

PPAR α -Dependent Effects of Palmitoylethanolamide Against Retinal Neovascularization and Fibrosis

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Received: November 1, 2019

Accepted: January 15, 2020

Published: April 16, 2020

Citation: Ye S, Chen Q, Jiang N, et al. PPAR α -dependent effects of palmitoylethanolamide against retinal neovascularization and fibrosis. *Invest Ophthalmol Vis Sci.* 2020;61(4):15.

<https://doi.org/10.1167/iovs.61.4.15>

PURPOSE. Pathological neovascularization and fibrosis are common pathological changes of many retinal diseases, such as proliferative retinopathy (PR) and age-related macular degeneration (AMD). Treatment modalities for these pathological changes are limited. The purpose of the present study was to test the effects of palmitoylethanolamide (PEA), an endocannabinoid mimetic amide, on retinal neovascularization and fibrosis and to determine its molecular mechanism of action.

METHODS. A rat Müller cell line (rMC-1), a mouse model of oxygen-induced retinopathy (OIR), and the very-low-density lipoprotein receptor (VLDLR) knockout mouse model were used. PEA was intraperitoneally injected or orally administered in animal models. Inflammation and profibrotic changes were evaluated by western blot analysis. Glial fibrillary acidic protein (GFAP) and peroxisome proliferator-activated receptor alpha (PPAR α) were measured by RT-PCR and western blot analysis.

RESULTS. Profibrotic changes were present in OIR and *Vldlr*^{-/-} retinas. PEA significantly alleviated inflammation and inhibited neovascularization in OIR and *Vldlr*^{-/-} retinas and suppressed profibrotic changes in OIR and *Vldlr*^{-/-} retinas. Moreover, PEA potently suppressed Müller gliosis in these retinas. In rMC-1 cells, PEA suppressed Müller gliosis, reduced inflammatory cytokines, and attenuated profibrotic changes. Further, both mRNA and protein levels of PPAR α were elevated in the retina under PEA treatment, and the effects of PEA were abolished in *Ppara*^{-/-} OIR mice.

CONCLUSIONS. PEA reduced retinal neovascularization and fibrotic changes and suppressed Müller gliosis in experimental PR and neovascular AMD by activating PPAR α . PEA may be a potential treatment for retinopathies with pathological neovascularization and fibrosis.

Keywords: palmitoylethanolamide, neovascularization, retinal fibrosis, proliferative retinopathy, neovascular age-related macular degeneration

Pathological neovascularization and subsequent fibrosis are common pathologies in many retinal diseases, such as proliferative retinopathy (PR) and age-related macular degeneration (AMD).¹ In developed countries, retinal neovascularization and fibrosis are considered to be the most common pathological changes in the retina that cause vision loss.² Following retinal injury, the release of chemotactic agents, proinflammatory cytokines, and vascular endothelial growth factor (VEGF) induces proliferation of endothelial cells and promotes the formation of new vessels. Meanwhile, the recruitment of inflammatory cells in the retina initiates a reparative process and facilitates synthesis and remodeling of new extracellular matrix (ECM). If the reparative process is not successful, the deposition of excess ECM may disturb the retinal architecture, interrupt proper cellular interaction, and cause retina contraction. Currently,

the therapeutic strategies for retinal neovascularization and fibrosis are limited. The exploration of new therapeutic treatments will greatly benefit patients with neovascular and fibrotic retinopathies.

It has been reported that palmitoylethanolamide (PEA), an endocannabinoid mimetic amide, has anti-inflammatory and anti-analgesic effects in the central nervous system (CNS).³ Recent studies have explored the application of PEA in glaucoma and diabetic retinopathy; for example, topical PEA suppresses the ocular surface inflammation associated with the long-term use of hypotensive eye drops in glaucoma patients.⁴ PEA attenuates retinal inflammation and reduces the expressions of the proinflammatory factors in streptozotocin-induced diabetic rats.⁵ However, the effects of PEA on retinal neovascularization are unknown.

In addition to the anti-inflammatory properties of PEA, antifibrotic effects for PEA have also been reported. A prior study identified that PEA inhibits liver fibrosis in a carbon tetrachloride-induced mouse model.⁶ In another study, PEA was found to reduce lung inflammation, pulmonary damage, and lung fibrosis in a mouse model of idiopathic pulmonary fibrosis.⁷ However, the effects of PEA on retinal fibrosis have not yet been investigated.

Large amounts of PEA are produced and secreted by glial cells in the brain.⁸ Studies have shown that PEA inhibits reactive astrogliosis induced by β -amyloid peptide in cultured astrocyte cells.⁹ A recent study also reported that PEA has therapeutic effects for Alzheimer's disease and brain gliosis, indicating a possible protective role of PEA in gliosis.¹⁰ Müller cells are glial-like cells in the retina that play an important role in maintaining retinal hemostasis.¹¹ Müller cell activation has been associated with initiation and development of many retinal diseases, including PR and AMD.^{12,13} Considering the suppressive effects of PEA on gliosis of the CNS, we hypothesize that PEA may also suppress retinal Müller cell activation.

In the present study, we evaluated the anti-angiogenic and antifibrotic effects of PEA in the retina using a mouse model of oxygen-induced retinopathy (OIR), an animal model for proliferative retinopathy,¹⁴ and the very-low-density lipoprotein receptor knockout (*Vldlr*^{-/-}) mouse, an animal model of neovascular AMD.¹⁵ We identified profibrotic changes in OIR and *Vldlr*^{-/-} retinas, suggesting the utility of these models for studying early retinal fibrotic changes. We also found that the Müller cells activated in these models were suppressed by PEA. Further, we confirmed the inhibitory effects of PEA on Müller cell activation using a cell culture model system. The mechanisms of action of PEA in the retina were also investigated. Our findings suggest that PEA inhibits retinal neovascularization and profibrotic changes and suppresses Müller cell activation in animal models of PR and neovascular AMD. PEA is a putative novel treatment for retinopathies with pathological angiogenesis and fibrosis.

MATERIALS AND METHODS

Animals

C57BL/6J mice were purchased from the Laboratory Animal Center of Xiamen University. Peroxisome proliferator-activated receptor alpha (PPAR α) knockout (*Ppara*^{-/-}) mice and VLDLR knockout (*Vldlr*^{-/-}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). In this study, *Vldlr*^{-/-} mice and control mice for *Vldlr*^{-/-} mice (littermate wild-type mice) were obtained by crossing C57BL/6J and *Vldlr*^{-/-} mice. All animals were housed in a specific-pathogen-free facility and maintained in 12-hour light/12-hour dark cycles. All procedures with animals in this study were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animal protocols were approved by the Xiamen University Experimental Animal Ethics Committee.

Cell Culture

The rat Müller cell line (rMC-1) cells were maintained in Dulbecco's Modified Eagle Medium basal medium supplemented with 10% fetal calf serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 10% penicillin streptomycin solution (Gibco).

Oxygen-Induced Retinopathy Model and PEA Treatment

Neonatal C57BL/6J mice (or *Ppara*^{-/-} mice) were exposed to 75% oxygen for 5 days from postnatal day 7 (P7) to P12.¹⁶ At P12, the mice were returned to room air (21% oxygen). As described previously,¹⁷ PEA was dissolved in solution: 0.01% dimethyl sulfoxide, 10% Tween 80 (Sigma-Aldrich, St. Louis, MO, USA), 20% polyethylene glycol 400, and 69.9% physiological saline. To investigate the effects of PEA on pathological angiogenesis, PEA (30 mg/kg) was injected intraperitoneally in OIR mice from P12 to P17 and in *Vldlr*^{-/-} mice from P12 to P26 daily. OIR mice (at P17) and *Vldlr*^{-/-} mice (at P26) were sacrificed, and retinas were dissected for further experiments. To study the effects of PEA on fibrotic changes in *Vldlr*^{-/-} mice, PEA (30 mg/kg) was administered to 2-month-old *Vldlr*^{-/-} mice by oral gavage for 30 days, and eyecups were collected for further experiments at P90.

Lectin Staining

Lectin staining of the flat-mounted retinas was conducted as described previously.¹⁸ Briefly, eyeballs were fixed with 4% paraformaldehyde for 1 hour. Retinas were dissected and then incubated with 0.5% Triton X-100 (Sigma-Aldrich) at 4°C overnight. After three washes with PBS, the retinas were incubated with isolectin G5-IB4 (Thermo Fisher Scientific) overnight at room temperature. Retinas were washed and flat-mounted for microscopy. Quantification of neovascularization vaso-obliteration was conducted as described previously.¹⁹

Immunofluorescent Staining

Retinal cryosections were fixed in acetone for 10 minutes and then incubated with antibody against glial fibrillary acidic protein (GFAP) (Abcam, Cambridge, United Kingdom) at 4°C overnight. After three washes with PBS, retinal sections were further incubated with Alexa Fluor 488-conjugated IgG (Thermo Fisher Scientific) for 1 hour. The sections were mounted with mounting medium (Vector Laboratories, Burlingame, CA, USA) and photographed with a fluorescent microscope (DM2500; Leica Microsystems, Wetzlar, Germany).

TUNEL Assay

The TUNEL assay was performed on retinal cryosections using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Western Blot Analysis

Western blot analysis was conducted as previously described.²⁰ Antibodies for GFAP, intercellular adhesion molecule 1 (ICAM-1), tumor necrosis factor alpha (TNF- α), and transforming growth factor beta (TGF- β) were obtained from Proteintech (Chicago, IL, USA). Antibodies for VEGF, TGF- β receptor II (TGF- β RII), Smad2/3, and fibronectin were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies for alpha smooth muscle actin (α -SMA) and β -actin were obtained from Sigma-Aldrich. An antibody for phospho-Smad2/3 (P-Smad2/3) was purchased from Cell Signaling (Danvers, MA, USA).

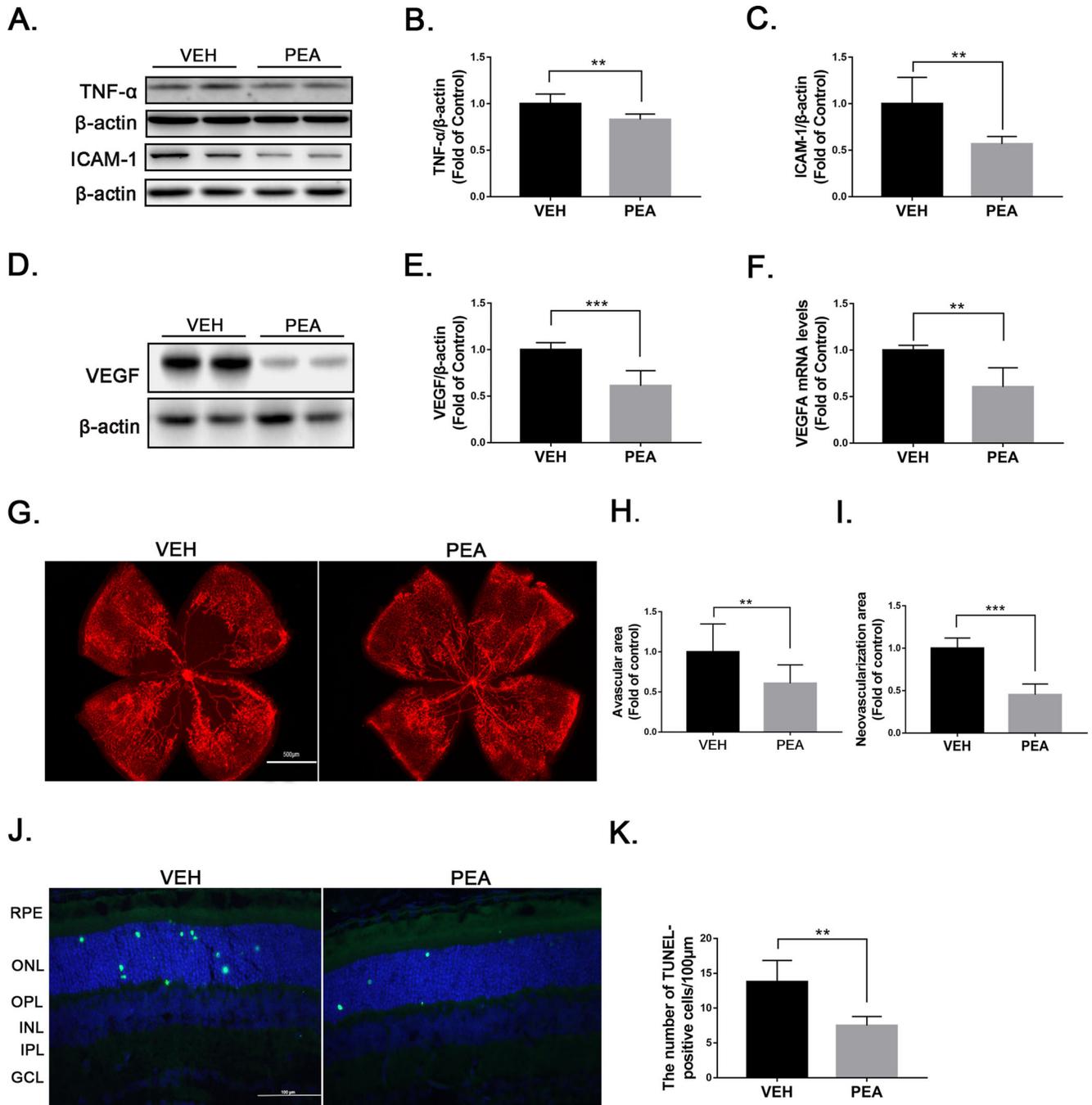


FIGURE 1. Anti-inflammatory and anti-angiogenic effects of PEA in OIR. (A) Representative images of western blotting for TNF- α and ICAM1 in OIR retinas treated with VEH or PEA. Protein levels of (B) TNF- α and (C) ICAM-1 were quantified by densitometry and normalized to β -actin levels ($n = 6$). (D) Representative images of western blotting for VEGF in OIR retinas treated with VEH or PEA. (E) Protein level of VEGF was quantified by densitometry and normalized to β -actin levels ($n = 6$). (F) Quantitative RT-PCR of *Vegfa* mRNA expression in VEH-OIR and PEA-OIR groups ($n = 5$). (G) Retinas from VEH- and PEA-treated OIR mice were flat-mounted and stained with isolectin GS-IB4 (red). Areas of (H) retinal vaso-obliteration and (I) neovascularization were quantified in VEH- and PEA-treated OIR retinas ($n = 8$). (J) Retinal cryosections from VEH- and PEA-treated OIR mice were stained with TUNEL (green) and 4',6-diamidino-2-phenylindole (DAPI) (blue). Representative images are shown. (K) Apoptotic cells were quantified and compared in VEH- and PEA-treated OIR mice. Data are presented as mean \pm SEM; ** $P < 0.01$, *** $P < 0.001$.

RNA Extraction and Quantitative RT-PCR Assay

Total RNA was extracted from retinas using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription

was performed according to the manufacturer's protocol (TaKaRa Bio, Inc., Shiga, Japan). The procedure for quantitative RT-PCR was described previously.²¹

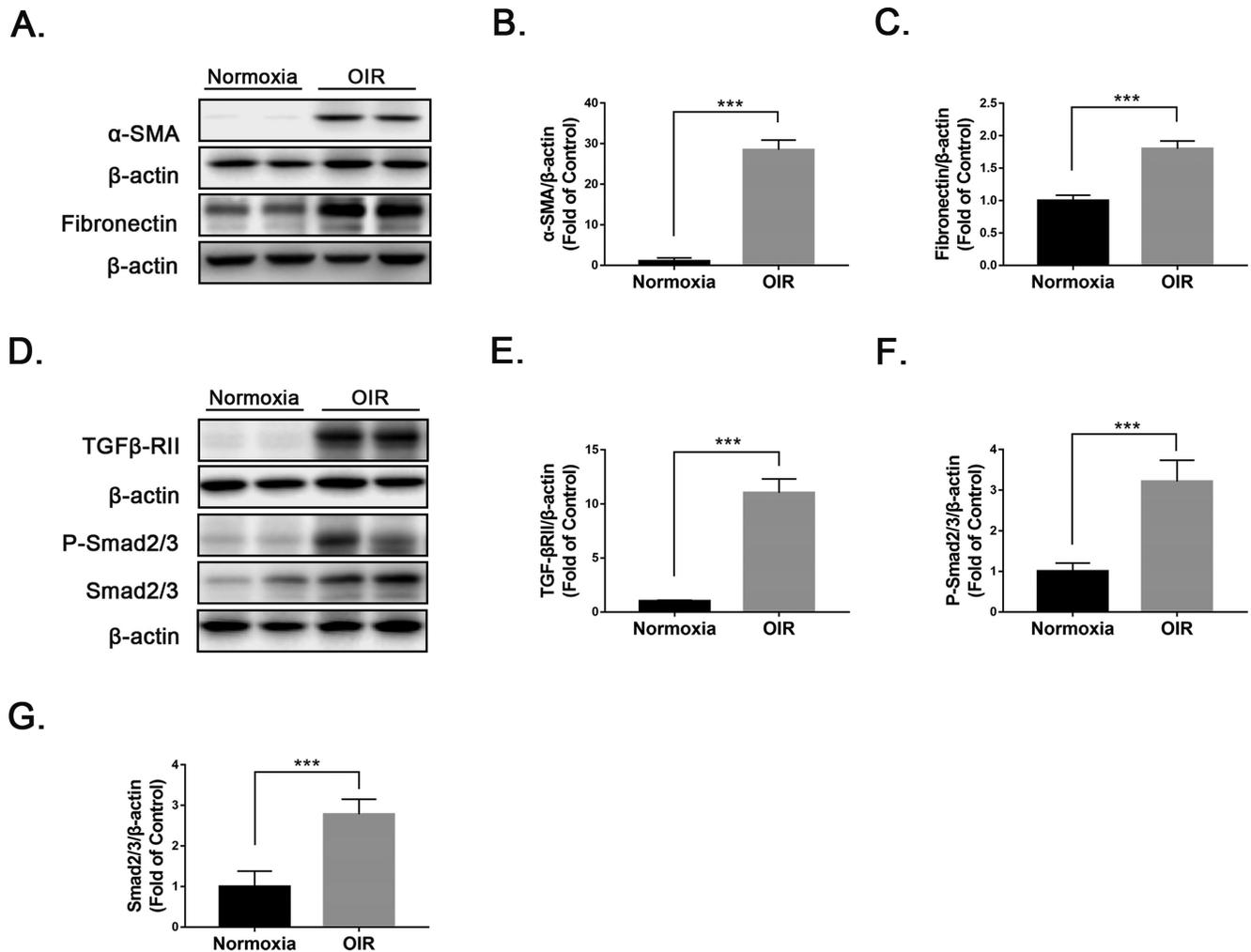


FIGURE 2. Presence of ECM remodeling and profibrotic changes in OIR retinas. (A–C) Protein levels of (A, B) α -SMA and (A, C) fibronectin were measured by western blot analysis and quantified by densitometry in wild-type (WT) and OIR retinas at P17 ($n = 6$). (D–G) Similarly, protein levels of (D, E) TGF β -RII, (D, F) P-Smad2/3, and (D, G) Smad2/3 were measured by western blot analysis and quantified by densitometry ($n = 6$). Data are presented as mean \pm SEM; *** $P < 0.001$.

Statistical Analysis

Prism 6 software (GraphPad, San Diego, CA, USA) was used to calculate a one-way ANOVA or a Student's t -test for statistical analyses. All values are expressed as mean \pm SEM. At least three independent measurements were conducted for each assay. A value of $P < 0.05$ was considered statistically significant.

RESULTS

PEA Attenuates Inflammation and Suppresses Neovascularization in OIR Retinas

To test the therapeutic effects of PEA in pathological retinal neovascularization, we used the OIR mouse model, a commonly used animal model of proliferative retinopathy.¹⁴ Multiple studies have demonstrated that levels of inflammatory cytokines such as TNF- α , ICAM-1, and VEGF are increased in OIR retinas.^{22,23} In the present study, PEA treatment significantly reduced protein levels of the inflammatory cytokines TNF- α and ICAM-1 in OIR retinas (Figs. 1A–1C). Also, both mRNA and protein levels of VEGF were

significantly decreased in PEA-treated OIR retinas compared to OIR retinas treated with vehicle (VEH) (Figs. 1D–1F). Lectin staining of flat-mounted OIR retinas revealed that PEA treatment decreased retinal areas of vaso-obliteration and neovascularization (Figs. 1G–1I), suggesting an anti-angiogenic effect of PEA in OIR retinas. Further, apoptotic cells in retinal sections were evaluated by TUNEL staining (Fig. 1J). PEA reduced apoptotic cells in OIR retinas (Fig. 1K), suggesting neuroprotective effects of PEA in this context. Taken together, these results indicate that PEA treatment attenuates retinal inflammation, neovascularization, and neuronal cell death in OIR retinas.

ECM Remodeling and Profibrotic Changes in OIR Retinas

Prior studies have demonstrated that pathological angiogenesis is accompanied by remodeling of the ECM.^{24,25} Under pathological conditions, excessive deposition of ECM can lead to fibrotic diseases and organ dysfunction.²⁶ To determine if early fibrotic changes were present in the

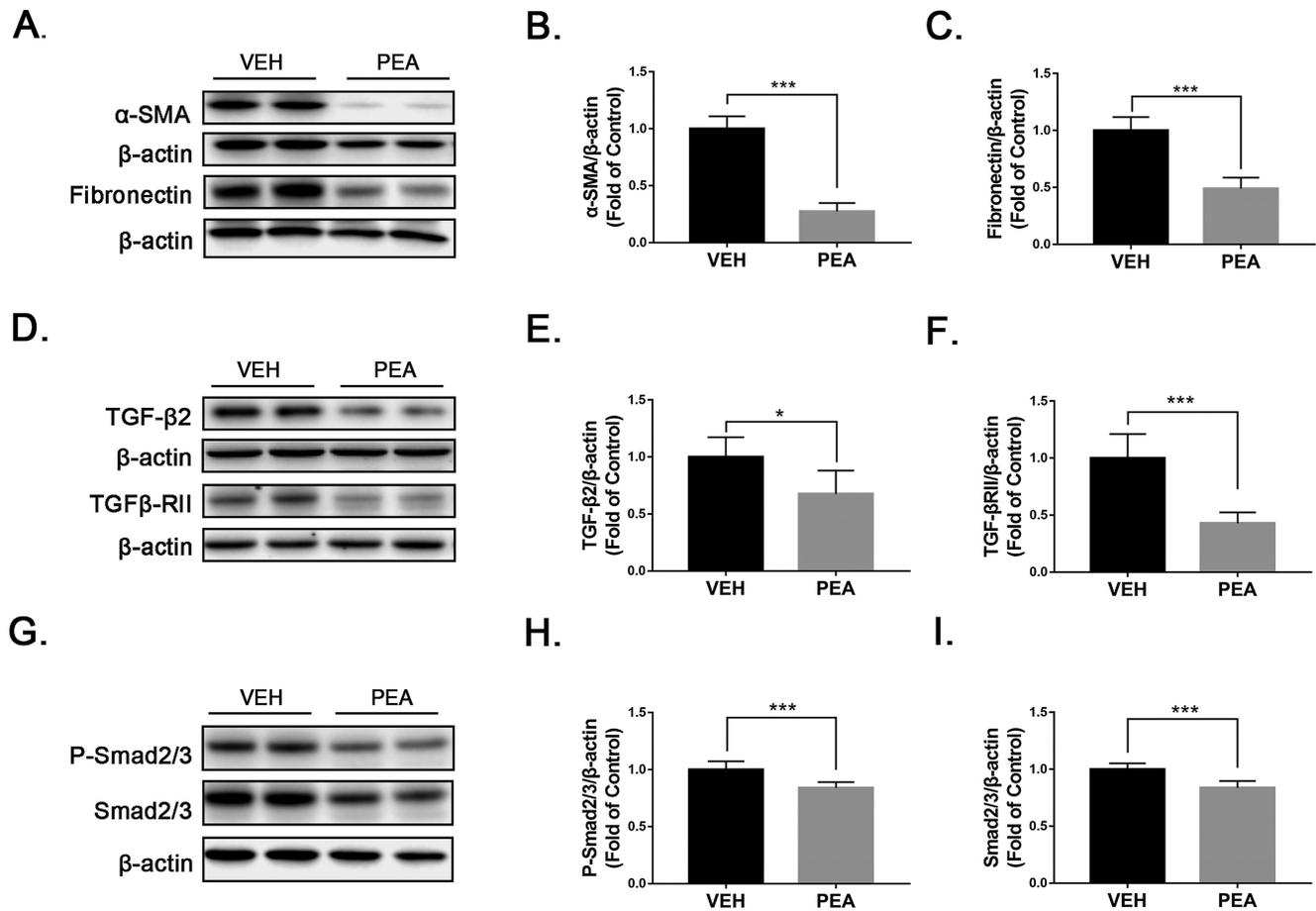


FIGURE 3. Antifibrotic effects of PEA in OIR retinas. (A–C) Protein levels of (A, B) α -SMA and (A, C) fibronectin were measured by western blot analysis and quantified by densitometry in VEH- and PEA-treated OIR retinas ($n = 6$). (D–I) Similarly, protein levels of (D, E) TGF- β 2, (D, F) TGF- β -RII, (G, H) P-Smad2/3, and (G, I) Smad2/3 were measured by western blot analysis and quantified by densitometry ($n = 6$). Data are presented as mean \pm SEM; * $P < 0.05$, *** $P < 0.001$.

OIR retinas, we measured the profibrotic marker α -SMA and a marker of ECM protein, fibronectin, in OIR retinas and normoxic retinas using western blot analysis (Fig. 2A). Protein levels of α -SMA and fibronectin were significantly upregulated in OIR retinas relative to normoxic retinas (Figs. 2B, 2C). In addition, protein levels of several key components of the TGF- β /Smad2/3 signaling pathway, TGF- β RII, P-Smad2/3, and Smad2/3 were significantly upregulated in OIR retinas compared with normoxic retinas (Figs. 2D–2G). Taken together, these results indicate the presence of ECM remodeling and profibrotic changes in OIR retinas.

PEA Inhibits Profibrotic Changes in OIR Retinas

Next, we tested whether PEA had protective effects against profibrotic changes in OIR retinas. Levels of α -SMA, fibronectin, and several components of the TGF- β /Smad2/3 pathway were measured by western blot analysis. PEA significantly suppressed upregulation of α -SMA (Figs. 3A, 3B) and fibronectin (Figs. 3A, 3C) in OIR retinas. Further, PEA treatment reversed upregulation of TGF- β 2 (Figs. 3D, 3E), TGF- β RII (Figs. 3D, 3F), P-Smad2/3 (Figs. 3G, 3H), and Smad2/3 (Figs. 3G, 3I) in OIR retinas. Taken together, these results

demonstrate that PEA suppresses profibrotic changes in OIR retinas.

PEA Attenuates Inflammation and Suppresses Neovascularization in *Vldlr*^{-/-} Retinas

We further assessed the anti-inflammatory and anti-angiogenic effects of PEA in the *Vldlr*^{-/-} mouse, a mouse model of neovascular AMD.^{15,27} Prior studies have demonstrated that protein levels of TNF- α , ICAM-1, and VEGF are elevated in *Vldlr*^{-/-} retinas compared to wild-type (WT) retinas.²⁸ After 2 weeks of treatment with PEA, protein expressions of the proinflammatory factors TNF- α (Figs. 4A, 4B), ICAM-1 (Figs. 4A, 4C), and VEGF (Figs. 4A, 4D) were significantly decreased in PEA-treated *Vldlr*^{-/-} retinas compared with VEH-treated *Vldlr*^{-/-} retinas. Lectin staining of whole-mounted retinas revealed less neovascular areas (Figs. 4E, 4F), suggesting that PEA suppresses pathological neovascularization in *Vldlr*^{-/-} retinas.

PEA Suppresses Fibrosis in *Vldlr*^{-/-} Retinas

Similarly, we evaluated the antifibrotic effects of PEA in *Vldlr*^{-/-} mice. At P90, the retinas of *Vldlr*^{-/-} mice exhibited increased levels of TGF- β 2, α -SMA, and fibronectin

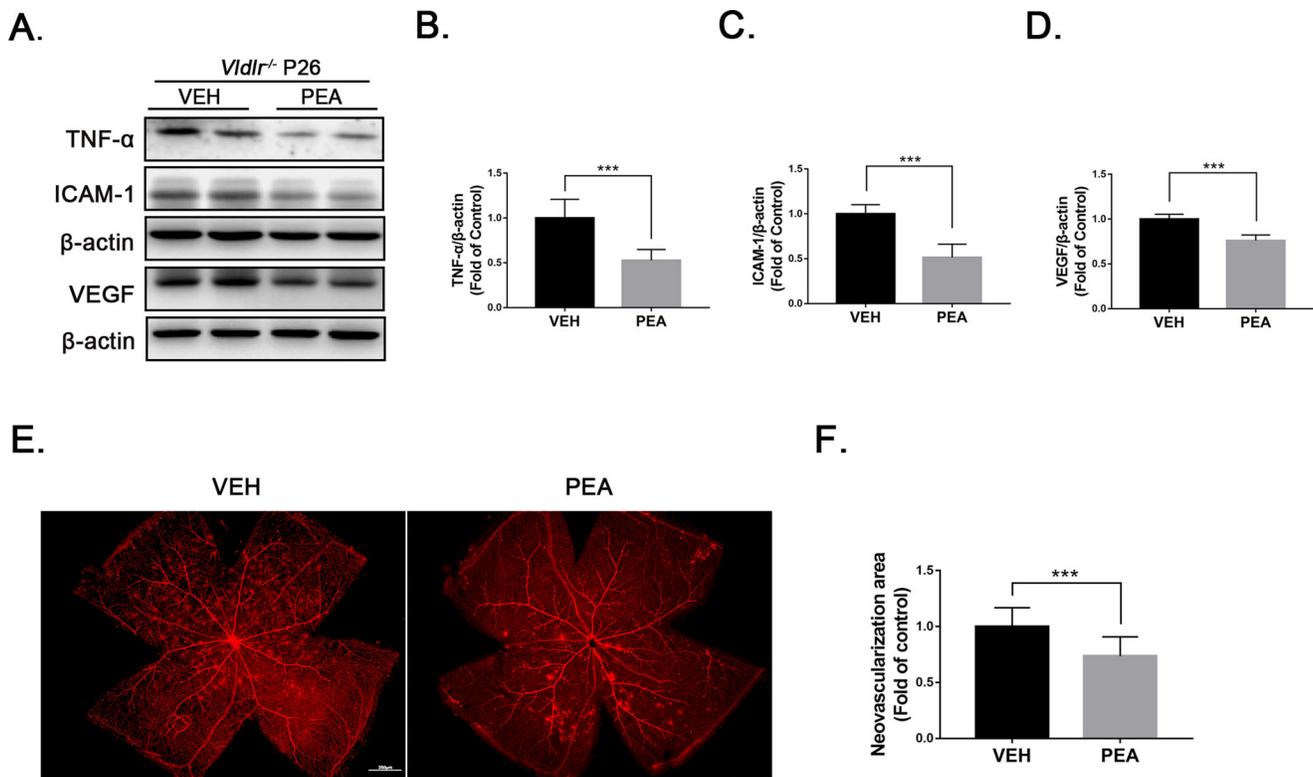


FIGURE 4. Anti-angiogenic effects of PEA in *Vldlr*^{-/-} retinas. (A) Protein levels of TNF- α , ICAM-1 and VEGF were measured by western blot analysis in the eyecups of *Vldlr*^{-/-} mice treated with VEH or PEA. (B) TNF- α , (C) ICAM-1, and (D) VEGF levels were quantified by densitometry ($n = 6$). (E) Retinas from VEH- and PEA-treated *Vldlr*^{-/-} mice were flat-mounted and stained with isolectin GS-IB4 (red). (F) Areas of neovascularization were quantified ($n = 8$). Data are presented as mean \pm SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

compared to age-matched WT controls, indicating the presence of fibrosis (Supplementary Fig. S1). After 1 month of PEA treatment, protein levels of α -SMA and fibronectin were decreased in PEA-treated *Vldlr*^{-/-} retinas (Figs. 5A–5C). The levels of TGF- β RII (Figs. 5D, 5E), P-Smad2/3 (Figs. 5D, 5F), and Smad2/3 (Figs. 5D, 5G) were significantly decreased in PEA-treated *Vldlr*^{-/-} retinas compared with VEH-treated *Vldlr*^{-/-} retinas, suggesting antifibrotic effects of PEA in the context of the *Vldlr*^{-/-} model.

PEA Inhibits Gliosis in OIR and *Vldlr*^{-/-} Retinas

Müller cells are the major glial cells in the retina; under stress conditions, massive retinal gliosis may occur.²⁹ Because it has been reported that PEA inhibits reactive gliosis in the CNS,^{9,10} we hypothesized that PEA could potentially inhibit Müller cell activation (gliosis) in the retina. GFAP, an indicator of glial activation, is dramatically increased in OIR retinas compared with normoxic retinas.²⁰ After PEA treatment, both mRNA and protein levels of GFAP were significantly decreased in OIR retinas (Figs. 6A–6C), suggesting that PEA suppresses gliosis in the context of OIR. In *Vldlr*^{-/-} retinas, protein levels of GFAP were significantly increased relative to WT retinas at P26 (Supplementary Figs. S2A, S2B) and at P90 (Supplementary Figs. S2C, S2D). GFAP levels were significantly decreased in PEA-treated *Vldlr*^{-/-} retinas relative to VEH-treated *Vldlr*^{-/-} retinas at P26 (Figs. 6D, 6E) and at P90 (Figs. 6D, 6F). Moreover, GFAP immunostaining revealed much fewer activated Müller cells (green) in PEA-treated OIR retinas relative to control OIR retinas (Fig. 6G).

Similarly, GFAP immunostaining showed fewer activated Müller cells (green) in *Vldlr*^{-/-} retinas at P26 (Fig. 6H) and at P90 (Fig. 6I) that were treated with PEA compared with retinas treated with VEH. Taken together, these results demonstrate that PEA inhibits Müller cell activation and suggest that the anti-angiogenic and antifibrotic effects of PEA could be due in part to suppression of Müller cell activation.

PEA Suppresses Gliosis and Inflammation in rMC-1 Cells

To further test the effects of PEA on the inhibition of Müller gliosis, we treated rMC-1 cells with tertiary-butyl hydrogen peroxide, a common inducer of cellular oxidative stress and inflammation.³⁰ Protein levels of GFAP were significantly decreased in rMC-1 cells treated with PEA relative to those treated with VEH (Figs. 7A, 7B). Further, protein levels of TNF- α (Figs. 7C, 7D), ICAM-1 (Figs. 7C, 7E), and VEGF (Figs. 7C, 7F) were significantly decreased in rMC-1 cells treated with PEA. Taken together, the results indicate that PEA suppresses Müller cell activation and the release of inflammatory and pro-angiogenic cytokines.

Antifibrotic Effects of PEA in TGF- β 2-Treated rMC-1 Cells

Müller cells are important sources of profibrotic cytokines.^{2,24} Müller cells secrete TGF- β 2 to promote fibrosis in proliferative retinopathy.³¹ Thus, we used TGF- β 2

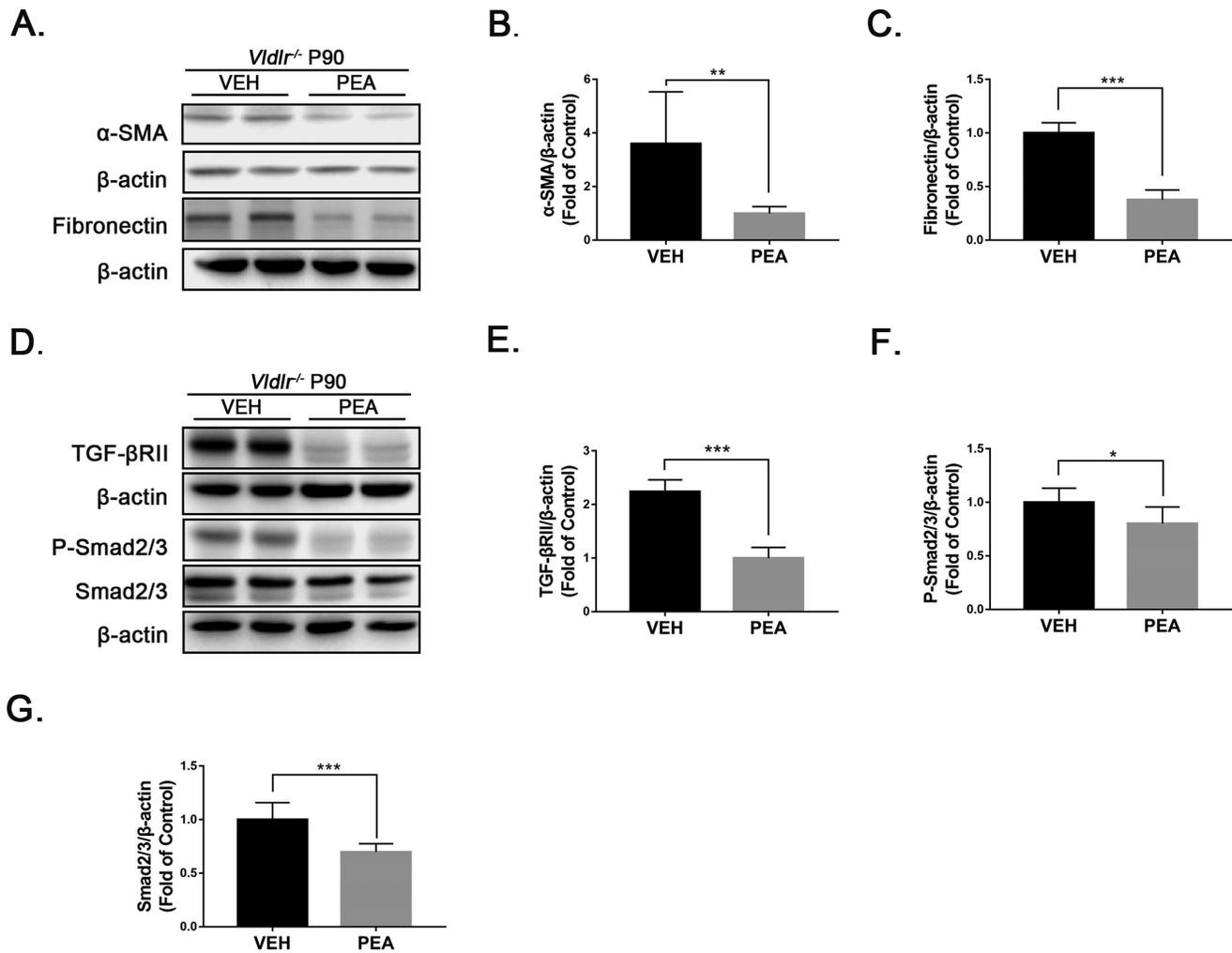


FIGURE 5. Antifibrotic effects of PEA in *Vldlr*^{-/-} retinas. (A) Protein levels of α -SMA and fibronectin were measured by western blot analysis in the eyecups of *Vldlr*^{-/-} mice treated with VEH or PEA. (B) α -SMA and (C) fibronectin were quantified by densitometry ($n = 6$). (D) Similarly, protein levels of TGF- β RII, P-Smad2/3, and Smad2/3 were measured by western blot analysis, and (E) TGF- β RII, (F) P-Smad2/3, and (G) Smad2/3 were quantified by densitometry ($n = 6$). Data are presented as mean \pm SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

to induce fibrosis and measured protein levels of profibrotic markers and signaling in Müller cells with and without PEA. Under TGF- β 2 treatment, protein levels of GFAP were significantly upregulated in rMC-1 cells, which was significantly attenuated by PEA treatment (Figs. 8A, 8B). TGF- β 2 induced expression of α -SMA and fibronectin in rMC-1 cells, which was suppressed by PEA treatment (Figs. 8C–8E). Similarly, TGF- β 2 induced upregulation of TGF- β RII, which was reversed by PEA (Figs. 8F, 8G). In addition, PEA also reversed the TGF- β 2-induced increases of P-Smad2/3 and Smad2/3 (Figs. 8F, 8H–8I) in rMC-1 cells, suggesting that PEA suppresses the TGF- β /Smad2/3 signaling pathway and alleviates profibrotic changes in Müller cells.

PPAR α -Dependent Anti-inflammatory and Antifibrotic Effects of PEA

Prior studies have suggested that the therapeutic effects of PEA are modulated in part by PPAR α activation.^{32,33} Therefore, we explored whether PEA inhibits retinal inflammation and fibrosis through activation of PPAR α . Indeed,

both mRNA and protein levels of PPAR α were increased in PEA-treated OIR retinas relative to VEH-treated OIR retinas (Figs. 9A–9C). In addition, PPAR α levels were elevated in *Vldlr*^{-/-} retinas treated with PEA at P26 (Figs. 9D, 9E) and P90 (Figs. 9F, 9G). To further verify the specific role of PPAR α in PEA-mediated suppression of inflammation and fibrosis, we subjected PPAR α knockout (*Ppar α* ^{-/-}) mice to OIR, treated them with PEA, and then measured protein levels of VEGF and TGF- β RII by western blot analysis (Figs. 9H, 9J). VEGF and TGF- β RII levels were not significantly changed by PEA in *Ppar α* ^{-/-} OIR mice (Figs. 9I, 9K), and retinal expression of GFAP was similar for VEH-treated and PEA-treated *Ppar α* ^{-/-} OIR mice (Figs. 9L, 9M). These results suggest that the protective effects of PEA in OIR are PPAR α dependent.

DISCUSSION

Pathological retinal neovascularization and fibrosis commonly lead to significant vision loss, but effective treatment modalities for these pathologies are limited. In the present study, we demonstrated that PEA, an endocannabinoid mimetic amide, has anti-angiogenic and

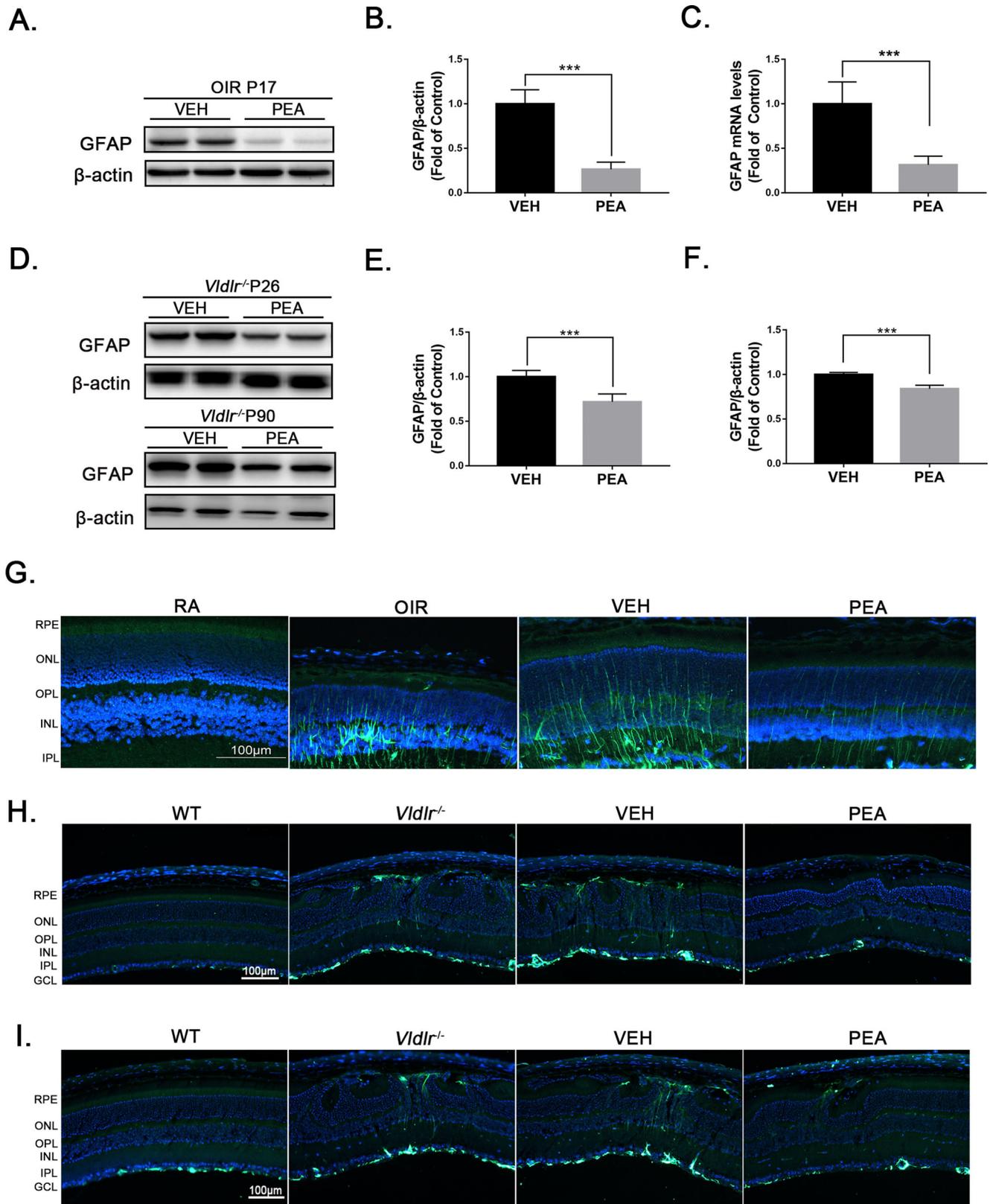


FIGURE 6. PEA inhibited gliosis in OIR and *Vidlr*^{-/-} retinas. **(A)** Protein levels of GFAP in the retinas of OIR mice treated with VEH or PEA were measured by western blot analysis. **(B)** Protein levels of GFAP were quantified by densitometry and normalized to β -actin levels ($n = 6$). **(C)** Quantitative RT-PCR of *Gfap* gene expression in the retina of VEH- and PEA-treated OIR mice ($n = 5$). **(D)** *Vidlr*^{-/-} mice at P14 were intraperitoneally injected with VEH or PEA for 2 weeks. Protein levels of GFAP were measured in *Vidlr*^{-/-} retinas at P26. In addition, *Vidlr*^{-/-} mice at P60 were orally administered PEA or VEH for 30 days. Protein levels of GFAP were then measured in the retina of these *Vidlr*^{-/-} mice at P90. **(E)** Levels of GFAP in *Vidlr*^{-/-} retinas at P26 were quantified by densitometry ($n = 6$). **(F)** Levels of GFAP in *Vidlr*^{-/-}

retinas at P90 were quantified by densitometry ($n = 6$). (G) Representative images of immunostaining for GFAP in the retinal sections of OIR mice treated with VEH or PEA. (H) Representative images of immunostaining for GFAP in the retinal sections of *Vldlr*^{-/-} mice at P26 treated with VEH or PEA. (I) Representative images of immunostaining for GFAP in the retinal sections of *Vldlr*^{-/-} mice at P90 treated with VEH or PEA. Data are presented as mean \pm SEM; *** $P < 0.001$.

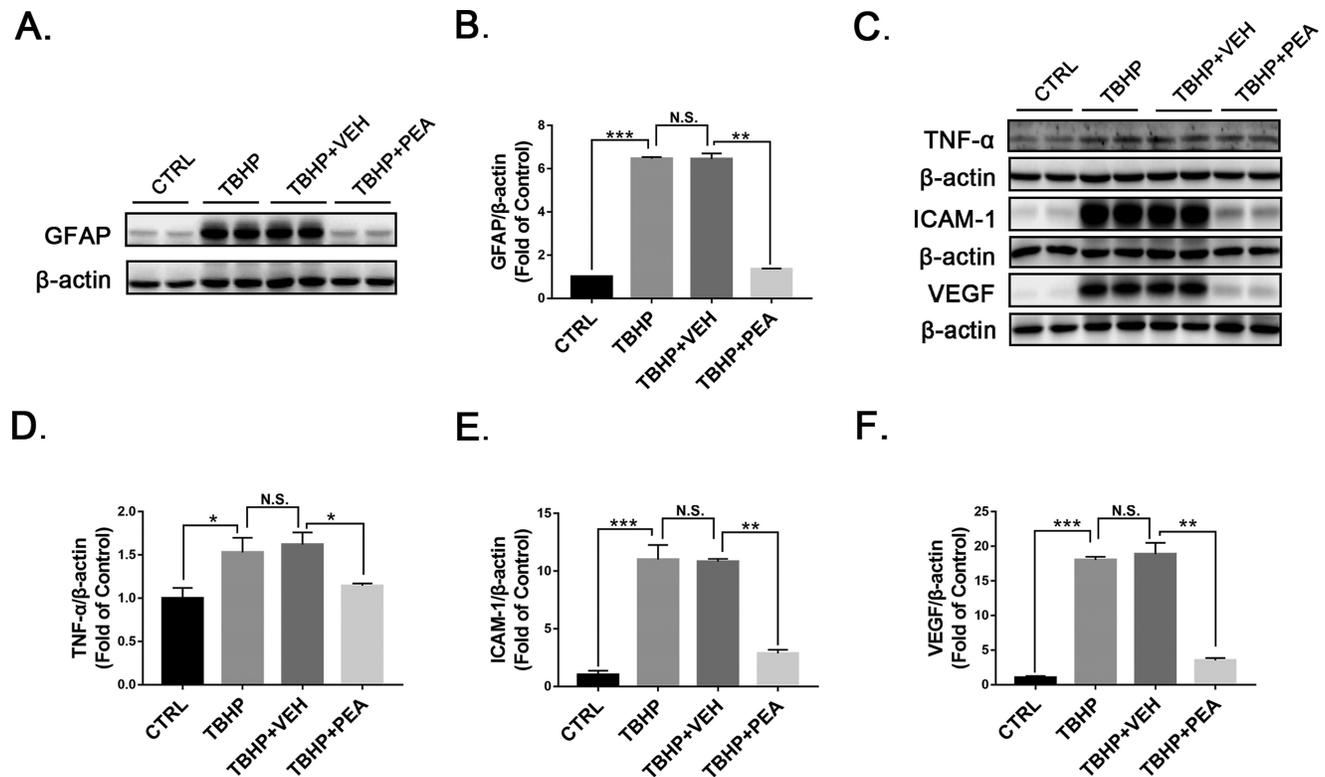


FIGURE 7. Effects of PEA in TBHP-treated rMC-1 cells. Representative images of western blotting for (A) GFAP and (C) TNF- α , ICAM-1, and VEGF in Müller cells treated with VEH or PEA in the presence of TBHP. Protein levels of (B) GFAP, (D) TNF- α , (E) ICAM-1, and (F) VEGF were quantified by densitometry and normalized to β -actin levels. Data are presented as mean \pm SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. TBHP, tertiary-butyl hydrogen peroxide.

antifibrotic effects in animal models of PR and neovascular AMD (as summarized in Supplementary Fig. S3). This is the first study to report that α -SMA and fibronectin, as well as TGF- β /Smad2/3 signaling, are increased in OIR and *Vldlr*^{-/-} retinas, suggesting the utility of these models for studying early retinal fibrotic changes. The present study is also the first to demonstrate that PEA treatment potentially inhibits retinal gliosis, angiogenesis, and fibrosis in OIR and *Vldlr*^{-/-} retinas, indicating that PEA may be a potential therapeutic drug for retinopathies with pathological neovascularization and fibrosis. Further, we found that the therapeutic effects of PEA in the retina are mediated in part by activation of PPAR α .

Two animal models, the OIR mouse model and the *Vldlr*^{-/-} mouse model, were used. OIR is a commonly used animal model for proliferative retinopathy.¹⁴ It is well characterized by retinal avascular regions and pathological neovascularization.¹⁶ However, there are no prior reports of fibrotic changes in this model. In the present study, we found that the profibrotic marker α -SMA and fibronectin, a marker of ECM remodeling, are significantly upregulated in OIR retinas. Meanwhile, profibrotic TGF- β /Smad2/3 signaling is also activated in OIR retinas. These findings indicate the possi-

ble new application of the OIR model to study early fibrotic changes in the retina, for which currently available animal models are lacking.

In *Vldlr*^{-/-} mice, the retinal vessels extend from the retinal capillary plexus of the outer proximal layer into the avascular photoreceptor layer,³⁴ mimicking the pathological process of retinal angiomatous proliferation in humans, which is present in approximately 10% of patients with newly diagnosed AMD.³⁵⁻³⁷ Thus, the *Vldlr*^{-/-} mouse is considered to be an animal model of neovascular AMD.^{27,38} In the present study, we observed that the proinflammatory cytokines TNF- α , ICAM-1, and VEGF were upregulated in *Vldlr*^{-/-} retinas, consistent with previous studies.^{27,28} Most importantly, we also identified that the profibrotic cytokines/markers TGF- β , α -SMA, and fibronectin were increased in these retinas, suggesting that the *Vldlr*^{-/-} mouse could also be used to study retinal fibrotic changes.

PEA, a naturally occurring *N*-acylethanolamine, has protective effects in animal models of neurodegenerative diseases.⁸ PEA is abundant in the CNS and may originate from glial cells.³⁹ In the eye, PEA is shown to be present in the retina and ciliary body,⁴⁰ but the function of PEA in the eye is not fully understood. Prior studies have reported

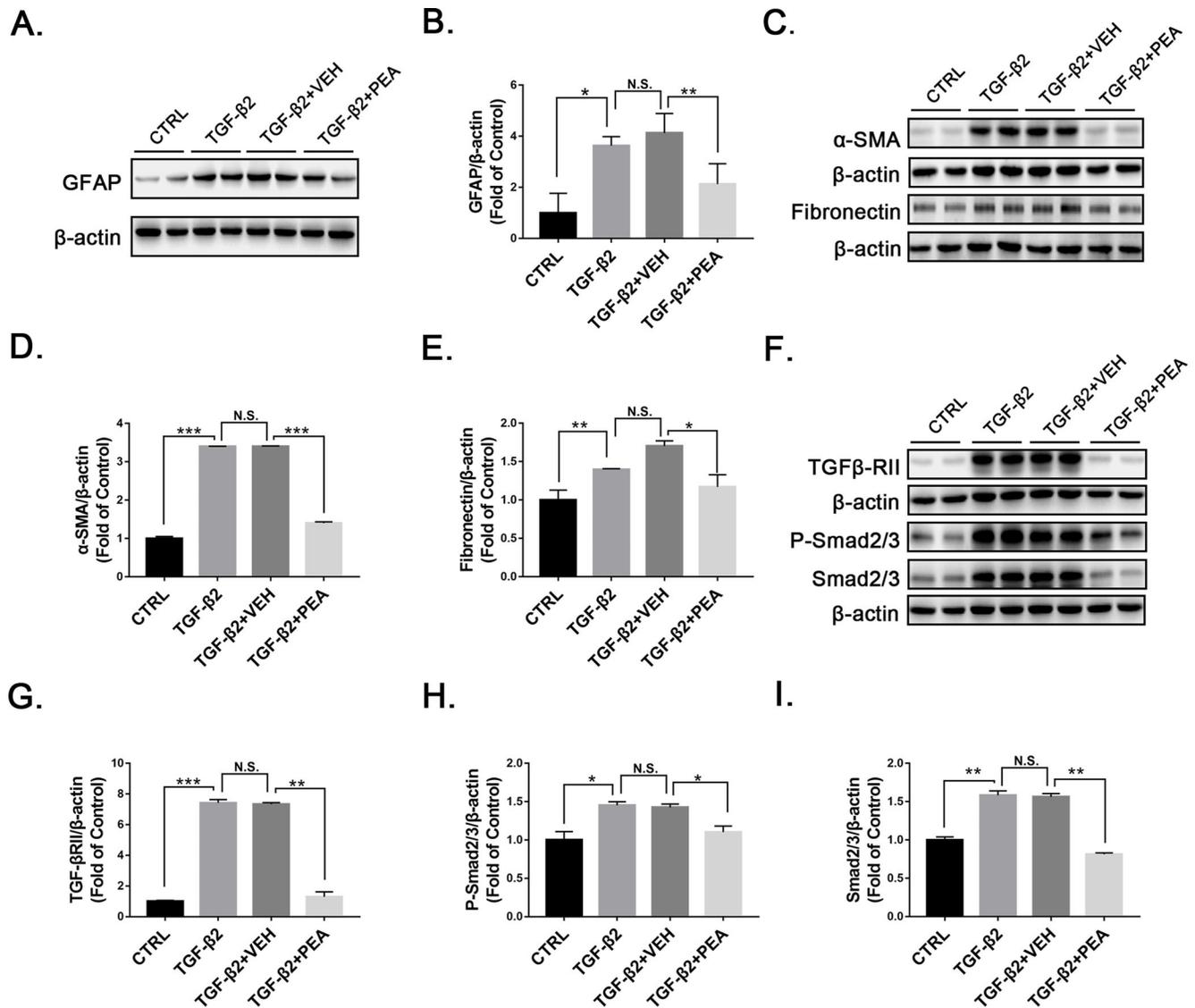


FIGURE 8. Effects of PEA in TGF- β 2-treated rMC-1 cells. (A) Representative images of western blotting for GFAP in rMC-1 cells treated with VEH or PEA in the presence of TGF- β 2. (B) Protein levels of GFAP were quantified by densitometry and normalized by β -actin levels. Similarly, protein levels of (C, D) α -SMA and (C, E) fibronectin were measured and quantified. Further, components of TGF- β /Smad2/3 signaling, (F, G) TGF- β RII, (F, H) P-Smad2/3, and (E, I) Smad2/3 were measured and quantified ($n = 6$). Data are presented as mean \pm SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

that PEA has beneficial effects in certain eye diseases, such as glaucoma⁴¹ and uveitis,⁴² due to the anti-inflammatory and neuroprotective properties of PEA. In this study, we explored the anti-angiogenic and antifibrotic effects of PEA in PR and AMD. PEA treatment reduced retinal expression of the inflammatory cytokines TNF- α , ICAM-1, and VEGF in OIR and *Vldlr*^{-/-} mice. These results are consistent with previous studies in other systems that have also identified anti-inflammatory effects of PEA.^{5,42} In addition to its anti-inflammatory effects, we also demonstrated that PEA could suppress pathological neovascularization in the retina.

This study explored the antifibrotic effects of PEA. The TGF- β /Smad2/3 pathway plays important roles in fibrotic disorders of the eye.⁴³ The pathway is initiated by TGF- β binding to TGF- β RII, which phosphorylates TGF- β RI; the phosphorylated TGF- β RI triggers the phosphorylation

of Smad2 and Smad3, which activates downstream gene expression and ultimately leads to tissue fibrosis.⁴⁴ In OIR and *Vldlr*^{-/-} retinas, PEA decreased protein levels of TGF- β 2, TGF- β RII, P-Smad2/3, and Smad2/3, suggesting suppression of the TGF- β /Smad2/3 pathway in these contexts. Previous studies have shown that elevated levels of TGF- β increase the synthesis and deposition of ECM proteins, such as fibronectin, and induce expression of α -SMA.^{39,45} In this study, PEA decreased the levels of fibronectin and α -SMA in both animal models, further supporting the antifibrotic effects of PEA. Interestingly, the PEA levels in OIR retinas and *Vldlr*^{-/-} retinas were not significantly changed compared to their controls (Supplementary Fig. S4). Because we have observed the beneficial effects of PEA treatment on OIR retinas and *Vldlr*^{-/-} retinas, it is possible that the amounts of PEA needed in the retina are increased when mice are in stressful conditions.

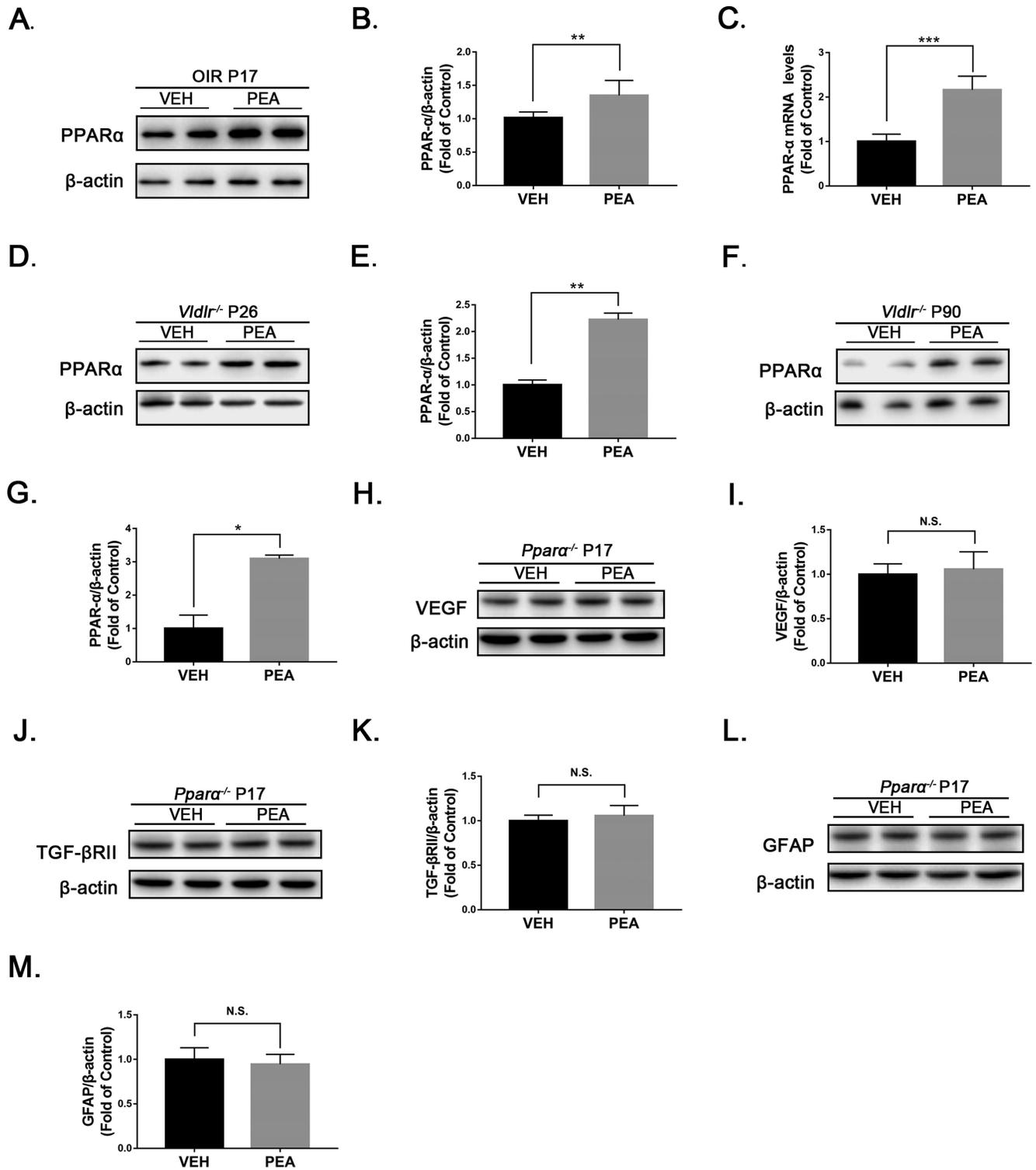


FIGURE 9. The effects of PEA were PPAR α dependent. **(A)** Representative images of western blotting for PPAR α in the retinas of OIR mice treated with VEH or PEA. **(B)** Protein levels of PPAR α were quantified by densitometry and normalized to β -actin levels ($n = 8$). **(C)** Quantitative RT-PCR of *Ppara* mRNA expression in PEA- and VEH-treated OIR retinas ($n = 5$). **(D–G)** Western blot analysis of PPAR α was performed in *Vidlr*^{-/-} mice treated with PEA or VEH at **(D, E)** P26 and **(F, G)** P90 ($n = 6$). **(H–M)** Further, OIR was induced in *Ppara*^{-/-} mice. At P17, protein levels of **(H)** VEGF, **(J)** TGF- β RII, and **(L)** GFAP were measured by western blot analysis in *Ppara*^{-/-} OIR retinas under VEH or PEA treatment. Protein levels of **(I)** VEGF, **(K)** TGF- β RII, and **(M)** GFAP were quantified by densitometry and normalized to β -actin levels ($n = 6$). Data are presented as mean \pm SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Müller cells are the major glial cell type of the retina, accounting for 90% of the glial population.⁴⁶ Müller cells produce pro-angiogenic and proinflammatory factors that may cause or exacerbate neovascularization, chronic retinal inflammation, and eventually cell death.^{12,13} In addition, Müller cells play a role similar to that of fibroblasts in non-CNS tissue and participate in the process of retinal fibrosis.^{13,24} In the present study, we demonstrated that PEA inhibits Müller gliosis in pathological retinal neovascularization. To further test the inhibitory effect of PEA in Müller gliosis, we investigated the effect of PEA using the cultured Müller cell line rMC-1. In rMC-1 cells, PEA inhibited Müller cell activation and reduced levels of proinflammatory cytokines and profibrotic markers. Our combined in vivo and in vitro findings suggest that the protective effects of PEA are mediated by suppression of Müller cell activation and the resultant release of proinflammatory and profibrotic cytokines.

Multiple studies have demonstrated the anti-inflammatory and anti-analgesic effects of PEA that occur through activation of PPAR α .^{47,48} Consistent with previous studies in other systems, we also found that the anti-angiogenic and antifibrotic effects of PEA are mediated by PPAR α and that these effects of PEA can be abolished in *Ppara*^{-/-} mice, further supporting the notion that the protective effects of PEA are PPAR α dependent.

In summary, we found that PEA suppressed retinal inflammation and neovascularization and reduced retinal fibrotic changes in experimental PR and neovascular AMDs. These findings suggest the potential utility of PEA for retinal neovascularization and retinal fibrosis. PEA is an endogenous amide with an excellent safety profile, and it has been used safely as an oral supplement.³ Further studies are warranted to evaluate the effects of PEA in other animal models of retinal fibrosis, potentially in small clinical trials.

Acknowledgments

The authors thank Vijay Sarthy of Northwestern University (Evanston, IL, USA) for providing the rMC-1 cell line. The authors also thank Elizabeth Pearsall of Massachusetts Eye & Ear (Boston, MA, USA) for her critical review of the manuscript.

Supported by grants from the National Science Foundation for Young Scientists of China (31807795) and the National Key R&D program of China (2018YFA0107302).

Disclosure: **S. Ye**, None; **Q. Chen**, None; **N. Jiang**, None; **X. Liang**, None; **J. Li**, None; **R. Zong**, None; **C. Huang**, None; **Y. Qiu**, None; **J.-X. Ma**, None; **Z. Liu**, None

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