Fluvastatin Downregulates VEGF-A Expression in TNF-α-Induced Retinal Vessel Tortuosity

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PURPOSE. To investigate the effect of tumor necrosis factor alpha (TNF-α) on the mouse retinal vasculature, function, and expression of vascular endothelial growth factor-A (VEGF-A) in the retina and retinal pigment epithelium (RPE) and to evaluate the protective effect of statin therapy (fluvastatin) on retinal vascular and functional changes.

METHODS. A single intravenous injection of murine TNF-α (8 μg/kg body weight) was administered to one group of mice (TNF group). In the second group of mice (TNF+Statin group), a single dose of TNF-α was followed by 28 days oral medication of fluvastatin (10 mg/kg/d), and an equivalent volume of saline was administered to the third group (Control group). After 28 days, electoretinography (ERG) and fundus photography were performed. Eyes were collected for cell and molecular studies. Transcript levels of VEGF-A in retina and RPE were quantified using real-time polymerase chain reaction, and protein expression was analyzed by Western blot and immunostaining.

RESULTS. TNF-α-injected mice showed retinal vessel tortuosity, structural change, and altered retinal function. Fluvastatin-treated mice exhibited retinal vascular, structural, and functional changes almost similar to those of the control group. VEGF-A expression was significantly upregulated in the retina and RPE of TNF-α-injected mice, and this was significantly downregulated in fluvastatin-treated mice.

CONCLUSIONS. This study shows that the TNF-α-induced inflammatory process results in the alteration of retinal microvasculature and function, and fluvastatin could be a potential therapy for treating/preventing retinal microvascular or inflammatory complications. (Invest Ophthalmol Vis Sci. 2011;52:7423–7431) DOI:10.1167/iovs.11-7912

Tumor necrosis factor alpha (TNF-α) is a major cytokine upregulated in various retinal vascular diseases such as diabetic retinopathy (DR),1,2 age-related macular degeneration (AMD),3,4 and uveitis.5 TNF-α acts as a potent mediator of inflammation, angiogenesis, and apoptosis, induces proangiogenic molecules, and indirectly stimulates retinal neovascularization.6,7 Studies have shown that upregulation of TNF-α leads to the loss of microvascular cells in the retina, and its inhibition by TNF-α receptor molecules/antibodies protects the retinal microvasculature.8–10 However, the exact role of TNF-α on retinal microvasculature structure and function remains unclear.

Vascular endothelial growth factor (VEGF) is another major cytokine involved in the development of retinal vascular diseases and is also a potent mediator of vascular remodeling and angiogenesis.11 Previous studies reported that VEGF and TNF-α show significant overlap with regard to their effects in the eye,12 and their levels have been found to be increased under pathophysiological conditions.1,2,11 Recently, drugs targeting TNF-α and VEGF have been used in patients with ocular inflammation with promising results.13,14 VEGF-A, which signals through VEGF-R1 and VEGF-R2, is involved in the pathogenesis of many ocular neovascular diseases15 and is the target of most current anti-VEGF treatments.16–18 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) are lipid-lowering drugs frequently used in dyslipidemia and type 2 diabetes.19 Several therapeutic effects of statins have been recognized, including anti-inflammation,20 antioxidative,21 antiangiogenesis,22 neuroprotection,23 and improvement of endothelial cell function.24 Fluvastatin, in addition to its lipid-lowering effect, is a semisynthetic statin that has been shown to improve vascular function in hypercholesterolemic patients.25 Studies have also suggested antiatherogenic, antithrombotic, anti-inflammatory, and antioxidant properties of fluvastatin.26,27 The vascular protective effect of fluvastatin has been extensively documented,20,28 and there is robust evidence that statins elicit important effects on the vascular endothelium, contributing to the reduction of morbidity and mortality of cardiovascular diseases.29

Recent studies have focused on the potential use of statins in preventing ocular inflammatory diseases. Clinical reports have suggested the use of statins for the management of DR20 and AMD.31 However, only few studies have examined the therapeutic effect of statin in preventing retinal microvascular complications.20,21,28 In vivo studies will be useful to investigate the role of fluvastatin at molecular and cellular levels before clinical trials of statins on major eye diseases. In this study, we investigated the effect of TNF-α on retinal microvasculature, retinal function, and cellular and molecular changes and determined whether these changes can be prevented by the administration of fluvastatin in mice.

MATERIALS AND METHODS

Experimental Groups

Wild-type C57BL/6 mice (8 weeks old) purchased from the National University of Singapore were used for the study. Mice were divided into three experimental groups: TNF, TNF+Statin, and Control group.

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Animals were monitored for 28 days, after which ERG and fundus imaging were carried out under anesthesia. Eyes were then collected for cell and molecular analysis.

**Fundus Imaging and Fundus Fluorescein Angiography**

Digital color fundus photography and fundus fluorescein angiography (FFA) were performed using a modified portable small animal fundus camera (Kowa Genesis, Tokyo, Japan) after topical administration of 1 drop each of 1% tropicamide (Alcon Laboratories, Inc., Tampa, FL) and phenylephrine (2.5%; Bausch and Lomb Pharmaceuticals, Inc., Tampa, FL) ophthalmic solutions for pupil dilation. Mice were anesthetized with a combination of ketamine (20 mg/kg body weight) and xylazine (2 mg/kg body weight). For FFA, mice were injected intraperitoneally with 10% sodium fluorescein dye at a dose of 0.01 mL/5-6 g body weight.
weight after pupil dilation. The passage of fluorescein through the retinal vascular system was serially recorded using a fundus camera equipped with exciter and barrier filters suitable for FFA. The images were used for examining retinal microvascular characteristics.

**Electroretinography**

Animals were dark-adapted overnight (12 hours), and the preparations for recordings were carried out under dim red light. Anesthesia and pupil dilation were induced as described. Animals were lightly secured to a stage with fastener strips across the upper and lower back to ensure a stable, reproducible position for ERG recordings. Body temperature was maintained between 37°C and 38°C with a pumped-water heating pad (TP500 T/Pump; Gaymar Industries, Orchard Park, NY) fixed to the top of the stage. ERGs were recorded (Espion; Diagnosis LLC, Redwood City, CA.) with corneal monopolar electrodes (Mayo, Aichi, Japan). A gold-cup electrode (Glass-Telefactor, West Warwick, RI) was placed in the mouth to serve as the reference electrode, and a silver-silver chloride electrode (Glass-Telefactor, West Warwick, RI) was placed in the tail to serve as the ground electrode. Recordings were performed at a wide range of stimulus intensities (∼3.3 to 1.0 log cd·s/m² in 0.3-log unit increments) in dark-adapted (scotopic) condition. The response at each intensity was an average of at least five trials. Signals were band-pass filtered from 1 to 100 Hz and were acquired at 1 kHz. The duration of the ERG recording session was approximately 30 minutes for each animal.

**Dissecting Retinal Tissue for Mounting**

Eyes were enucleated, and whole eyes were fixed in 4% paraformaldehyde (PFA) for 4 hours. Retinas were carefully removed by using a modification of the method of Chan-Ling et al.53 Briefly, under a dissecting microscope, an incision was made at the limbus, and the cornea was circumcised from the sclera. The lens was gently removed without disturbing the retina. The remaining eyecup was transferred to 1× phosphate-buffered saline (PBS), and the full extent of the retina was eased from the sclera using fine forceps. The retina was then placed onto a microscope slide and flattened by making four incisions, each 90° apart, beginning at the ora serrata and extending centrally from the equator, stopping short of the optic nerve opening.

**Isolectin Staining**

The flattened retinas were made permeable in ice-cold 70% vol/vol ethanol for 20 minutes and then in PBS/1% Triton X-100 for 30 minutes. Retinas were incubated with AlexaFluor 568–conjugated *Griffonia simplicifolia* isoelectin B4 (5 μg/mL; Invitrogen-Molecular Probes, Eugene, OR) in 1× PBS overnight at 4°C for staining of the vasculature. Then retinas were rinsed three times in 1× PBS for 10 minutes each and mounted in antifade medium containing DAPI (4,6-diamidino-2-phenylindole; Santa Cruz Biotechnology, Inc.) to visualize the cell nucleus. Sections incubated with 4% BSA with omitted primary antibody were used as a negative control.

A fluorescence microscope (Axioplan 2; Carl Zeiss Meditec GmbH, Oberkochen, Germany) was used to examine the slides, and images were captured. Experiments were repeated in duplicate from three different samples.

**Real-Time–Polymerase Chain Reaction**

The whole retina and retinal pigment epithelium (RPE) were removed from the control and experimental mice after 28 days and then immediately frozen in liquid nitrogen. The retina and RPE were isolated as described previously.54 Total RNA was extracted from the retina and RPE using reagent (TRizol; Invitrogen), and the amount of total RNA was determined by spectrophotometry.

![Graph A](image1.png)  
**Figure 2.** Increased ERG a- and b-wave amplitude in TNF-α–induced mice. The bar graph represents the changes in the a-wave amplitude (A) and b-wave amplitude of TNF-α–injected and fluvastatin-treated groups (B). The a-wave demonstrates the function of retinal photoreceptors, and the b-wave represents the function of inner retinal layers (bipolar and amacrine cells). The abnormal a- and b-wave amplitudes were observed in TNF-α–injected mice, which indicates the alterations of retinal function. Fluvastatin-treated group and saline-injected control mice retina show the normal pattern of a- and b-wave amplitude ERG recordings. Data represent the mean ± SD. *P < 0.05.
was quantified using a spectrometer (NanoDrop; Wilmington, DE). cDNA was synthesized using cDNA synthesis kit (Bio-Rad; Hercules, CA). Quantitative RT-PCR was carried out on a PCR amplification system (Light Cycler; Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) using a hot start reaction kit (Fast Start DNA Master Plus SYBR Green 1 kit; Roche Diagnostics) and specific primers for VEGF-A (forward, 5′ CAC AGC AGA TGT GAA TGc AG 3′; reverse, 5′ TTT ACA CGT CTG CGG ATC TT 3′). Expression of the VEGF-A gene was measured in triplicate and was normalized using the GAPDH gene as an endogenous internal control. Gene expression of VEGF-A in control and experimental groups was quantified using the 2−ΔΔCt method.26

Western Blot Analysis
The mouse retina and RPE were ground to a fine powder in liquid nitrogen and homogenized in ice-cold radio-immunoprecipitation assay buffer. Total protein concentration was determined using a direct colorimetric protein assay reagent (Bio-Rad). Fifty micrograms of the proteins was separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. The membrane was blocked in 5% (wt/vol) milk for 1 hour and washed briefly with TBS-Tween (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 0.05% Tween-20). It was then incubated with a primary antibody (VEGF-A; Santa Cruz Biotechnology Inc.) diluted in 1× PBS for 1 hour and washed briefly with TBS-Tween. Secondary anti–rabbit antibody (anti-rabbit IgG HRP; Thermo Fisher Scientific Inc.) was then added at a dilution of 1:15,000 in 1× PBS for 1 hour, followed by three 5-minute washes. β-Tubulin (Sigma-Aldrich, St. Louis, MO) was used as the housekeeping protein. Protein bands were visualized and quantified.

Statistical Analysis
Data were assessed using the Student’s t-test and ANOVA. They are expressed as mean ± SD and are representative of evaluations in a minimum of six mice. P < 0.05 was considered statistically significant.

RESULTS

TNF-α Induces Retinal Vessel Tortuosity
Color fundus photographs and FFA showing retinal vessels characteristics of control, TNF-α, and statin-treated groups of mice 28 days after treatment are shown in Figure 1. Retinal vessel tortuosity was clearly observed in mice with TNF-α but not in the TNF+Statin or control group. No fluorescein leakage was observed with FFA in all the groups.

ERG Changes in TNF- and Statin-Treated Mice
Average a- and b-wave amplitudes obtained at the flash intensity of 1.0 log cd · s/m² for each experimental group are presented in Figure 2. Both a- and b-wave amplitudes were significantly increased (P < 0.05) in the TNF group compared with the control group. A similar increase in amplitude was also observed at all levels of flash intensity (data not shown). The a- and b-wave amplitudes of the fluvastatin group, however, were similar to those of the control.

Isolectin Staining
Whole-mount G. simplicifolia isolecitin B4 labeling of the retinal vasculature of the control, TNF-α, and statin groups of mice was performed to study changes in the microvasculature of the retina. Isolectin staining demonstrated increased or altered retinal arteriolar microvasculature in the TNF-α-injected group compared with those seen in the control group. The fluvastatin-treated group showed retinal vasculature similar to that of the control group (Fig. 3).

Histology and Immunohistochemistry
Staining of retinal sections with hematoxylin and eosin demonstrated disordered arrangements of the retinal ganglion cell layer in the TNF-α-injected mice, whereas the fluvastatin-treated group showed well-organized ganglion cell layer (Fig. 4A). The retinal thickness was significantly reduced in the TNF group compared with the fluvastatin- and saline-treated group (Fig. 4B, P < 0.05). Thus, TNF-α induced structural change to the mouse retina, and this change was shown to be reversed to near control by fluvastatin therapy. Immunofluorescence labeling using VEGF-A antibody showed increased expression of VEGF-A in the retina, especially in the photoreceptor layer of TNF-α-treated mice compared with control. Fluvastatin treatment decreased the expression of VEGF-A to near control in the retina. There is an increased expression of VEGF-A in the RPE of TNF-α-treated mice, whereas the fluvastatin-treated group showed well-organized ganglion cell layer similar to that of the control group (Fig. 3).
tatin—treated group showed reduced expression of VEGF-A in the RPE compared with control (Fig. 5).

Fluvastatin Downregulates VEGF-A Gene Expression in Retina and RPE

Increased VEGF-A expression has been shown to be a primary factor in retinal microvascular complications. Hence, using RT-PCR, we determined VEGF-A gene expression in the retina and in the RPE (which is the major source of VEGF in the retina) of the control, TNF-α, and statin groups of mice. Results showed a significant upregulation ($P < 0.05$) of VEGF-A gene expression in the retinas of TNF-α—treated mice; this was near baseline in the fluvastatin-treated group ($P < 0.05$) compared with the control group. In the RPE, the relative fold change of VEGF-A gene expression was significantly ($P < 0.05$) upregulated in the TNF-α—treated mice but was significantly downregulated ($P < 0.05$) in the statin-treated mice compared with control (Figs. 6A, 6B).

VEGF-A Protein Expression in Retina and RPE by Western Blot Analysis

As did VEGF-A gene expression, Western blot analysis demonstrated significant increases in VEGF-A protein in the retinas of the TNF group of mice, whereas this was significantly reduced in the fluvastatin-treated group and was similar in the control group. In the RPE, VEGF-A protein expression was increased significantly in the TNF-α—treated group and showed decreased expression in the statin-treated group (Figs. 6C, 6D). β-Tubulin was used as the loading control.

DISCUSSION

TNF-α plays a major role in various retinal vascular diseases such as DR and AMD through a range of pathogenic pathways such as endothelial and retinal cell injury, apoptosis, angiogenesis, and vascular leakage. There is evidence that TNF-α...
inhibition by TNF-α receptor molecules results in the protection of the retinal microvasculature. One of the major findings of the present study was that the proinflammatory cytokine TNF-α induced retinal vessel tortuosity in mice at 28 days after the administration of a single dose of murine TNF-α. Retinal vascular tortuosity is an important marker of vascular disease not only in the eye but also in other systems. Clinical studies have shown that increased retinal vessel tortuosity is associated with cardiovascular disease risk factors, hyperglycemia, and DR and that it may be related to endothelial dysfunction.

An experimental study by Moe et al. reported that TNF-α induced retinal arterial dilation and ventricular remodeling in mice 28 days after administration. Previous reports indicate that the angiogenic process can cause dilation and tortuosity of retinal blood vessels before the onset of neovascularization and before TNF-α has been shown to be angiogenic in vivo.

In this study, we demonstrated the role of TNF-α in retinal vessel tortuosity. TNF-α also induced increased retinal arteriolar microvasculature, as observed with isoelectin staining of the retina. In addition, changes in retinal function were observed in mice with TNF-α, whereas the statin-treated group showed retinal function compatible to that of the control group. Changes in ERG a-wave amplitude are linked to photoreceptor function in the retina, and b-wave amplitude is linked to photoreceptor function in the middle retinal cell layers, primarily the bipolar cells. Increased ERG amplitudes have been reported in many inflammatory conditions and were observed in this study in TNF-α–treated mice. The exact mechanism of enhanced ERG amplitudes is unclear, but they indicate the presence of altered retinal function. Previous studies reported that TNF-α circulating levels are predominantly increased in
inflammatory diseases such DR and ischemic retinopathy, which lead to retinal dysfunction.18,46 The protective effect of simvastatin on the retinal function and retinal ganglion cells of the rat after ischemia has been reported very recently.55 Similarly, the present study results provide evidence that TNF-α-induced inflammatory processes in the mouse retina cause retinal cell damage. Ganglion cell layer, inner nuclear layer, and inner plexiform layer thickness were reduced by systemic TNF-α treatment. This result indicates that TNF-α promotes injury or apoptosis to the retinal cells. The retinal apoptotic process may occur either because TNF-α creates hypoxia to the retinal cells or because of chronic inflammation. The activity of fluvasatin was found to cause no adverse effects in retinal function, as demonstrated with our ERG data. Fluvastatin therapy also reversed TNF-α-induced structural changes in the ganglion cell layer of the retina. Thus, our study also reveals the beneficial effect of fluvastatin therapy on retinal vasculature, function, and structure in mice.

Furthermore, retinal vascular, structural, and functional changes were associated with the increased expression of VEGF-A in the retina and the RPE. VEGF-A expression was more prominent in the photoreceptor layer of the TNF-α-injected group. This finding supports the hypothesis that TNF-α induces ischemia or hypoxic stimulus in the photoreceptor layer that may contribute to hypoxia-related angiogenesis or retinal hypoxia, which increases VEGF-A production.48–50 VEGF is a potent stimulator of inflammation, angiogenesis, and vascular remodeling. Both VEGF and TNF-α levels have been found to be increased under pathophysiological conditions.1,2,11 TNF-α has been shown to be proangiogenic in vivo,41 possibly through the stimulation of VEGF induction.51 VEGF is known to affect vascular development and has been demonstrated to be an early event in the development of retinal vessel tortuosity and dilation.52 Our studies also demonstrated an increased expression of VEGF-A in TNF-α-induced retinal vessel tortuosity. Previous studies reported TNF-α-induced expression of VEGF receptors in cultured vascular endothelial cells53 and inhibition of TNF-α expression by anti-VEGF treatment.54 Because TNF-α is able to increase levels of VEGF,55,56 studies suggest that VEGF could contribute to mediating the effects of TNF-α in the retina.57 Thus, our findings also indicate that TNF-α-induced vascular and functional changes in the retina might be mediated through the increased expression of VEGF-A. Studies in humans and experimental animal models have reported that statins are highly effective in preventing vascular inflammation and improving vascular endothelial cell function.58,59 Evidence has shown that statins enhance retinal capillary endothelial cell survival and modulate angiogenic repair, thereby preventing preretinal neovascularization.22 Studies have revealed that fluvasatin may prevent vascular dysfunction20 by inhibiting inflammatory cell-endothelial cell interactions.60 Hence, the protective effect of statin therapy on vasculature has been attributed to their antioxidant, anti-inflammatory, and antiangiogenic properties and to their lipid lowering effect.61 Experimental studies have reported the anti-inflammatory effect of statin through the attenuation of TNF-α expression.52 In this study, we assessed the beneficial effect of fluvasatin therapy in preventing TNF-α-induced retinal complications in mice. Previous studies also indicate that statins induce greater protection against vascular risk than that expected by cholesterol reduction.63 Retinal flat-mount images of isolectin staining showed altered retinal vessel morphology by systemic administration of TNF-α that was reversed to near control by fluvasatin therapy. Thus, our findings revealed that fluvasatin therapy could inhibit TNF-α-induced alterations in retinal microvasculature, structure, and function and may be a useful drug for treating microvascular complications.

It has become evident during the past decade that VEGF receptors are critical targets for developing new drugs to suppress a range of diseases, particularly ocular inflammations.14 Statins have antiangiogenic effects, which are associated with alterations in VEGF signaling.59,65 Treatment with statins has been shown to reduce VEGF in the serum and plasma of hypercholesterolemic patients.54,60 Experimental studies have
also demonstrated that statin effects in preventing retinal vascular dysfunction are associated with the blockade of VEGF expression.\textsuperscript{61,66} Recent studies in animal models have reported the antiangiogenic effect of fluvastatin by reducing VEGF expression in the retina.\textsuperscript{20} Neutralizing VEGF decreases retinal vessel tortuosity and dilation.\textsuperscript{67} In the present study, fluvastatin treatment downregulated TNF-α-induced expression of VEGF-A in the retina and RPE, thereby inhibiting the TNF-α-induced changes in the retina.

The protective effect of statin therapy on retinal vasculature through the downregulation of VEGF may be associated with their antioxidant/anti-inflammatory properties. Oxidative stress plays an important role in triggering retinal vascular inflammatory processes. Previous studies demonstrated that NADPH oxidase-derived reactive oxygen species are important for hypoxia/diabetes-induced increases in VEGF.\textsuperscript{61,66,69} The retinal microvascular and functional changes observed in the present study may be due to hypoxia or to an inflammatory process induced by TNF-α in the retina. We speculate that the vasculoprotective effect of fluvastatin through the downregulation of VEGF may be associated with the inhibition of hypoxia-induced oxidative stress in the retina, which must be explored further.

In summary, our study demonstrates the following. First, TNF-α induces increased retinal vessel tortuosity and functional alterations, possibly mediated through increased expression of VEGF-A. Second, fluvastatin therapy reduces the retinal vascular changes induced by TNF-α. Third, the protective effect of fluvastatin therapy on TNF-α-induced retinal complications might be mediated through downregulation of VEGF-A. Thus, our findings indicate that fluvastatin may have anti-VEGF activity and may have potential for treating or preventing major retinal vascular diseases, such as DR and AMD.

Acknowledgments

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