Fenofibrate Exerts Protective Effects in Diabetic Retinopathy via Inhibition of the ANGPTL3 Pathway

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PurposE. Fenofibrate has been demonstrated to exert a promising therapeutic effect against diabetic retinopathy. Angiopoietin-like 3 (ANGPTL3) has been shown to exert significant pathogenic effects on vascular endothelial cells, which are critically involved in the pathogenesis of diabetic retinopathy. The present study aimed to investigate the link between the therapeutic effects of fenofibrate and the pathogenic effects of ANGPTL3 in diabetic retinopathy.

Methods. Diabetic and control rats were randomly assigned to the following treatments: intravitreal injection with ANGPTL3 small interfering RNA (siRNA), recombinant human (rh)ANGPTL3, fed with normal feeds, or fenofibrate-containing feeds for 8 weeks. Human retinal microvascular endothelial cells (HRMECs) were exposed to normal glucose or high glucose levels with ANGPTL3 siRNA, ANGPTL3 RNA overexpression, blank vector, cinglentide, or fenofibrate treatment. Expression levels of ANGPTL3, IL-1b, IL-6, Bax, P53, VEGF, and integrin αVβ3 in the retinas of rats and HRMECs were examined by Western blotting and real-time PCR. The apoptosis rates of HRMECs were examined using a TUNEL apoptosis assay kit.

Results. Expression levels of ANGPTL3, IL-1β, IL-6, Bax, P53, VEGF and integrin αVβ3 were found to be upregulated after high-glucose stimulation or ANGPTL3 overexpression in HRMECs or diabetic retinal tissue. However, expression levels of the above markers were downregulated following fenofibrate intervention, blockage of integrin αVβ3 receptor, or ANGPTL3 siRNA interference.

Conclusions. We identified fenofibrate exerts its protective effects by inhibiting ANGPTL3-induced apoptosis and inflammation in diabetic retinopathy, which is a novel mechanism. Keywords: diabetic retinopathy, fenofibrate, ANGPTL3, integrin, inflammation, apoptosis

Fenofibrate is a selective peroxisome proliferator-activated receptor-α (PPAR-α) agonist that is widely clinically used as a lipid-lowering agent because of its pharmacologic effects, including reduction of triglyceride (TG) levels and low-density cholesterol (LDL) and elevation of high-density cholesterol (HDL) levels. Additionally, two large randomized clinical trials, namely, the ACCORD study and FIELD study, demonstrated the beneficial effects of fenofibrate on diabetic microvascular complications.1,2 Moreover, fenofibrate can significantly reduce the requirement for laser photocoagulation relative to the control group in both diabetic macular edema and proliferative diabetic retinopathy (PDR).3 Notably, the therapeutic effect of fenofibrate was found to be independent of its lipid-lowering effects. In particular, similar cholesterol and triglycerides levels were observed between the fenofibrate-treated and control groups in the FIELD study.3 Studies have implicated that inflammatory and apoptotic cytokines mediate the beneficial effects of fenofibrate.3,4 However, the mechanisms underlying the effects of fenofibrate remain to be fully understood.

Angiopoietin-like protein 3 (ANGPTL3), an adipokine, is a member of the ANGPTL family. ANGPTL3 is structurally similar to angiopoietin and has been demonstrated to promote both neovascularization and hyperlipidemia,5,6 which are mediated by the N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain. In addition, ANGPTL3 was revealed to not only increase the permeability of glomerular endothelial cells via the integrinαVβ3 receptor,8 but also to induce apoptosis in podocytes,9 a vital pathophysiologic process in diabetic nephropathy. Despite the above-mentioned effects of ANGPTL3, we found no evidence implicating ANGPTL3 in the pathogenesis of diabetic retinopathy (DR). Importantly, as a lipid-lowering agent, determining whether the protective effects of fenofibrate on DR are mediated by ANGPTL3 inhibition has significant implications for the development of potential therapeutic methods in the treatment of diabetic retinopathy.

In a recent study, we showed that ANGPTL4 regulates diabetic retinal inflammation and angiogenesis by activating profilin-1 both in vivo and in vitro.10 In the present study, we demonstrated a novel mechanism by which ANGPTL3 promotes apoptosis and inflammation in diabetic retinopathy by interacting with the integrin αVβ3 receptor. Moreover, our results suggested that fenofibrate treatment significantly prevented high glucosel-induced apoptosis and inflammation both in vitro and in vivo by inhibiting ANGPTL3 activity.

Methods
All experiments in this study complied with the requirements of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision

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Research. All chemicals were of reagent grade quality and were purchased from Sigma Chemicals (St. Louis, MO, USA) unless stated otherwise.

**Cell Culture**

Human retinal microvascular endothelial cells (HRMECs) and attachment factor were purchased from Applied Cell Biology Research Institute (Kirkland, WA, USA) and maintained in EGM2-MV medium (Lonza Group AG, Basel, Switzerland) containing 5% fetal bovine serum (Lonza, Inc., Allendale, NJ, USA) in flasks coated with the attachment factor. Cells subjected to three to five passages were plated at 2500 cells/cm² in six-well plates (Corning, Acton, MA, USA) and exposed to high glucose concentration (30 mmol/L), normal glucose concentration (5 mmol/L), normal glucose plus ANGPTL3 overexpression, high glucose plus siRNA knockdown of the ANGPTL3 gene, high glucose concentration plus cilengitide (10 μM), high glucose concentration plus fenofibrate (100 μM), normal glucose concentration with ANGPTL3 overexpression plus fenofibrate, and normal glucose concentration with ANGPTL3 overexpression plus cilengitide. Cells were incubated high glucose concentration after 48 hours, subsequently transfected with RNA plasmid for 48 hours, or treated with fenofibrate or cilengitide for 48 hours.

**RNA Interference and Transfection in HRMECs**

The small human interfering RNA targeting ANGPTL3 (siRNA ANGPTL3), ANGPTL3 (OE ANGPTL3) mRNA overexpression, and blank vehicle plasmid were chemically synthesized by Shanghai Asia Vehicle Co., Ltd. (Shanghai, China). The siRNA sequence used for ANGPTL3 knockdown was 5'-CTACCTCCAGTTGGAAGATTGG-3'. The constructed vectors were transfected into cells using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. The transfected cells were then subjected to puromycin (10 μg/mL) selection for 5 days. Puromycin-resistant colonies were then selected and expanded. ANGPTL3 expression levels in the selected clones were evaluated by quantitative PCR (qPCR) and Western blotting.

**Animals**

Eight-weeks-old male Sprague-Dawley rats weighing ~200 g (Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China) were randomly assigned (six to eight rats per group) to the following treatment groups: intraperitoneal injection with streptozotocin (STZ 60 mg/kg), STZ plus intraocular injection with ANGPTL3 siRNA, STZ plus fenofibrate feeding, intraperitoneal injection with citrate buffer alone (NG rats), intraperitoneal injection with citrate buffer with intraocular rhANGPTL3 injection, and intraperitoneal injection with citrate buffer with intraocular rhANGPTL3 injection plus fenofibrate feeding. All rats were provided free access to standard rat food or fenofibrate diet and drinking water. Retinal tissues of the eyes were extracted under deep anesthesia at the end of the experiments.

**Fenofibrate Diet for Diabetic Rats**

Fenofibrate was mixed with AIN93M diet at a ratio of 800 mg:1 kg for diabetic rats (Trophic Animal Feed High-tech Co., Ltd., Nantong, China).

**Real-Time RT-PCR**

Total RNA was extracted from rat retinal tissue and HRMECs using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Real-time PCR was performed using an all-in-one qPCR kit (GeneCopoeia, Rockville, MD, USA) according to the manufacturer’s instructions. The primer sequences (sense/antisense) used are shown in the Table. Relative quantification of the signals was performed by normalizing the signals of different genes to that of the β-actin signal. Results were calculated as fold change relative to the control group.

**Western Blotting**

Extracted proteins (50 μg) obtained from each sample (HRMECs and retinas) were subjected to SDS-PAGE in a Bio-Rad miniature slab gel apparatus and electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked in a 5% fat-free dried-milk solution and incubated overnight with partially purified rat or human anti-IL-1β.
monoclonal antibody (mAb; Proteintech, Rosemont, IL, USA, 16806-1-AP; Abcam, Cambridge, UK, ab9722), anti–IL-6 mAb (Bioworld Technology, Saint Louis Park, MN, USA, BS6419), anti-P53 mAb (Proteintech, 10442-1-AP), anti-Bax mAb (Cell Signaling Technology, Danvers, MA, USA, 14796), anti-ANGPTL3 mAb (Proteintech, 11964-1-AP; Abcam, ab175288), anti-integrin αV mAb (Abcam, ab150361), and integrin β3 mAb (Proteintech, 18309-1-AP). β-actin levels were detected using a mAb (Sigma-Aldrich, St. Louis, MO, USA, A2066) and used as an internal control to confirm equivalent total protein loading. Signal intensities in the control lanes were arbitrarily assigned a value of 1.0. Western blots were repeated three to five times.

**RESULTS**

Anti-Inflammatory and Antiapoptotic Effects of Fenofibrate Both In Vivo and In Vitro

Western blotting and RT-PCR results revealed that IL-1β, IL-6, Bax, P53, and VEGF expression levels were significantly decreased in the retinas of rats treated with fenofibrate compared to diabetic control rats. **P < 0.01 DM versus DM+F, *P < 0.05 DM versus DM+F.**

**TUNEL Assay**

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% sodium citrate and 0.1% Triton X-100. DNA fragmentation was determined by TUNEL assay according to the manufacturer’s instructions (Roche Applied Science, Mannheim, Germany). Fluorescence images were captured using an EVOS fluorescence microscope (AMG, Bothell, WA, USA). The number of apoptotic cells was calculated using Graphpad Prism 5.0 software system (GraphPad, San Diego, CA, USA).

**Statistical Analysis**

Experimental data were expressed as means ± SD. Group means were compared using 1-way ANOVA using the Graphpad Prism 5.0 software system (GraphPad, San Diego, CA, USA) and the statistical software program SPSS 17.0 for Windows (Chicago, IL, USA). *P < 0.05 was considered significant in all cases.
were downregulated after fenofibrate treatment of the retinal tissues of diabetic rats (Figs. 1A, 1B) and retinal endothelial cells (Figs. 1C, 1D). The apoptosis rates in HRMECs were evaluated with the TUNEL assay, as shown in Figure 1E. Apoptosis rates were lower in the fenofibrate-treated group than those in the high-glucose stimulation group.

**Elevation of ANGPTL3 Levels Stimulates Apoptosis and Inflammation After High-Glucose Stimulation in the Retinas of Diabetic Rats**

IL-1β and IL-6 play crucial roles in the inflammatory process. P53 and Bax play major roles in apoptosis, which are in turn critical in pathogenesis in diabetic retinopathy. As shown in Figure 2, our results suggested that the protein (Fig. 2A) and mRNA (Fig. 2B) levels of ANGPTL3, IL-1β, IL-6, Bax, P53, and VEGF were upregulated in the retinas of diabetic rats relative to those of normal rats (DM versus NG). Furthermore, we examined whether ANGPTL3 promotes inflammation and apoptosis by performing both intraocular rhANGPTL3 injection in normal rats and siRNA-mediated downregulation of ANGPTL3 expression in diabetic rats. As shown in Figures 2A and 2B, IL-1β, IL-6, Bax, P53, and VEGF levels were upregulated after intraocular rhANGPTL3 protein injection in normal rats (rhANG versus NG). Both protein and mRNA levels of IL-1β, IL-6, Bax, P53, and VEGF were found to be downregulated after ANGPTL3 downregulation (DM versus DM + si).

**Pathogenic Effects of ANGPTL3 in the Endothelial Cell of Retinal Vessels**

Given the above-mentioned hypotheses, we evaluated the expression levels of genes involved in the inflammatory and apoptotic processes in HRMECs. As shown in Figure 2, both protein (Fig. 2C) and mRNA (Fig. 2D) levels of IL-1β, IL-6, Bax, P53, and VEGF were upregulated following high-glucose stimulation (HG versus NG) and overexpression of the ANGPTL3 gene (OE versus NG) but were downregulated after siRNA knockdown of ANGPTL3 expression (HG versus HG + si). The same results were obtained based on the TUNEL assay (Fig. 2E).
Overall, our findings suggested that elevation of \textit{ANGPTL3} levels can stimulate inflammation and apoptosis in diabetes both in vivo and in vitro.

\textbf{ANGPTL3 Stimulates the Apoptotic and Inflammatory Reactions via the Integrin $\alpha_V\beta_3$ Receptor}

\textit{ANGPTL3} is known to induce endothelial cell adhesion and migration, which leads to angiogenesis by interacting with the integrin $\alpha_V\beta_3$ receptor in vivo. Given the pathogenic effects of \textit{ANGPTL3}, we investigated the target receptor pathway of \textit{ANGPTL3}. As shown in Figure 3, our results indicated that both high-glucose treatment and \textit{ANGPTL3} overexpression upregulate the expression of integrin $\alpha_V\beta_3$ receptor in HRMECs (HG versus NG, OE versus NG), which can be inhibited by downregulating the \textit{ANGPTL3} gene (HG versus HG+si). To assess whether \textit{ANGPTL3} interacts with the integrin$\alpha_V\beta_3$ receptor to trigger a series of downstream reactions, we evaluated inflammation and apoptosis following treatment with the integrin $\alpha_V\beta_3$ inhibitor cilenitide. The above results indicated that inhibition of the integrin $\alpha_V\beta_3$ receptor (Figs. 3C–3E) can attenuate inflammation and apoptosis under both high-glucose stimulation and \textit{ANGPTL3} overexpression conditions. In conclusion, our results showed that \textit{ANGPTL3} can promote inflammation and apoptosis via the integrin $\alpha_V\beta_3$ receptor. In addition, the effects of \textit{ANGPTL3} can be blocked by treatment with cilenitide, an inhibitor of the integrin $\alpha_V\beta_3$ receptor.

\textbf{Fenofibrate Can Inhibit ANGPTL3-Induced Inflammation and Apoptosis in HRMECs}

Our previous findings showed that \textit{ANGPTL3} can stimulate inflammation and apoptosis in diabetic retinopathy. Therefore, we examined whether fenofibrate treatment can inhibit the pathogenic effects of \textit{ANGPTL3}. We first evaluated the \textit{ANGPTL3} levels, inflammation, apoptosis, and expression levels of integrin $\alpha_V$ and $\beta_3$ receptors in the retina of diabetic rats. As shown in Figure 4, both protein (Figs. 4A, 4B) and mRNA (Fig. 4C) expression levels of \textit{ANGPTL3}, IL-1$\beta$, IL-6, Bax, P53, VEGF, integrin $\alpha_V$, and integrin $\beta_3$ receptors were upregulated in diabetic rats (DM versus NG) and intraocular injection with rhuANGPTL3 of rats with normal glucose level (rhANG versus NG), and downregulated expression of the above markers were observed after fenofibrate treatment (DM versus DM+F, rhANG versus rhANG+F). We observed the same effects in HRMECs among the following groups: high-glucose (HG) condition, \textit{ANGPTL3} overexpression under normal glucose environment (OE), high-glucose treatment with fenofibrate (HG+F), \textit{ANGPTL3} overexpression in normal glucose environment with fenofibrate (OE+F), and normal glucose (NG). As shown in Figures 4D–4F expression levels of \textit{ANGPTL3}, IL-1$\beta$, IL-6, Bax, P53, VEGF, integrin $\alpha_V$, and integrin $\alpha_V$.
FIGURE 4. Downregulated expression of ANGPTL3, inflammation and apoptosis markers, and VEGF and integrin αVβ3 receptors both in vivo and in vitro (A–C). Western blotting (A), quantification (B), and real-time RT-PCR analysis of the expression levels of (C) IL-1β, IL-6, Bax, P53, VEGF, ANGPTL3, and integrin αVβ3 receptors in the retinas of rats in the following treatment groups: diabetic rats (DM), diabetic rats treated with fenofibrate (DM+F), intra-ocular injection with rhANGPTL3 rats with normal glucose (rhANG), intra-ocular injection with rhANGPTL3 rats with normal glucose plus fenofibrate (rhANG+F), rats exposed to normal glucose levels (NG). **P < 0.01 DM versus DM+F, *P < 0.05 DM versus DM+F, ###P < 0.01 rhANG versus rhANG+F, #P < 0.05 rhANG versus rhANG+F. D–F. Western blotting (D), quantification (E), and real-time RT-PCR analysis (F).
β3 receptors were upregulated under high-glucose stimulation (HG versus NG) and ANGPTL3 overexpression (OE versus NG). On the other hand, fenofibrate treatment downregulated the expression of the above markers (HG versus HG+F OE versus OE+F).

Furthermore, results of the TUNEL assay (Fig. 5) demonstrated increased apoptosis rates in HRMECs under both high-glucose stimulation and ANGPTL3 overexpression (HG versus NG, OE versus NG). Fenofibrate treatment was found to reduce apoptosis rates (HG versus HG+F OE versus OE+F). In conclusion, fenofibrate can inhibit ANGPTL3-induced inflammation and apoptosis in HRMECs.

**DISCUSSION**

Our results indicated that ANGPTL3 upregulation promotes inflammation and apoptosis, which can be inhibited by treatment with fenofibrate, a widely used lipid-lowering agent, both in vivo and in vitro. Furthermore, our results revealed that ANGPTL3 exerts pathogenic effects via interaction with the integrin αVβ3 receptor in HRMECs.

Accumulating evidence has documented the positive correlation between diabetes and plasma ANGPTL3 levels.\(^1\)\(^3\)

Our current findings showed that ANGPTL3 expression was upregulated under high glucose stimulation in HRMECs and in the retinas of diabetic rats. To investigate the relationship between ANGPTL3 and diabetic retinopathy, we measured the protein and RNA expression levels of genes involved in inflammation and apoptosis in both HRMECs and rat retinas before and after ANGPTL3 siRNA intervention under high-glucose conditions. On the other hand, apoptosis rates were evaluated using the TUNEL assay. Results showed that IL-6, IL-1β, P53, Bax, and VEGF levels were upregulated under both high glucose conditions and high ANGPTL3 overexpression. By contrast, expression levels of the above markers were downregulated after ANGPTL3 siRNA interference both in vivo and in vitro. The same results were observed based on the TUNEL assay. Therefore, ANGPTL3 upregulation in diabetic retinopathy will stimulate inflammation and apoptosis.

The mechanisms by which ANGPTL3 initiates the inflammatory and apoptotic processes remain poorly understood. Recent studies showed that ANGPTL3 can promote the podocyte foot processes via the integrin β3 receptor and followed activation of FAK/PI3K signaling pathway, which ultimately leads to proteinuria.\(^1\)\(^4\)\(^-\)\(^7\)\(^1\)\(^7\) ANGPTL3 can also promote the motility and permeability of podocytes by upregulating nephrin expression, which plays a role in the pathophysiology of proteinuria.\(^1\)\(^8\)\(^-\)\(^9\) To elucidate the mechanisms underlying the above-mentioned pathogenetic effects, we measured integrin αVβ3 receptor levels in both HRMECs and retinal tissue in diabetic rats. Consequently, elevated expression of integrin αVβ3 receptor was observed after exposure to high-glucose conditions and rhANGPTL3 stimulation in HRMECs. Expression of the integrin αVβ3 receptor was observed following ANGPTL3 overexpression, thereby demonstrating that ANGPTL3 promoted inflammation and apoptosis via integrin αVβ3 receptor. Furthermore, IL-6, IL-1β, P53, Bax, and VEGF levels were downregulated under treatment with cilengitide, an inhibitor of integrin αVβ3 receptor, thereby verifying the mechanisms underlying the pathogenetic effects of ANGPTL3.

Fenofibrate, a widely used lipid-lowering agent, has been demonstrated to exert therapeutic effects in diabetic retinopathy. In humans, fenofibrate was revealed to significantly reduce the requirement for laser photocoagulation in both diabetic macular edema and proliferative diabetic retinopathy (PDR) compared with the control group. Furthermore, fenofibrate can slow down the progression of diabetic retinopathy.\(^4\) Recent studies have demonstrated that fenofibrate exerts anti-inflammatory effects in diabetic retinopathy by PPARα activation\(^2\)\(^0\) and by inhibiting LPS-induced IL-6 expression in mouse aortic explants.\(^2\)\(^1\) In addition, fenofibrate can inhibit fibroblast growth factor–induced proliferation in bovine capillary endothelial cells and VEGF-induced proliferation and migration in human umbilical vein endothelial cells.\(^2\)\(^2\) Furthermore, our previous findings showed that fenofibrate can suppress the inflammatory reaction in diabetic retinopathy via the sirtuin 1-dependent signaling pathway in endothelial cells.\(^2\)\(^3\) Therefore, we aimed to investigate whether fenofibrate

**Figure 5.** TUNEL assay for determining apoptosis rates in HRMECs in the following treatment groups: high glucose (HG), high glucose plus fenofibrate (HG+F), normal glucose (NG), ANGPTL3 overexpression in HRMECs under normal glucose levels (OE), ANGPTL3 overexpression plus fenofibrate in HRMECs under normal glucose levels (OE+F), and normal glucose levels (NG). **P < 0.01 HG versus HG+F #P < 0.01 OE versus OE+F #P < 0.05 HG versus HG+F #P < 0.05 OE versus OE+F

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influences ANGPTL3 and its downstream effects. Consequently, our study showed that fenofibrate suppressed the ANGPTL3-induced apoptosis and inflammation both in vitro and in vivo. Our study is the first to suggest that fenofibrate suppresses cellular inflammation and apoptosis by inhibiting ANGPTL3. The use of fenofibrate for the management of diabetic retinopathy appeared to provide beneficial effects against the complications of diabetes mellitus. The molecular pathways underlying this interaction remain to be investigated.

To our knowledge, our study is the first to establish a link between fenofibrate and ANGPTL3 in the pathophysiology of diabetes. First, fenofibrate has been demonstrated to exert therapeutic effects in diabetic retinopathy by inhibiting inflammation (based on IL-6 and IL-1β) and apoptosis (based on Bax and P53). Second, ANGPTL3 upregulation was found to be associated with the inflammatory and apoptotic processes, which were mediated by the interaction with the integrin αvβ3 receptor. Therefore, we measured integrin αvβ3 levels following downregulation of ANGPTL3 expression and the inflammation and apoptosis after treatment with cilengitide, an inhibitor of the integrin αvβ3 receptor. Results showed that the expression levels of the above-mentioned markers were downregulated by cilengitide intervention. Hence, the integrin αvβ3 receptor acts as a vital link between ANGPTL3 and diabetic retinopathy. Finally, we investigated whether ANGPTL3 is associated with the therapeutic effects of fenofibrate. Our results showed that fenofibrate can attenuate the pathogenic effects of ANGPTL3 in diabetic retinopathy.

In conclusion, our study revealed that ANGPTL3 stimulates inflammation and apoptosis in diabetic retinopathy. Using in vivo and in vitro models, we demonstrated that ANGPTL3 exerts its pathogenic effects via integrin αvβ3 receptors, which can be blocked by cilengitide, an inhibitor of integrin αvβ3 receptor. We also provided evidence that fenofibrate suppresses ANGPTL3-induced inflammation and apoptosis. Our findings suggested that ANGPTL3 is a potential therapeutic target for the treatment of diabetic retinopathy. Additionally, the use of fenofibrate in managing diabetic retinopathy provides more benefits for microvascular and macrovascular complications of diabetes mellitus regardless of its lipid-lowering effect.

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