). The retinæ were then flat mounted, and adherent leukocytes in the vasculature were counted under a fluorescence microscope by an operator masked to treatment allocation.

ELISA for retinal monocyte chemoattractant protein-1 and soluble ICAM-1. The eyecups or retinæ were homogenized and centrifuged. Monocyte chemoattractant protein-1 (MCP-1) (Assay Design, Ann Arbor, MI) and soluble ICAM-1 (sICAM-1) levels (R&D Systems, Minneapolis, MN) were measured using ELISA according to the manufacturer's instructions and normalized by total protein concentration in the retina.

Triglyceride measurement. The measurement of triglyceride concentrations in plasma followed a manufacturer's procedure (Triglyceride Determine Kit; Sigma-Aldrich). In brief, blood was collected and centrifuged at 700 *g* for 10 min at 4°C. The triglyceride in the plasma was initiated enzymatically by adding lipase and incubated at 30°C for 10 min to convert triglyceride to free fatty acids and glycerol. The released glycerol was subsequently measured by a coupled enzymatic reaction system with a colorimetric readout at 540 nm. To determine the total triglyceride level, the glycerol was continuously catalyzed at 37°C for 15 min by a reconstituted triglyceride reagent including ATP, glycerol kinase, glycerol phosphate oxidase, and peroxidase; the released quinoneimine dye, directly proportional to the triglyceride concentration of the sample, was measured and recorded at 540 nm.

Retinal endothelial cell tube formation. Primary bovine retinal endothelial cells (RECs) at passage five were used throughout the study. After pooling of 50 μ L ice-cold Matrigel into the 12-well plates at 37°C for 30 min to solidify, RECs were overlaid onto the Matrigel with or without 50 μ m fenofibrate in the media, and tube formation was examined at 6 h by an operator masked to treatment identity.

Endothelial cell scratch wound assay. Eighty-percent confluent RECs were wounded by drawing a line with a sterile 200- μ L pipette tip across the monolayer surface. The cells were then cultured for 24 h. The average linear migration rate was calculated by tracing the border of the cell monolayer on both sides of the wound at 0 and 24 h, measuring the cell-free area over a fixed length along the wound, by an operator masked to cell treatment.

Transwell inserts cell migration assay. The undersurfaces of Transwell motility chamber inserts of a 96-well Transwell (Neuro Probe, Inc., Gaithersburg, MD) were coated with or without 10 mg/mL mouse cellular fibronectin, and lipophilic carbocyanines (DiI)-labeled RECs (Invitrogen, Grand Island, NY) were cultured in the upper chamber of the inserts in the presence of fenofibrate at various concentrations (0, 50, 100, and 200 μ mol/L). After 6 h incubation, the cells on the upper surface of the membrane were removed, and the fluorescence in the cell monolayer on the other side was determined.

Statistical analysis. Quantitative data were analyzed and compared using Student *t* test for comparison of two groups and one-way ANOVA for studies of more than two groups. Statistical significance in multiple groups was determined by Tukey post hoc analysis, and statistical significance was set at *P* < 0.05.

RESULTS

Fenofibrate attenuates retinal vascular permeability in type 1 diabetes models. To determine if fenofibrate decreases retinal vascular leakage in type 1 diabetic rodents, STZ-induced diabetic rats at 1 week after diabetes onset were fed chow containing fenofibrate for 7 weeks. Controls were age-matched nondiabetic rats and diabetic rats fed with regular chow. Retinal vascular leakage was evaluated using Evans blue dye as tracer with normalization to total retinal protein concentration. Oral fenofibrate treatment significantly reduced retinal vascular leakage in STZ-diabetic

rats, compared with the untreated diabetic rats, to a level similar to nondiabetic rats (Fig. 1A). Similarly, 3 weeks of oral fenofibrate treatment of Akita mice, a genetic model of type 1 diabetes, also significantly reduced retinal vascular leakage in diabetic mice to a level similar to the age-matched nondiabetic mice (Fig. 1B).

Fenofibrate reduces retinal vascular leukostasis in type 1 diabetic rats. We examined fenofibrate effects on leukocyte adherence (leukostasis) in the retinal microvasculature in STZ-induced diabetic rats. Diabetic and nondiabetic rats were fed chow containing fenofibrate for 7 weeks, and retinal leukostasis was examined. Unlike the retinal vasculature in nondiabetic rats (Fig. 2A and B), multiple adherent leukocytes were observed in the retinal vasculature of untreated diabetic rats (Fig. 2C and D), but there were fewer leukocytes in the fenofibrate-treated diabetic rats (Fig. 2E and F). There were significantly fewer adherent leukocytes in the fenofibrate-fed diabetic rats compared with untreated diabetic rats ($P < 0.001$), to a level comparable to the retinae of nondiabetic rats (Fig. 2G).

Fenofibrate attenuates overexpression of inflammatory factors in the retinae of type 1 diabetic animals. Levels of ICAM-1 and MCP-1, which promote leukostasis and leukocyte infiltration (41), were measured by Western

blot analysis and ELISA in the retinae of age-matched Akita mice fed standard chow (control) and those fed fenofibrate chow. As shown in Fig. 3A and B, fenofibrate treatment significantly reduced retinal levels of MCP-1 and ICAM-1. As shown by immunohistochemistry, fenofibrate also decreased levels of NF- κ B and attenuated NF- κ B nuclear translocation in the retina of Akita mice (Fig. 3C–H). **Intraocular administration of fenofibrate reduces retinal vascular leakage in diabetic and OIR rats.** As fenofibrate decreases hepatic VLDL production (42), we determined if fenofibrate's effects on DR were via its systemic effects or via direct effects on the retina. We injected 5 μ L of 125 μ mol/L fenofibrate into the vitreous in one eye and the same volume of vehicle (10% rat normal serum in DMSO) into the contralateral eye of STZ-induced diabetic rats. As shown by the retinal permeability assay, intraocular injection of fenofibrate significantly reduced retinal vascular leakage in the diabetic rats, when compared with the vehicle control (Fig. 4A).

Similarly, 3 μ L of 125 μ mol/L fenofibrate was injected into the vitreous of OIR rats at age P12, a model of ischemia-induced retinopathy. As shown by retinal vascular permeability assay at P16, intraocular injection of fenofibrate significantly reduced vascular leakage in the retina, compared

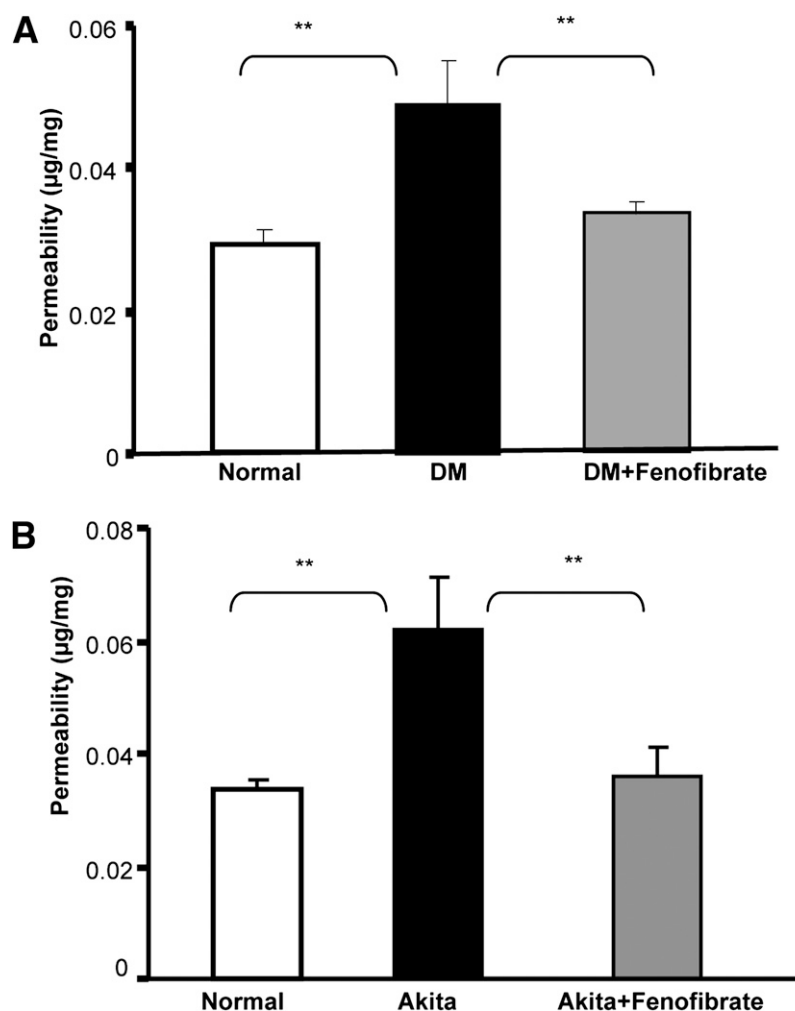


FIG. 1. Fenofibrate reduces retinal vascular leakage in type 1 diabetic animals. **A:** STZ-induced diabetic rats at 1 week after diabetes onset were fed chow with fenofibrate for 7 weeks. **B:** Akita mice at the first week after diabetes onset were fed chow with fenofibrate for 3 weeks. Controls were nondiabetic and untreated diabetic animals fed standard chow. Retinal vascular leakage in both of the STZ-diabetic rats and Akita mice was quantified by vascular permeability assay using Evans blue dye as a tracer (mean \pm SD; $n = 7$). ** $P < 0.01$. DM, diabetes.

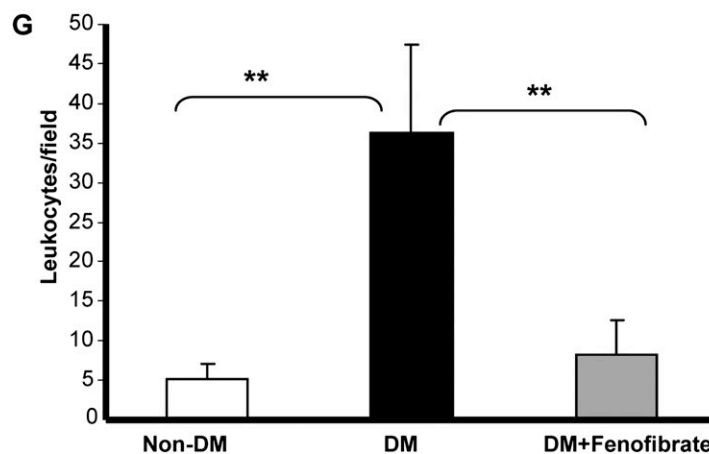
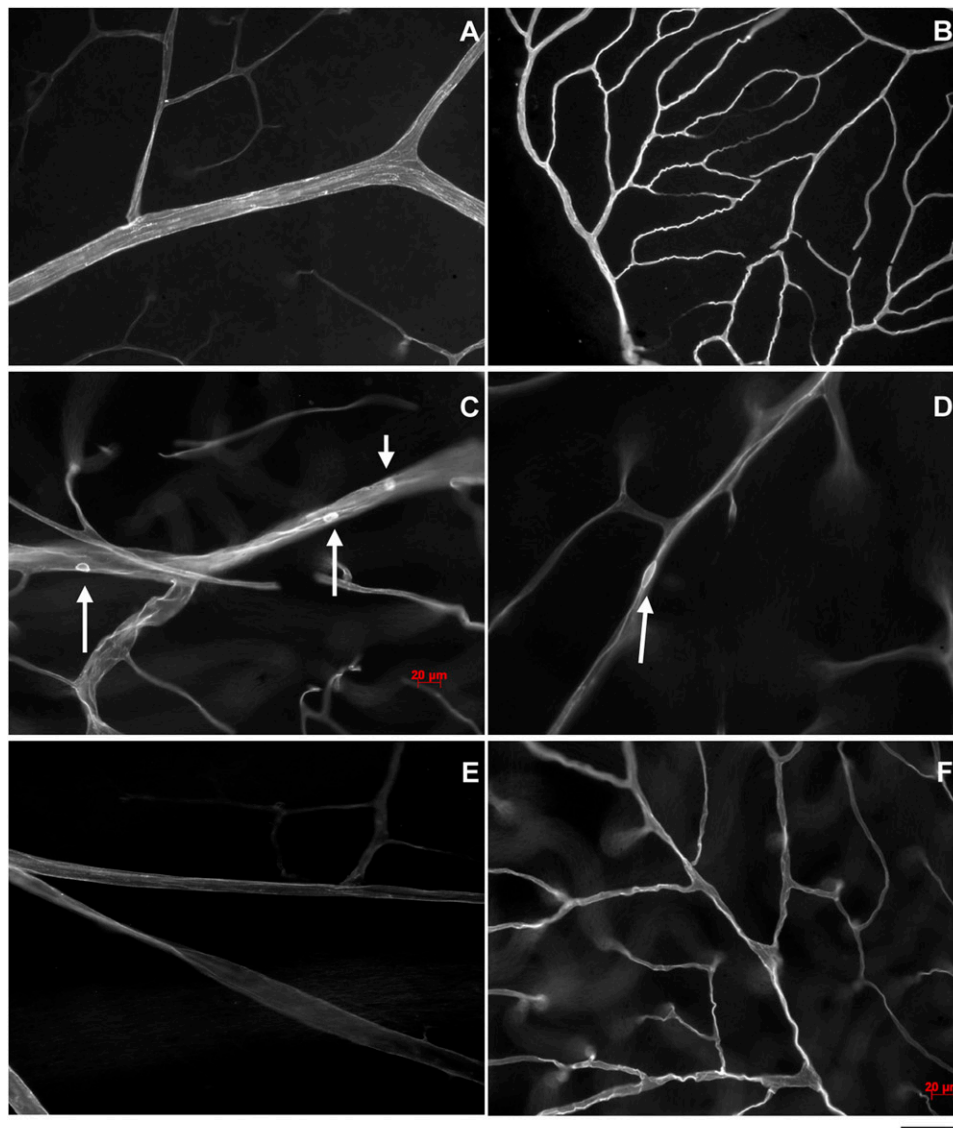


FIG. 2. Fenofibrate decreases retinal vascular leukostasis in type 1 diabetic animals. The retinal vascular endothelium and adherent leukocytes were stained with FITC-conjugated concanavalin-A after the removal of circulating leukocytes. The retinae were then flat mounted, and adherent leukocytes were visualized by fluorescence microscopy. Representative images of retinal flat mounts from nondiabetic rats (*A* and *B*), untreated STZ-induced diabetic rats (*C* and *D*), and diabetic rats treated with fenofibrate for 7 weeks (*E* and *F*) are shown. Arrows indicate adherent leukocytes. Scale bar, 50 μ m. *G*: Quantification of leukocytes showed that fenofibrate significantly reduced adherent leukocytes in the diabetic rats (mean \pm SD; $n = 7$). $**P < 0.05$. DM, diabetes. (A high-quality digital representation of this figure is available in the online issue.)

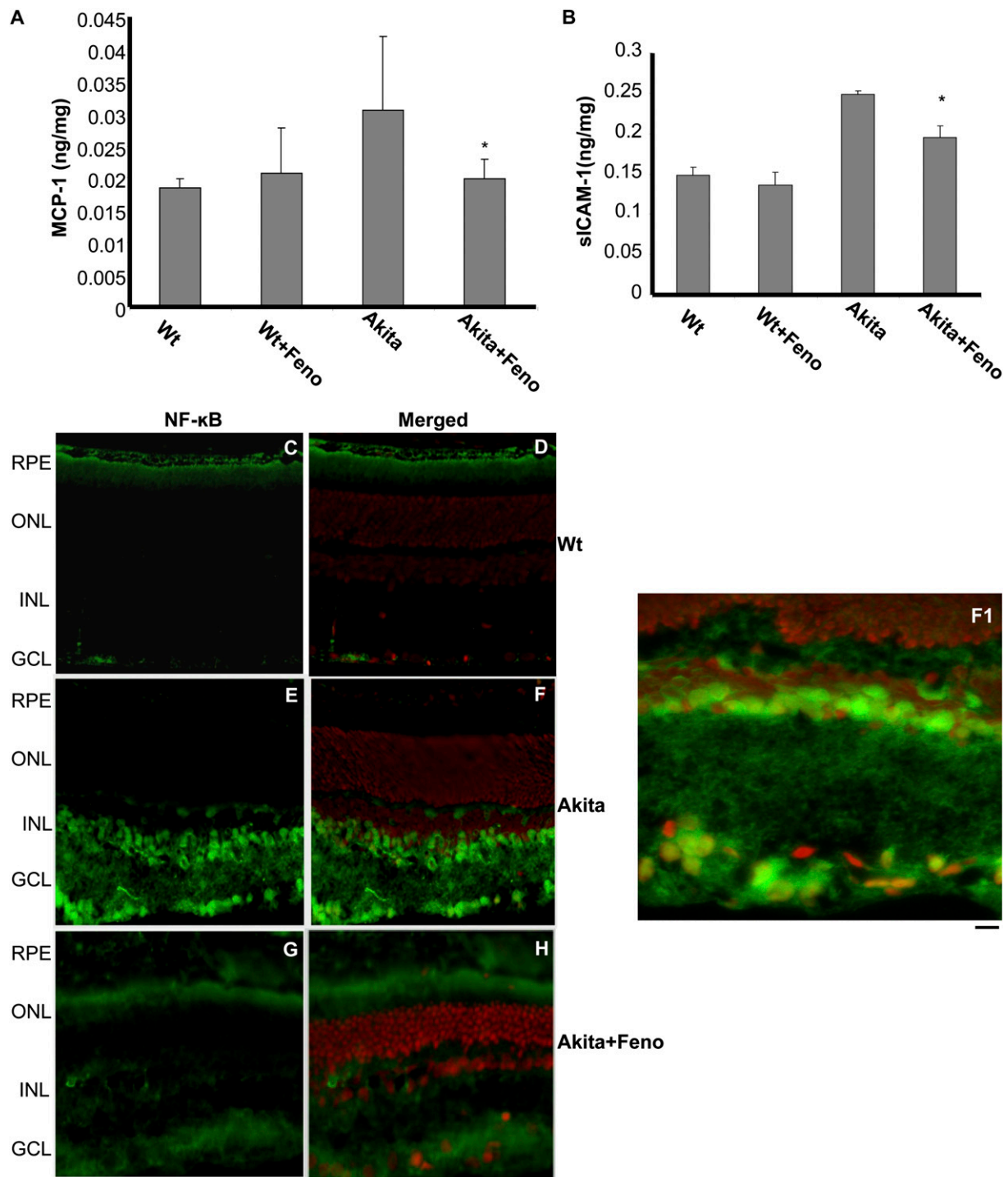


FIG. 3. Fenofibrate suppresses the diabetes-induced overexpression of pathogenic factors. *A* and *B*: MCP-1 and sICAM-1 levels were measured using ELISA in retinae from 3-month-old Wt and Akita mice fed chow with or without fenofibrate (Feno) for 3 weeks and expressed as ng/mg of total retinal protein (mean \pm SD; $n = 3$). * $P < 0.05$. Representative immunostaining images of NF- κ B (green) on retinal cross-sections from Wt mice (*C* and *D*), Akita mice (*E* and *F*), and Akita mice fed with fenofibrate (*G* and *H*). The nuclei were counterstained with DAPI (red). *F1*: High magnification of *F*, to view nuclear translocation of NF- κ B. Note that nuclear translocation of NF- κ B is seen as orange-colored nuclei as a result of merged red (DAPI) and green (NF- κ B) signals. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. Scale bar: 10 μ m in *F1* and 20 μ m in all other panels. (A high-quality digital representation of this figure is available in the online issue.)

with the contralateral eye injected with vehicle only (Fig. 4*B*).

Intraocular injection of fenofibrate attenuates retinal NV in OIR rats. To evaluate the effect of fenofibrate on retinal NV, fenofibrate was injected into the vitreous of OIR

rats (3 μ L of 125 μ mol/L fenofibrate) at P12. Fluorescein angiography at P18 showed that the fenofibrate-treated eyes developed less severe retinal NV (Fig. 5), compared with the contralateral eyes injected with the vehicle only. Quantification of preretinal NV cells on cross-sections of OIR eyes

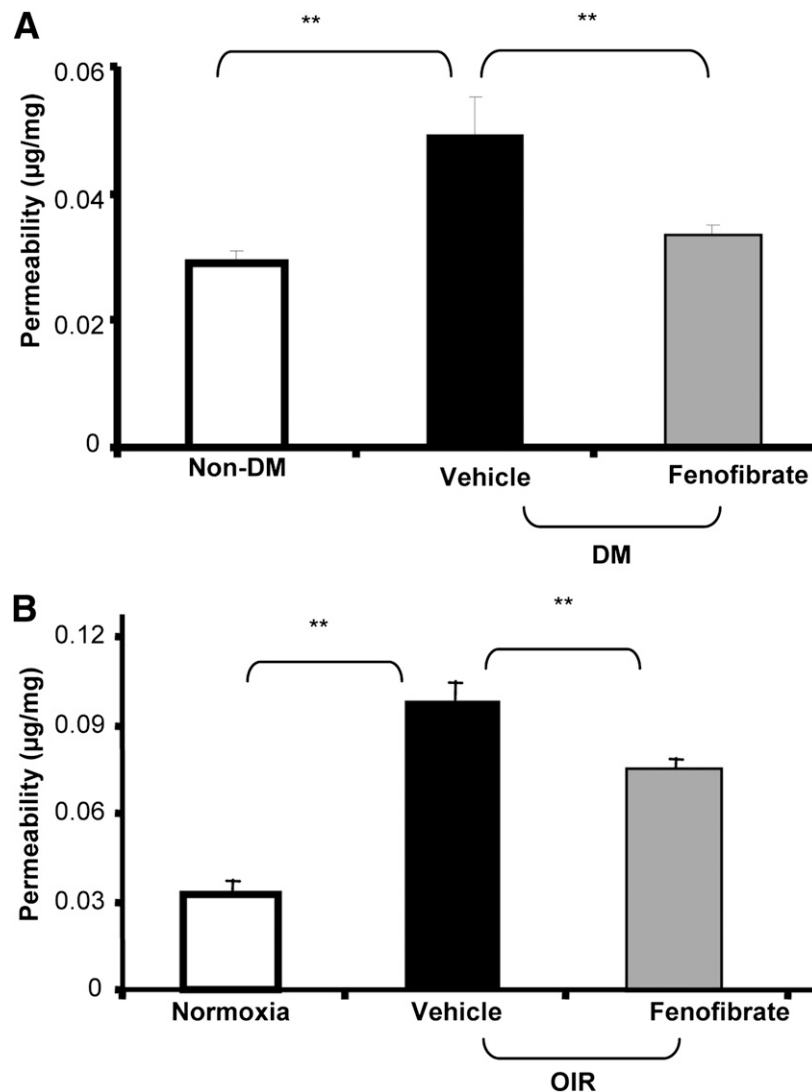


FIG. 4. Intraocular injection of fenofibrate reduces retinal vascular leakage in STZ-induced diabetic rats and in OIR rats. *A*: STZ-induced diabetic rats were injected with 5 μL of 125 $\mu\text{mol}/\text{L}$ fenofibrate into the vitreous of one eye and the same volume of vehicle into the contralateral eye, 6 weeks after diabetes onset. Four days after the injection, retinal vascular leakage was quantified by the vascular permeability assay (mean \pm SD; $n = 5$). *B*: OIR rats were injected with 3 μL of 125 $\mu\text{mol}/\text{L}$ fenofibrate into the right vitreous cavity at P12 (immediately after they were removed from 75% oxygen), and the same volume of vehicle was injected into the left vitreous cavity as control. At P16, retinal vascular leakage was quantified by the vascular permeability assay (mean \pm SD; $n = 5$). ** $P < 0.01$. DM, diabetes.

showed that the eyes injected with fenofibrate developed significantly fewer preretinal vascular cells, relative to the vehicle control in the contralateral eyes (Fig. 5C–E), supporting an inhibitory effect of fenofibrate on ischemia-induced NV.

Intraocular injection of fenofibrate ameliorates retinal inflammation in OIR rats. To evaluate the direct effects of fenofibrate on VEGF overexpression, fenofibrate was injected into the vitreous of OIR rats at P12, and vehicle was injected into the contralateral eyes; retinal VEGF levels were measured at P16. As shown by Western blot analysis, fenofibrate greatly decreased retinal VEGF levels in the OIR rats, compared with vehicle control (Fig. 6A). Similarly, immunostaining showed that the immunosignals of VEGF and hypoxia-inducible factor-1 α (HIF-1 α), a transcription factor activating VEGF in ischemic conditions, were decreased by fenofibrate in the inner retina of OIR rats (Fig. 6B–G). These results indicate that intraocular administration of fenofibrate attenuated ischemia-induced HIF-1 activation and VEGF overexpression in the retina.

Fenofibrate inhibits REC tube formation and migration. In the tube formation assay, RECs cultured in the absence of fenofibrate aggregated to form a tube-like pattern on Matrigel, whereas RECs exposed to 50 $\mu\text{mol}/\text{L}$ fenofibrate did not form the tube-like structures (Fig. 7A and B).

REC migration was also evaluated using the scratch wound healing assay in primary REC monolayers, which showed that fenofibrate-treated RECs had substantially decreased motility, as measured 24 h after wounding (Fig. 7C and D). Additionally, the Transwell cell migration assay demonstrated that the number of RECs that migrated through the filter was significantly decreased by fenofibrate, compared with the vehicle-only control (Fig. 7E–G). These results support that fenofibrate inhibits REC migration.

The therapeutic effects of fenofibrate on DR in type 1 diabetes models are PPAR α dependent. To investigate if the therapeutic effects of fenofibrate on DR are via a PPAR α -dependent mechanism, we activated PPAR α by intravitreal injection of GW590735 (2 μL of 100 nmol/L),

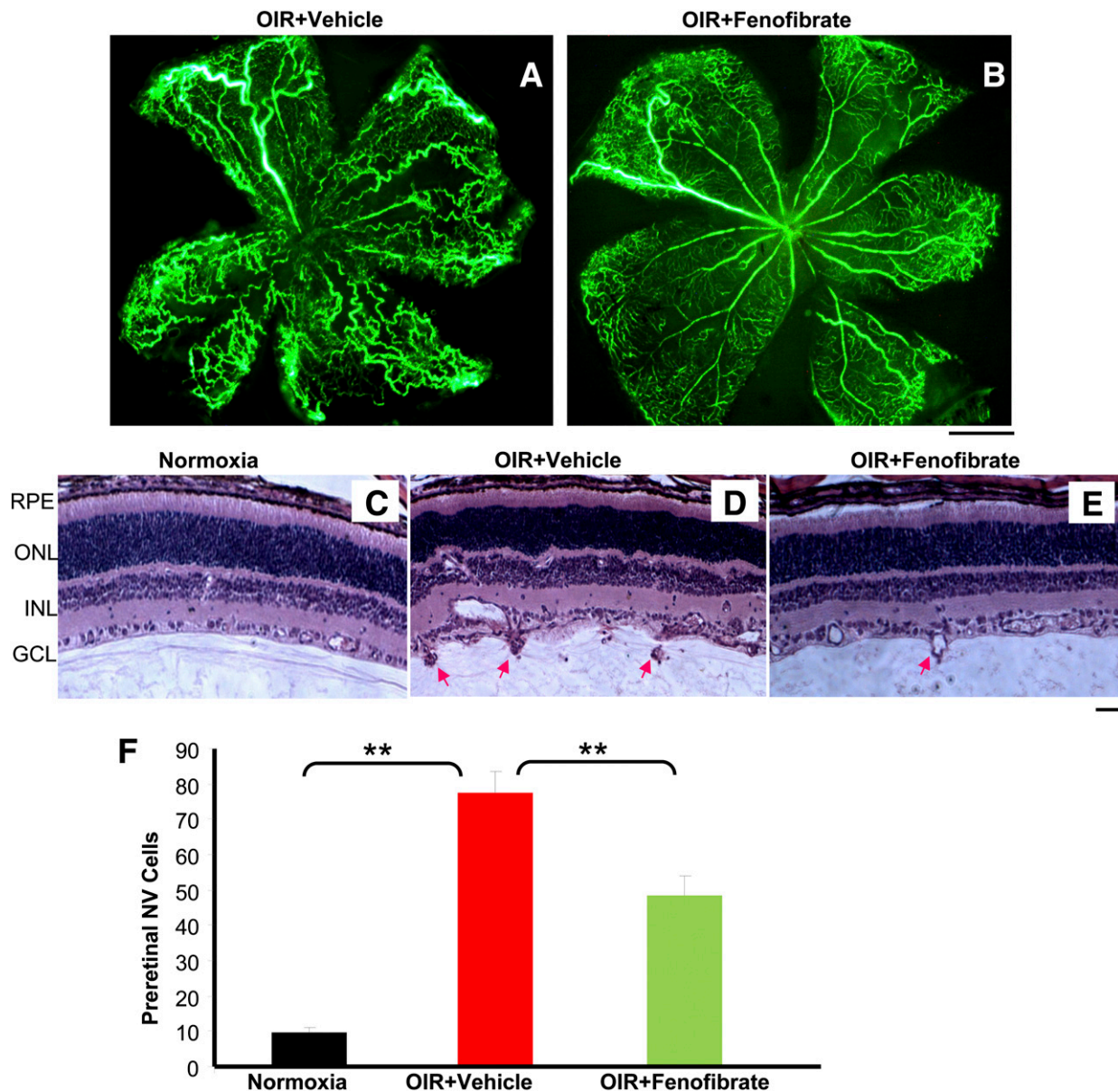


FIG. 5. Intraocular delivery of fenofibrate ameliorates ischemia-induced retinal NV in OIR rats. Rats were exposed to 75% oxygen from P7 to P12. The rats were returned to room air and received an intravitreal injection of 3 μ L per eye of 125 μ mol/L fenofibrate into the vitreous cavity of the right eye, and the same amount of vehicle into the left vitreous cavity as control at P12. **A** and **B**: At P18, retinal vasculature was visualized by fluorescein angiography. **C–E**: The eyes were fixed, sectioned, and stained with hematoxylin and eosin at P18. Representative sections from rats at normoxia (**C**), OIR rats injected with vehicle (**D**), and OIR rats with fenofibrate injection (**E**). Red arrows indicate preretinal vascular cells. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. Scale bar, 40 μ m. **F**: Preretinal vascular cells were counted in eight noncontinuous sections per eye and averaged as described in RESEARCH DESIGN AND METHODS. The average numbers of preretinal vascular cells (mean \pm SD; $n = 6$) were compared between the eyes injected with fenofibrate and those with vehicle using paired Student t test. $**P < 0.01$. (A high-quality digital representation of this figure is available in the online issue.)

another PPAR α agonist with a chemical structure different from fenofibrate, into diabetic rats. The permeability assay showed that GW590735 significantly reduced retinal vascular leakage in diabetic rats (Fig. 8A).

We also used *Ppara*^{-/-} mice for the OIR and diabetic models to evaluate fenofibrate's efficacy. Wt and *Ppara*^{-/-} mice with 4 weeks of STZ-induced diabetes and age-matched Wt controls were fed fenofibrate chow (as described above) for another 6 weeks. The permeability assay showed that fenofibrate significantly reduced retinal vascular leakage in Wt mice with diabetes, but not in diabetic *Ppara*^{-/-} mice (Fig. 8B). In the *Ppara*^{-/-} mice with OIR, an intravitreal injection of fenofibrate did not decrease retinal

VEGF levels (Fig. 8C), demonstrating that the beneficial effects of fenofibrate on DR are through PPAR α activation.

DISCUSSION

Two independent, large clinical studies demonstrated substantial protective effects of fenofibrate against diabetic eye complications in type 2 diabetic patients (29,30). Here we provide the first evidence that fenofibrate also has therapeutic effects on DR in two type 1 diabetes models and on ischemia-induced retinal NV. Furthermore, we have demonstrated that the therapeutic effect of fenofibrate on DR can be achieved by intravitreal injection, suggesting

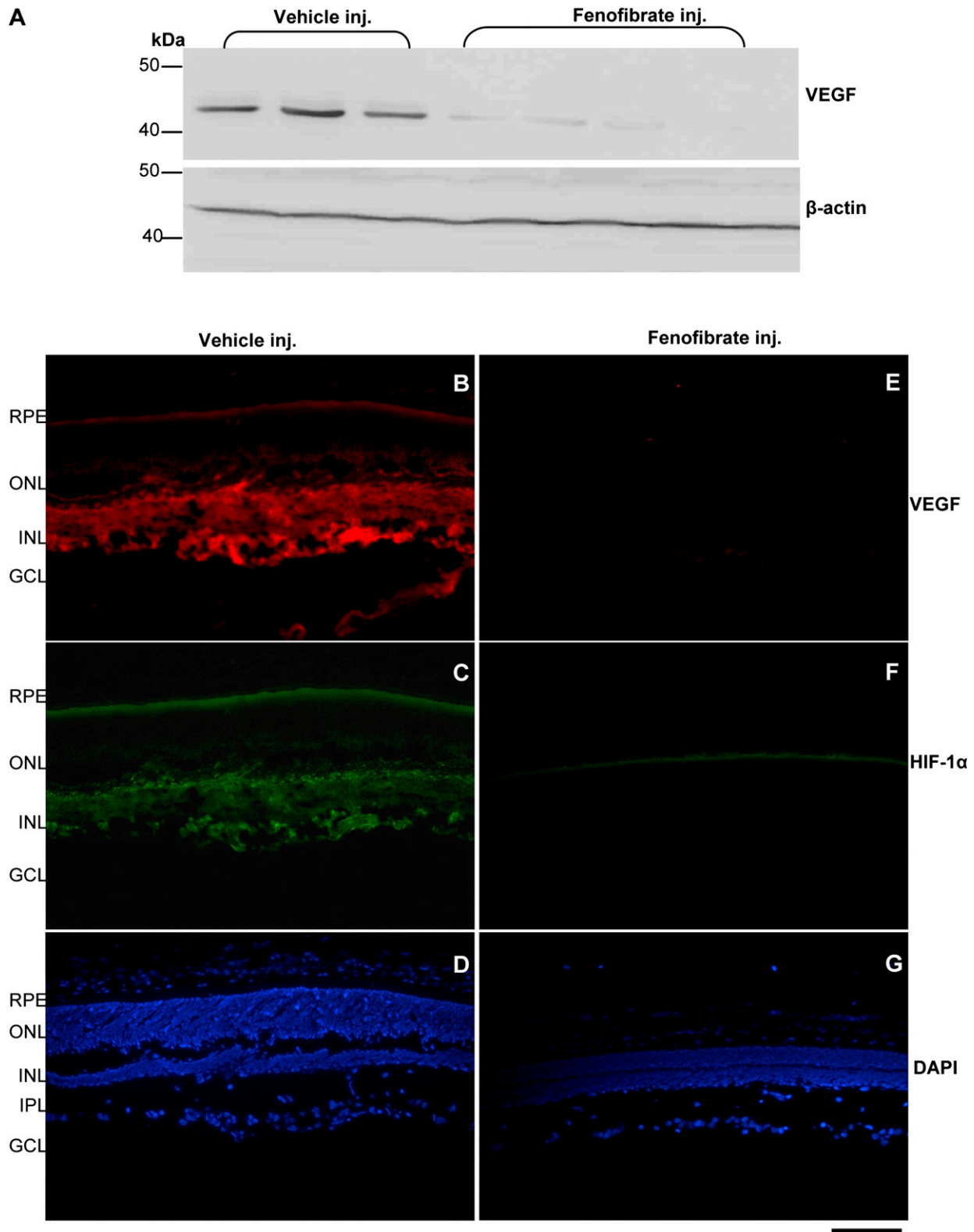


FIG. 6. Intraocular injection (inj.) of fenofibrate downregulates VEGF in the retinae of OIR rats. At P12, OIR rats were injected with 3 μ L per eye of 125 μ mol/L fenofibrate into the right vitreous, and the same amount of vehicle into the left vitreous as control. **A:** At P16, equal amounts (50 μ g) of retinal proteins were blotted with an antibody against VEGF, with β -actin as a loading control. Each lane represents an individual rat. **B–G:** Ocular cross-sections from OIR rats (P16) injected with fenofibrate (**E–G**) and those with vehicle (**B–D**) were immunostained with antibodies specific for VEGF (red) and HIF-1 α (green). Nuclei were counterstained with DAPI (blue). In the inner retina, the VEGF and HIF-1 α signals in the fenofibrate-injected eyes were lower than in the vehicle-injected eyes. Scale bar, 50 μ m. RPE, retinal pigment epithelium; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. (A high-quality digital representation of this figure is available in the online issue.)

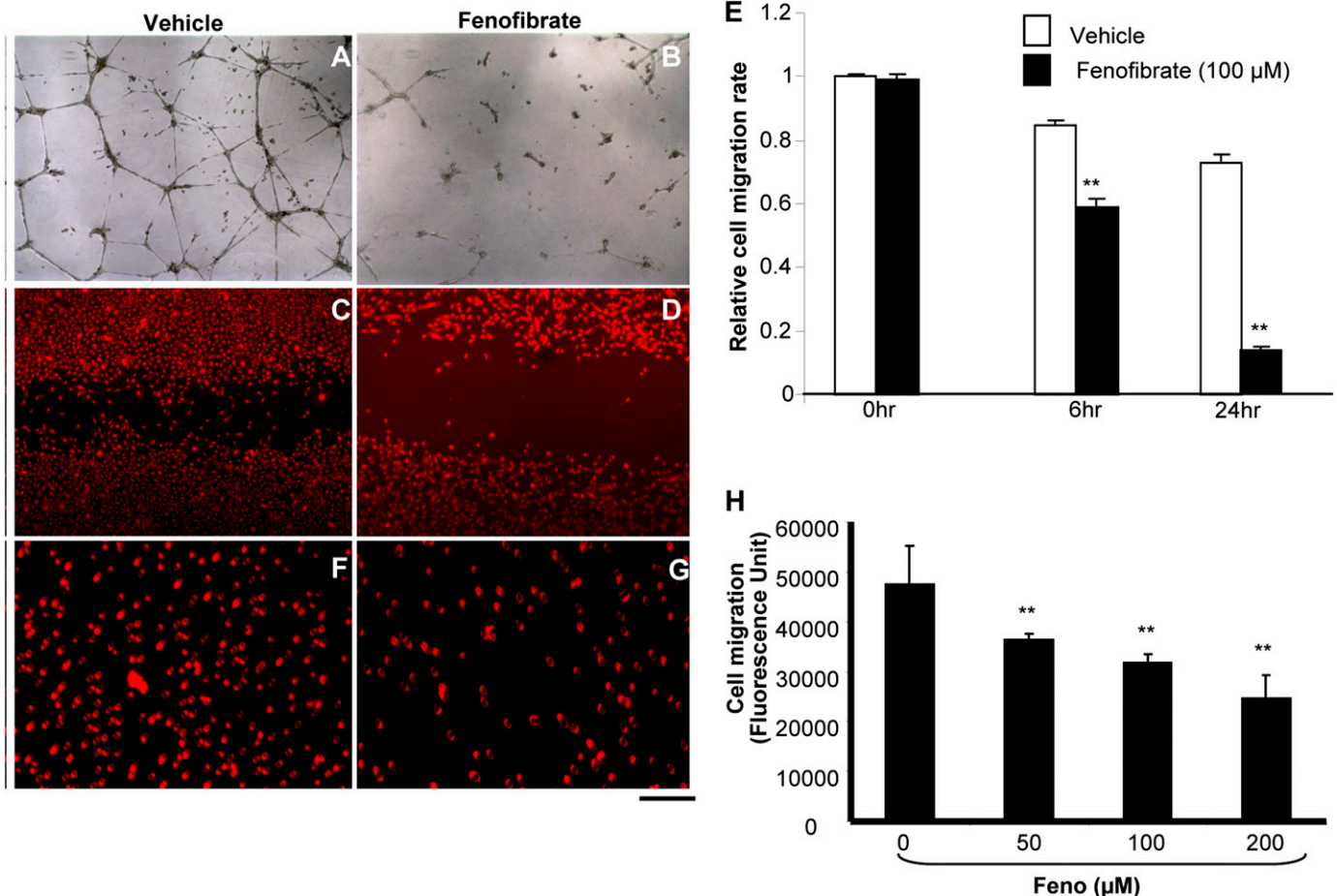


FIG. 7. Fenofibrate blocks REC migration and tube formation and prevents high glucose-induced oxidative stress. *A* and *B*: Primary REC treated with 50 $\mu\text{mol/L}$ fenofibrate or the same volume of vehicle were cultured on Matrigel for 6 h. In the absence of fenofibrate, RECs formed a capillary-like pattern (*A*), which was blocked by fenofibrate (*B*). *C* and *D*: RECs were cultured in the presence or absence of 50 $\mu\text{mol/L}$ fenofibrate on a gelatin-coated plate, and an acellular area was generated by a scratch. As visualized 24 h after the scratch wounding, the scratch width in the control cells was smaller than in the wounded cells treated with fenofibrate. *E*: Cell migration was quantified by changes in the acellular area. Relative to the vehicle control, fenofibrate significantly reduced REC migration (mean \pm SD; $n = 3$). ** $P < 0.01$. *F–H*: The undersurfaces of 96-well Transwell motility chamber inserts were coated with 10 mg/mL mouse cellular fibronectin. RECs were seeded in the upper chambers of the Transwell and cultured in the presence (*G*) or absence (*F*) of various concentrations of fenofibrate for 6 h. The cells that migrated to the undersurface were quantified after removal of cells from the upper surface. Scale bar, 100 μm . *H*: Relative to the control cells, fenofibrate (Feno) significantly reduced REC migration to the undersurface of the Transwell motility chamber (mean \pm SD; $n = 3$). ** $P < 0.01$. (A high-quality digital representation of this figure is available in the online issue.)

that the drug's protective effects are independent of its systemic effects. Toward its mechanism of action, we have shown that the anti-inflammatory effect of fenofibrate may be through downregulation of ICAM-1 and MCP-1 expression and inhibition of NF- κ B signaling in the diabetic retina. We have also shown that the anti-NV effect of fenofibrate may be ascribed to its inhibition of hypoxia-induced activation of the HIF-1 pathway and, subsequently, attenuation of VEGF overexpression. More importantly, our results using another PPAR α agonist, a PPAR α antagonist, and Ppara $^{-/-}$ mice suggest that the therapeutic effects of fenofibrate on DR occur through a PPAR α -dependent mechanism.

Chronic inflammation, including increased vascular leukostasis, which damages the retinal endothelium and promotes vascular leakage, has been shown to play a major pathogenic role in DR. Leukostasis can also lead to retinal capillary closure, causing nonperfusion of vessels and local ischemia, which subsequently induces overexpression of VEGF and other proinflammatory factors and promotes further vascular leakage, leading to clinical DR and DME (43,44). Our results demonstrate that fenofibrate significantly

decreases retinal leukostasis and retinal levels of VEGF and proinflammatory factors ICAM-1 and MCP-1 in two type 1 diabetes models, STZ-induced diabetic rats and Akita mice. NF- κ B signaling plays a key role in upregulation of inflammatory factors in DR (45). HIF-1 is a major transcription factor activating VEGF expression in ischemic conditions, such as in diabetic retina, which contributes to vascular leakage and NV in DR (46). Our results show that fenofibrate inhibits activation of both NF- κ B and HIF-1 signaling, which may account for its anti-inflammatory and anti-NV effects in DR models.

Retinal vascular leakage is a major cause of DME. Our in vivo retinal vascular permeability assays have shown that oral administration of fenofibrate significantly reduces retinal vascular leakage in both STZ-diabetic rats and Akita mice, without significantly lowering blood glucose levels and body weights (Supplementary Fig. 4). In the human clinical trials, such as the FIELD study (30), fenofibrate use was not associated with significant differences in HbA $_{1c}$ or body weight relative to placebo use. Furthermore, intraocular injection of fenofibrate also attenuated retinal vascular leakage in OIR

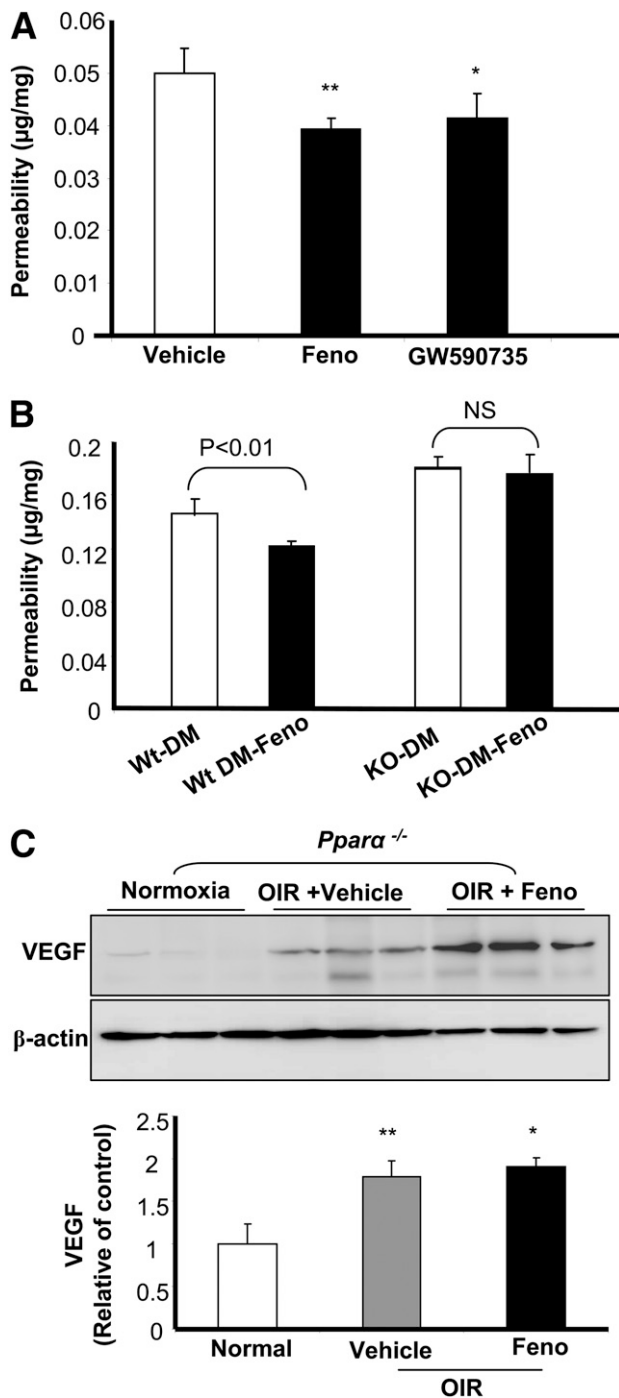


FIG. 8. Therapeutic effects of fenofibrate on DR are PPAR α dependent. **A:** STZ-induced diabetic rats at 4 weeks after diabetes onset received an intravitreal injection of 2 μ L of GW590735 (100 nmol/L). The same volume of vehicle and fenofibrate (Feno) (50 μ mol/L) were used as a negative and a positive control, respectively. Retinal vascular leakage was quantified by vascular permeability assay using Evans blue dye as a tracer (mean \pm SD; $n = 7$). ** $P < 0.01$; * $P < 0.05$. **B:** STZ-induced diabetic Wt mice or *Ppara*^{-/-} mice at 4 weeks after diabetes onset were fed chow with or without 120 mg/kg/d fenofibrate for 6 weeks. Retinal vascular leakage was quantified by the vascular permeability assay (mean \pm SD; $n = 7$). **C:** Newborn *Ppara*^{-/-} mice were exposed to 75% oxygen from P7 to P12. At P12, the OIR mice received an intravitreal injection of 3 μ L per eye of 125 μ mol/L fenofibrate. Age-matched *Ppara*^{-/-} mice maintained at constant room air were used as controls. The retina was dissected at P16 and homogenized. The same amount of retinal proteins from each mouse was used for Western blot analysis of VEGF, which was semiquantified by densitometry and normalized by β -actin levels. DM, diabetes; KO, knockout.

rats. These results support that fenofibrate may have therapeutic effects on DME in type 1 diabetes. These results are also in keeping with the report that an early fibrate, clofibrate, reduces retinal hard exudates (47).

To confirm that the beneficial effects of fenofibrate on retinal inflammation and retinal vascular leakage are a local ocular effect and not secondary to its systemic effects, we injected fenofibrate into the vitreous of STZ-diabetic and OIR rats. Direct ocular delivery of fenofibrate decreased retinal inflammation and retinal vascular leakage to a level comparable to the nondiabetic control rats. Ocular injection of fenofibrate also ameliorated retinal NV in the OIR model, a model of ischemia-induced retinal NV without diabetes and dyslipidemia. These results indicate that the drug targets for fenofibrate are present in and/or are accessible via the retina. Our studies provide supportive evidence of fenofibrate's benefit on DR in humans being chiefly due to direct ocular effects on ocular tissues rather than a consequence of systemic effects, such as decreased hepatic VLDL production and clearance. This notion is consistent with the findings from the FIELD and ACCORD studies that the ocular benefits of fenofibrate did not correlate with changes in the lipid profile (30,33).

Fenofibrate has been used clinically for many years to treat dyslipidemia (16,17), but the recent FIELD and ACCORD clinical trials both reported the surprising findings of fenofibrate benefit on DR in type 2 diabetic patients (30,33). A natural question is whether the beneficial effects of fenofibrate on DR are through activation of PPAR α or are off-target effects. To address this intriguing question, we conducted several experiments, including the use of another PPAR α agonist, a PPAR α antagonist, and PPAR α knockout mice. First, intraocular delivery of another PPAR α agonist that has a chemical structure distinct from fenofibrate reduced retinal vascular leakage in a DR model, similar to that of fenofibrate. Second, the beneficial effects of fenofibrate on DR were diminished by a PPAR α antagonist (Supplementary Fig. 2). Third, the therapeutic effects of fenofibrate were abolished when PPAR α was deficient, as in the *Ppara*^{-/-} mice. Taken together, these observations provide evidence that the beneficial effects of fenofibrate on DR are through PPAR α activation.

The present series of studies show that oral and intraocular administration of fenofibrate are promising therapeutics for the treatment or prevention of DR in type 1 diabetes. The exciting finding of direct ocular effects of fenofibrate on DR and DME in both types of diabetes is crucially important, since the FIELD trial is the first clinical study to identify an oral drug, other than oral hypoglycemic agents, that has clinical benefit on DR in any form of diabetes. Compared with anti-VEGF compounds, fenofibrate has several advantages, including low costs, oral administration, low toxicity, and protection against diabetic nephropathy (48,49) and amputation in diabetic patients (32,48). As not all patients can tolerate oral fenofibrate, ocular administration may be advantageous. The current study, with multiple model validation, is the first to reveal that fenofibrate's benefit on DME and DR is via direct effects on retinal inflammation, retinal vascular leakage, and retinal NV, and that it is relevant to type 1 diabetes as well (50). However, many questions, such as the mechanism of action of fenofibrate and the signaling pathway mediating the effect of PPAR α on inflammation and angiogenesis, remain to be fully elucidated. Future studies should include clinical studies of fenofibrate in type 1 diabetes patients and basic science studies of the

underlying cell signaling and molecular mechanisms by which fenofibrate ameliorates DR. Elucidation of the molecular mechanisms responsible for fenofibrate's effect may reveal a promising drug target and herald the opportunity for new classes of agents effective on DR and DME.

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Y.C. researched data, wrote the manuscript, and designed the study. Y.H., M.L., and R.M. researched data. A.J.J., A.C.K., and T.J.L. contributed to discussion and reviewed and edited the manuscript. J-x.M. designed the study and wrote the manuscript. J-x.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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