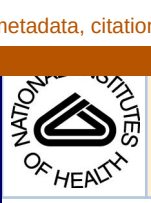

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Pigment Epithelium-Derived Factor Inhibits Retinal Microvascular Dysfunction Induced By 12/15-Lipoxygenase-Derived Eicosanoids

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Abstract

We recently demonstrated that 12/15-lipoxygenase (LOX) derived metabolites, hydroxyeicosatetraenoic acids (HETEs), contribute to diabetic retinopathy (DR) via NADPH oxidase (NOX) and disruption of the balance in retinal levels of the vascular endothelial growth factor (VEGF) and Pigment Epithelium-Derived Factor (PEDF). Here, we test whether PEDF ameliorates retinal vascular injury induced by HETEs and the underlying mechanisms. Furthermore, we pursue the causal relationship between LOX-NOX system and regulation of PEDF expression during DR. For these purposes, we used an experimental eye model in which normal mice were injected intravitreally with 12/15HETE with/without PEDF. Thereafter, Fluorescein Angiography (FA) was used to evaluate the vascular leakage, followed by Optical coherence tomography (OCT) to assess the presence of angiogenesis. FA and OCT reported an increased vascular leakage and pre-retinal neovascularization, respectively, in response to 12-HETE that were not observed in PEDF-treated group. Moreover, PEDF significantly attenuated the increased levels of vascular cell and intercellular adhesion molecules, VCAM-1 and ICAM-1, elicited by 12-HETE injection. Accordingly, the direct relationship between HETE and PEDF has

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been explored through *in-vitro* studies using Müller cells (rMCs) and human retinal endothelial cells (HRECs). The results showed that HETEs triggered the secretion of TNF-α and IL-6, as well as activation of NFκB in rMCs and significantly increased permeability and reduced zonula occludens protein-1 (ZO-1) immunoreactivity in HRECs. All these effects were prevented in PEDF-treated cells. Furthermore, interest in PEDF regulation during DR has been expanded to include NOX system. Retinal PEDF was significantly restored in diabetic mice treated with NOX inhibitor, apocynin, or lacking NOX2 up to 80% of the control level. Collectively, our findings suggest that interfering with LOX-NOX signaling opens up a new direction for treating DR by restoring endogenous PEDF that carries out multilevel vascular protective functions.

Keywords

Diabetic Retinopathy; PEDF; 12/15-Lipoxygenase; 12/15 HETEs; Retinal vascular leakage; and retinal inflammation

INTRODUCTION

Diabetic retinopathy (DR) is a potentially sight-threatening disease characterized by retinal barrier breakdown leading to macular edema and induction of retinal neovascularization (RNV). Although the pathogenesis of DR is multi-factorial, including inflammation, oxidative injury, and endothelial structural as well as functional abnormalities, several studies have consistently reported that the disruption of the delicate balance between the levels of vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF) is a pathophysiological hallmark in the development of DR [1–3]. Consequently, restoration of this balance is a potential therapeutic target that would substantially ameliorate DR and the subsequent vision loss.

PEDF is a multifunctional, pleiotropic secretory glycoprotein first isolated from cultures of fetal retinal pigment epithelial cells [4] and is also expressed in different cell types of the eye [5]. PEDF has been reported to elicit antiangiogenic, antioxidative, and anti-inflammatory properties [6, 7]; suggesting its use in the treatment of DR. Earlier clinical studies demonstrated an inverse relationship between endogenous PEDF level and the development of proliferative diabetic retinopathy (PDR) [8, 9]. Additionally, lower vitreous level of PEDF has been found to be associated with early phase of experimental DR [10], and that systemic or local delivery of recombinant PEDF protein or viral vector-mediated PEDF gene therapy successfully inhibited RNV and reduced retinal vascular permeability in an adult and chronic model of retinal neovascularization [11–13]. However, to maintain the sustained intraocular delivery of PEDF is a critical challenge. As such, restoration of endogenous PEDF offers an alternative dimension for DR therapy. To accomplish this objective the mechanisms by which diabetes suppresses PEDF must be dissected out.

Our recent study suggested that 12/15-lipoxygenase (LOX)-derived eicosanoids, 12-and 15-hydroxyeicosatetraenoic acids or (HETEs), contribute to DR via disrupting the retinal balance between VEGF and PEDF [14, 15]. In addition, we have shown that NADPH oxidase (NOX), a major source of oxidative stress during DR [16], is a downstream mediator from HETEs [15]. The goals of the present study are to test if exogenous PEDF

protects retina against 12/15-HETEs-induced inflammation and microvascular injury such as hyperpermeability and neovascularization and to investigate whether NADPH oxidase has a regulatory effect on PEDF level in retina during diabetes.

MATERIALS AND METHODS

Animal Preparation and Experimental Design

All procedures with animals were performed in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare publication, NIH 80–23) and Georgia Regents University guidelines. Eight-week-old male NOX2, a catalytic subunit of NADPH oxidase, knockout ($NOX2^{-/-}$) and corresponding littermate controls, wild-type (Wt) mice in C57BL/6J background (Jackson Laboratory, Bar Harbor, ME), were matched according to sex, age, and weight. All animals were maintained in clear plastic cages, subjected to standard 12-hour light/12-hour dark light cycles in the animal room at regulated temperature (22 to 24°C), and allowed to eat and drink *ad libitum*. Light levels at the bottom of cages were controlled at 1.5-foot candles (16.1 lux) to avoid the possibility of light damage in the retina.

Animals were given intraperitoneal (I.P) injections of freshly prepared streptozotocin (STZ, 45 mg/kg, Enzo Life Sciences, Farmingdale, NY) dissolved in 0.9% NaCl after four hour-fasting for 5 consecutive days. Diabetes was verified by blood glucose readings with mean of 412±30 mg/dL for diabetic mice versus 169±10 mg/dL for all non-diabetic controls. In pharmacologic studies, age-, weight- and sex-matched C57BL/6J mice were rendered diabetic and then treated with or without apocynin (Sigma, St. Louis, MO, 10 mg/kg) in drinking water for the duration of the study (n = 4–6/group). The dose of apocynin was chosen according to our previous studies [16, 17]. Five weeks after establishment of diabetes, retina samples were used for PEDF analysis by Western blot and frozen eye sections were prepared to examine PEDF distribution in retina by immunofluorescence. Average body weight was equal in all groups (Wt, 24.94 g ±1.6 SD Vs $NOX2^{-/-}$, 25.2 g ± 2.3 SD) prior to the induction of diabetes and increased slightly in the control non diabetic controls to be 27.8 g ±1.9 SD for Wt and 28.3 g ± 2.5 SD for $NOX2^{-/-}$, compared with the three diabetic groups which did not change during the course of diabetes.

For intravitreal injections, the procedure was essentially the same as previously described [18]. Briefly, each mouse received an intramuscular injection of rodent anesthesia cocktail (ketamine 100 mg/kg, xylazine 10 mg/kg) in 0.1–0.2 ml of phosphate-buffered saline (PBS). Anesthetized mice were given eye drops of topical anesthesia (proparacaine HCl, Alcon, Fort Worth, Texas) as well as pupil dilator (tropicamide 1%, Alcon). 5-, 12-, or 15-HETE (Cayman Chemical, Ann Arbor, MI) was dissolved in ethanol and a working solution of 10× was prepared by diluting 0.32 μl of stock solution (312 μM) to 100 μl with PBS. Assuming the vitreous volume of mouse eye is ~ 5–7 μl [19], then by injecting 0.5 μl of this working solution in the presence of 0.5 μl containing 2 μg/μl PEDF purified as described previously [20] or PBS-vehicle, an 0.1 μM vitreal concentration of HETEs with or without 1 μg PEDF/mouse eye was obtained. The vitreal concentration of ethanol was 0.032%. To ensure the proper delivery and even distribution of the intravitreally injected compounds, all solutions for intravitreal injection contained 5 μg/ml of Fast Green FCF (Sigma). The volume of the

injected solution apparently did not cause significant pressure-induced retinal damage, because 0.032% ethanol-PBS-injected control eyes showed normal retinal morphology with no apparent apoptosis within 7 days. Intravitreal injections were performed using a 10-μL Hamilton syringe adapted with a 32-gauge microneedle. The needle tip was inserted into the superior hemisphere of the eye, at a 45° angle through the sclera into the vitreous body under the guidance of Stemi DV4 Stereo Microscope (Carl Zeiss, Inc., Thornwood, NY). This route of administration avoided retinal detachment or injury to eye structures, including the iris and lens, which release factors that induce neuronal survival [21]. Neomycin/polymyxin B/bacitracin (Alcon) ophthalmic ointment was applied to the injected eyes. The mice recovered spontaneously from the anesthesia and then were sent back to the animal room with food and water *ad libitum*. The doses of 12/15-HETEs were chosen according to what detected previously in the vitreous of patients with DR, 50 ng/mL (~0.1–0.2 uM) [14], while the dose of PEDF was chosen according to what reported previously that PEDF efficiently inhibited ischemia-induced RNV and hyper-permeability at a dose of ~1–2 μg/eye [20, 22].

Fluorescein Angiography (FA)

To evaluate retinal vasculature and permeability in vivo, one week after intravitreal injections, mice were anesthetized using intramuscular injection of rodent anesthesia cocktail described above. Pupils were dilated using 1% tropicamide eye drop. The mouse was placed on the imaging platform of the Phoenix Micron III retinal imaging microscope (Phoenix Research Laboratories, Pleasanton, CA), and Goniovisc 2.5% (hypromellose; Sigma Pharmaceuticals, LLC, Monticello, IA) was applied liberally to keep the eye moist during imaging. Mice were administered 10 to 20 μL 10% fluorescein sodium (Apollo Ophthalmics, Newport Beach, CA), and rapid acquisition of fluorescent images ensued for ~5 minutes. Fluorescein leakage manifests as indistinct vascular borders progressing to diffusely hazy fluorescence. Fluorescein leakage was compared between different groups by quantifying the uorescence intensities collected after 1, 2, and 3 minutes following uorescein injection using ImageJ software (National Institutes of Health, Bethesda, MD).

Optical coherence tomography (OCT)

To assess the presence of angiogenesis, imaging of eyes was performed 3 weeks after intravitreal injections by using OCT imaging device coupled with Color Doppler imaging (Envisu OCT, Bioptigen, Inc., Morrisville, NC) with a 25-diopter lens fitted on a 30-degree angle lens. The pupils of anesthetized mice were dilated with 1% tropicamide eye drops before image acquisition. Lubricant Eye Gel (GenTeal; Novartis Pharmaceuticals, East Hanover, NJ) was used throughout the procedure to maintain corneal moisture and clarity. Three repeated-volume intensity projections were acquired for each eye. The images consisted of 100 averaged B-scans. Collection of the data permitted three-dimensional renderings of the retina. Doppler imaging was performed during the scan to visualize blood vessels.

Immunofluorescence

Immunofluorescence analysis was performed using frozen retinal sections. Briefly, cryostat sections (10 μm) were fixed in 4% paraformaldehyde, blocked with 10% normal goat serum (NGS) and then incubated overnight at 4°C with primary antibody for PEDF (Millipore, Temecula, CA) or biotinylated isolectin B4 (Vector Laboratories, CA). Thereafter, sections were briefly washed with PBS and incubated with Texas red-labeled secondary antibody or Texas red-labeled streptavidin. Slides were examined by confocal microscopy (LSM 510, Carl Zeiss). Specificity of the reaction was confirmed by omitting the primary antibody. Images were collected from five sections per mouse of at least three mice in each group and then analyzed using Image J software to quantify the intensity of immunostaining.

Microvascular human retinal endothelial cells (HRECs)

HRECs (Cell Systems Corporation (CSC), Kirkland, WA, USA), passages 6–8, were grown on gelatin-coated dishes and maintained in M199 media supplemented with CSC endothelial growth factors, 1% penicillin/streptomycin, and 10% fetal bovine serum (FBS, Atlantic Biological, Norcross, GA, USA). After the cells formed a complete confluent layer, the cells were shifted to 1% FBS overnight, then treated with 0.1 μM of 15-HETE with or without PEDF (100 nM)-pretreatment. The experiment was terminated after 24 hours, then cells were fixed with 2% paraformaldehyde for 10 minutes and blocked with normal goat serum for one hour followed by incubation with zonula occludens (ZO)-1 antibody (Invitrogen, Eugene, OR, 1:100) overnight at 4°C before incubation with Oregon green labeled secondary antibody (1:500, Invitrogen, Eugene, OR). Images then were collected and color intensity was measured by Image J.

Assessment of HREC barrier function

HRECs were seeded on collagen/fibronectin coated membranes with 0.4 μm pores (Transwell; Corning Costar), in normal media. After becoming completely confluent cells were shifted to 1% serum media overnight then were treated by 0.1 μM 15-HETE in the presence or absence of PEDF (100 nM)-pretreatment in the apical chambers for 24 hours. Thereafter, fluorescein isothiocyanate (FITC)-dextran (1 mg/ml, 70 KD, Sigma) was added to the apical chambers followed by obtaining aliquots from the basolateral chamber every 30 min for 4 h then measuring the fluorescence intensity with a plate reader. The rate of diffusive flux (Po) was calculated by the following formula [23]:

$$\mathrm{Po}=[(\mathrm{FA}/\Delta \mathrm{t})\mathrm{V_A}]/(\mathrm{F_L A}).$$

Where Po is in centimeters per second; FA is basolateral fluorescence; F_L is apical fluorescence; Δt is change in time; A is the surface area of the filter (in square centimeters); and V_A is the volume of the basolateral chamber (in cubic centimeters).

Rat Retinal Müller cells (rMC-1)

rMC-1 was obtained as a gift from Dr. V. Sarthy (Northwestern University, Chicago, IL). The cells were maintained in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM: F12, Mediatech Cellgro, Herndon, VA) supplemented with, 1% penicillin/

streptomycin, and 10% FBS. To determine the effect of 12-HETE on Interleukin (IL)-6 and Tumor Necrosis Factor (TNF)-α expression in rMC-1, 12-HETE was added to the cultured rMC-1 (0.1 μM in serum-free DMEM:F12) with/without PEDF. By the end of the experiment (after 72 h), rMC-conditioned media were collected and processed for enzyme-linked immunosorbent assay (ELISA) to detect IL-6 as well as TNF-α, while cell homogenates were analyzed by Western blotting for NF-κB.

Enzyme-linked immunosorbent assay (ELISA) for IL-6 and TNF-α

IL-6 and TNF-α levels in the supernatants of culture media were estimated with ELISA kits (R & D, Minneapolis, MN) per the manufacturer's instructions. Standards and samples were added and bound by the immobilized antibody. After washing, an enzyme-linked polyclonal antibody specific for the cytokine was added to the wells followed by a substrate solution yielding a colored product. The intensity of the color was measured at 450 nm and corrected at 540 nm.

Protein extraction and Western blot analysis

Washed cultured cells as well as retinal tissues were lysed in modified RIPA buffer (Upstate, Lake Placid, NY), supplemented with 40 mM NaF, 2mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, and 1:100 (v/v) of proteinase inhibitor cocktail (Sigma). Insoluble material was removed by centrifugation at 12,000 xg at 4°C for 30 min. Protein was determined by DC Protein Assay (Bio-Rad, Hercules, CA) and 50 μg was boiled in Laemmli sample buffer, separated by SDS-PAGE on a gradient gel (4 to 20%, Pierce, Rockford, IL), transferred to nitrocellulose membrane and incubated with specific antibodies. Antibodies for PEDF, β-actin (Sigma), ICAM-1, (Santa Cruz Biotechnologies, Santa Cruz, CA), VCAM-1, pNFκB, and NFκB (Cell Signaling Technology, Beverly, MA), were detected with a horseradish peroxidase-conjugated antibody and enhanced chemiluminescence (Amersham, Pittsburgh, PA). Intensity of immunoreactivity was measured by densitometry using Image J software.

Data Analysis

The results are expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Tukey Kramer post-hoc test for multiple group comparisons. A confidence level of $P<0.05$ was considered statistically significant.

RESULTS

Effect of PEDF on retinal vascular abnormalities induced by 12/15-hydroxyeicosatetraenoic acids (HETEs)

12/15-LOX-derived metabolites have been shown to contribute significantly to DR via disrupting the balance between VEGF and PEDF [14]. In the same study we have reported that treatment of rMC1 with 12-HETE reduced the expression of PEDF and increased VEGF production. Therefore, we questioned whether restoring PEDF using exogenous-PEDF peptide would ameliorate retinal vascular injury induced by these eicosanoids. Toward this goal, initially we used an experimental eye model in which normal mice were injected intravitreally with the most common metabolite analogs derived from LOX (5-, 12-, 15-

HETE) or solvent control. One week later, FA was used to evaluate retinal vascular leakage. As shown in Figure 1, 12-HETE-injected eyes had the most increased fluorescein leakage among the three types of HETE- injected mice compared with the solvent-injected controls. Secondly, the co-administration of PEDF with 12-HETE significantly reduced the 12-HETE-induced retinal vascular leakage, Figure 1.

This finding of PEDF's protective effect against increased retinal permeability in mice receiving 12-HETE has been substantiated in human retinal endothelial monolayer treated with 15-HETE. Of note, species-specific difference between orthologous LOX isoforms has been described for the murine 12-LOX, which is an arachidonate 15-LOX in human. This difference indicates that care should be taken if experimental data on LOX metabolism are being transferred from one species to another [24, 25]. Accordingly, we have used 12-HETE in all murine experiments versus 15-HETE in all human cell experiments. With HREC model, we examined whether 15-HETE induces permeability changes to FITC dextran flux through the confluent monolayer. We noticed that 15-HETE-treated HRECs became significantly permeable to FITC-dextran as indicated by increasing diffusive flux (Po) for FITC-dextran. However, PEDF significantly reduced the effect of 15-HETE on FITC-dextran leakage (Figure 2A). Additionally, we investigated if 15-HETE modulates the expression of ZO-1, a tight junction protein that is crucial in maintaining the barrier function. As shown in Figure 2B and quantified in 2C, a smooth and continuous staining for ZO-1 along the intercellular borders of endothelial cells was seen in vehicle-treated controls. However, the exposure of HRECs to 15-HETE caused a noticeable alteration of ZO-1 spreading at cellular border, displaying less immunoreactivity. These effects induced by 15-HETEs were significantly reversed by pre-treatment with PEDF.

Following this further, we used OCT to evaluate the angiogenic activity of 12-HETE in the presence or absence of PEDF. Normally there are no blood vessels located in the vitreous cavity or pre-retinal space. As shown in Figure 3A, OCT imaging of mouse retina after 3 weeks from the 12-HETE injection showed pre-retinal neovascularization as confirmed by Color Doppler Imaging, hematoxylin and eosin (H&E) staining (Figure 3B), three dimensional OCT imaging (Figure 3C), and immunofluorescence with isolectin B4 as a marker of capillary endothelial cells (Figure 3D). On the other hand, these new vessels were not observed in PEDF-treated group, implying its anti-angiogenic activity.

Effect of PEDF on retinal inflammatory response induced by 12-HETE

An increase in retinal vascular permeability is a hallmark of inflammation. Given this fact, we next sought to determine whether the beneficial effect of PEDF on 12-HETE-induced retinal vascular leakage would associate with a reduction in the inflammatory response. In this regard, we have determined the effect of PEDF on 12-HETE-induced increases in levels of VCAM-1 and ICAM-1, well established markers of endothelial dysfunction in inflammatory conditions [26]. Western blot analysis showed significant increases in the expression of VCAM-1 and ICAM-1 in 12-HETE-injected eyes compared with vehicle-injected controls. Co-administration of PEDF significantly attenuated the increases in VCAM-1 and ICAM-1 levels elicited by 12-HETE injection (Figure 4 A and B).

Next we aimed to explore a potential mechanism by which PEDF signaling regulates the retinal inflammation induced by 12-HETE. To do this, additional studies using retinal Müller cells (rMCs) treated with 12-HETE were performed. rMCs were chosen here because they have been recognized as a potential source contributing to the retinal inflammatory outcome during diabetes [27]. rMCs have been shown to be activated by hyperglycemia to produce inflammatory cytokines in NFκB-dependent mechanism [28]. With the use of this model, we first verified whether 12-HETE could stimulate inflammatory cytokine production such as IL-6 and TNF-α from rMCs. IL-6 and TNF-α, were selected as biomarkers of inflammation because they have been shown to be more consistently elevated in DR contributing to blood-retinal barrier (BBB) permeability changes and their proinflammatory immune functions [29–32]. As shown in Figure 5 A and B, there were prominent increases in IL-6 as well as TNF-α production in the conditioned media of rMCs incubated with of 12-HETE (0.1 μM) compared with the vehicle-treated cells. Secondly, we characterized the effect of PEDF on pro-inflammatory cytokine levels produced by 12-HETE in rMCs. As shown in Figure 5, PEDF potently inhibited the 12-HETE-induced IL-6 and TNF-α expression in rMCs. Lastly, we pursued the molecular mechanisms by which PEDF mediating its anti-inflammatory effects in 12-HETE-treated rMCs. Since we have shown previously that 12-HETE generates ROS [15] which modulate a number of gene expression through the activation of transcriptional factor NF-κB [33], we tested the effect of 12-HETE on NF-κB activation and the modulation effects of PEDF. As shown in Figure 5C, 12-HETE had significantly increased the level of pNF-κB compared to the control group. PEDF on the other hand, was found to partially, but significantly, block the effect of 12-HETE on NF-κB activation. Collectively, these results suggest that the retinal protective effect of PEDF is mediated by abrogating the inflammatory response induced by 12-HETE via suppression of NF-κB pathway.

Role of NADPH oxidase in diabetes-induced retinal PEDF suppression

After having shown the salutary effects of PEDF on the impairment of retinal vascular functions induced by 12-HETE injection, interest in its regulation during DR has been expanded to include LOX-NADPH oxidase pathway. 12/15-LOX has been suggested as a potential contributor to vascular hyperpermeability during DR via NADPH oxidase dependent mechanism [15]. Whether or not LOX-NADPH oxidase pathway modulates PEDF expression during diabetes was also examined here. To test our hypothesis, we treated diabetic and non-diabetic mice with/without NADPH oxidase inhibitor, apocynin, and determined its effect on diabetes-induced retinal PEDF suppression. Western blot analysis followed by densitometric analysis showed that level of PEDF was decreased by 50% in the retina of 5-weeks diabetic mice compared with non-diabetic retinas (Figure 6A). Treatment of the diabetic animals with apocynin significantly restored the normal level of PEDF in the diabetic mice up to 80% of the control level. Moreover, PEDF-like epitopes were featured within the retinal tissue of normal mice to be localized in inner retina as well as in retinal pigment epithelium (RPE) layer. However, PEDF immunoreactivity observed in inner retina was significantly decreased by diabetes and the treatment with apocynin resulted in a marked recovery of its immunoreactivity within diabetic retina (Figure 6B).

To further assess the role of NADPH oxidase in mediating PEDF suppression during diabetes, we studied the effects of NOX2 ablation on diabetes-induced retinal PEDF suppression. Comparative analysis of PEDF level in retina samples revealed that PEDF in the diabetic $NOX2^{-/-}$ mice was increased significantly compared with the diabetic Wt littermates and brought back closer to normal level (Figure 6A). Moreover, PEDF immunofluorescence reactivity in the retinas of diabetic $NOX2^{-/-}$ mice was greatly improved and back to 80% of normal level compared with their age-matched diabetic Wt littermates (Figure 6B). Taken together, these results clearly demonstrate that NOX pathway plays a crucial role in mediating retinal PEDF-suppression associated with diabetes.

DISCUSSION

Although the protective effect of PEDF in DR is generally considered to be a potential therapeutic strategy, its regulation and mechanism of action are not yet well identified. In this study, we put forward a new concept pertaining to the protective role of PEDF against 12/15-LOX derived hydroxyeicosanoids-induced retinal endothelial dysfunctions. Furthermore, our study provides a preclinical evidence for the involvement of NADPH oxidase pathway in the regulation of PEDF level during DR.

In recent years, a growing body of evidence has highlighted the role of 12/15-LOX pathway as a contributing factor to various retinal vascular diseases including DR. Our recent studies reported that retinal expression and activity of 12/15- LOX are associated with increased vascular leakage and NV, the hallmark features of DR. Moreover, inhibition of 12/15-LOX demonstrated beneficial effects in attenuating the early inflammatory response and RNV in experimental models of DR and oxygen-induced retinopathy, respectively [14, 15]. Furthermore, the *in vitro* studies on retinal vascular endothelial cells incubated with 12/15 HETEs have shown increases in ROS generation, NOX2 expression, and VEGF-R2 phosphorylation together with decreased pSHP1 expression [15]. As such, targeting 12/15-LOX signaling system by an agent carries out multilevel protective functions may open up a new direction for treating DR.

PEDF is a versatile multifunctional endogenous protein involved in a variety of biochemical functions. PEDF has been documented by numerous studies to inhibit endothelial cell proliferation and migration which can prevent the growth of new blood vessels in retina during pathological conditions [34–36]. PEDF also reduced VEGF-induced hyper-permeability in retinal microvascular endothelial cells [22, 37]. Beyond PEDF's antiangiogenic activity, PEDF has been well recognized for its neuroprotective, anti-inflammatory, and antioxidant properties [22, 38–40]. Given these functions, PEDF represents a potential intervention therapy for DR. These beneficial effects of PEDF were correlated with the decreased levels of retinal inflammatory factors, including VEGF, VEGF receptor-2, MCP-1, TNF-α, and ICAM-1 [22]. In concordance with these previous studies, we reported similar findings for the beneficial effects of PEDF in decreasing abnormalities in retinal microcirculation functions. However, this study is the first to extend the knowledge of PEDF's anti-permeability, anti-angiogenic, and anti-inflammatory effects to include its ability to counteract retinal endothelial dysfunctions in response to 12/15-HETEs.

Accordingly, the direct relationship between HETEs and PEDF has been explored through *in vitro* studies using two critical cell types of the eye, rMCs and HRECs. The results of these experiments demonstrated that 12/15-HETEs trigger the secretion of TNF-α as well as IL-6 from rMCs and this inflammatory response is associated with activation of NFκB in rMCs and alteration of ZO-1 protein dynamics in HRECs. NF-κB is a pleiotropic regulator of many pro-inflammatory cytokines that has been found to be activated by a variety of stimuli, including diabetic stress [41, 42]. All these effects were prevented in PEDF-treated cells.

Despite the aforementioned rescue effects of PEDF, its application is limited by its short half-life, unstable pharmacology, and administration pathway. The gene therapy method, such as adeno-associated virus vector-(AAV) mediated PEDF, has been used to overcome the limitations accompanied the topical application of recombinant PEDF [44–46]. However, because of possible immunogenicity properties associated with AAV, PEDF application is still limited [47]. These limitations prompted us to find an efficient way to maintain PEDF expression and action in the diabetic retina. This approach requires deciphering the molecular events underlying the disruption of PEDF expression during diabetes.

Previous studies showed that PEDF suppressed the VEGF-, advanced glycation end products-, and diabetes-induced leukostasis and vascular permeability[6, 37, 48] via inhibition of NADPH oxidase-mediated ROS generation and NFκB signaling pathway [49–51]. However, the reverse relationship between NADPH oxidase and PEDF is not yet clear and needs extensive investigation. Previously, we have shown that 12-HETE attenuated the levels of PEDF. Furthermore, we have shown that NADPH oxidase is a major downstream mediator from HETEs. These findings, together with our observations that NOX2 deletion resulted in a marked recovery of PEDF's immunoreactivity within diabetic retina, have underpinned the notion that LOX-NOX pathway plays a crucial role in retinal PEDF-suppression during diabetes.

In summary, taken together our previous and current studies provide new insights into the mechanisms of how diabetes affects the expression of PEDF in the retina, demonstrating that signaling through LOX-NOX pathway is a critical pathway for down-regulating PEDF. Additionally, our results suggest that interfering with the LOX-NOX pathway may represent a novel mechanism by which PEDF exerts its broad effects against DR and other retinal microvascular dysfunctions associated with increased eicosanoids. Figure 7 illustrates the hypothetical mechanisms in the causal inference between these biomolecular pathways in DR.

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List of Nonstandard Abbreviations

DR	diabetic retinopathy
12/15-LOX	12/15-Lipoxygenase
NOX	NADPH oxidase
HETEs	12- and 15-hydroxyeicosatetraenoic acids
PEDF	pigment epithelium-derived factor
TNF-α	tumor necrosis factor-α
VEGF	vascular endothelial growth factor
ELISA	Enzyme-linked immunosorbent assay
ROS	reactive oxygen species
STZ	Streptozotocin
HRECs	human retinal endothelial cells
rMCs	retinal Müller cells

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Highlights

- 12/15-HETEs induce a phenotype of DR, increased permeability and neovascularization.
- PEDF inhibits 12/15HETEs-induced retinal microvascular dysfunctions.
- NADPH oxidase regulates retinal PEDF level during diabetes.

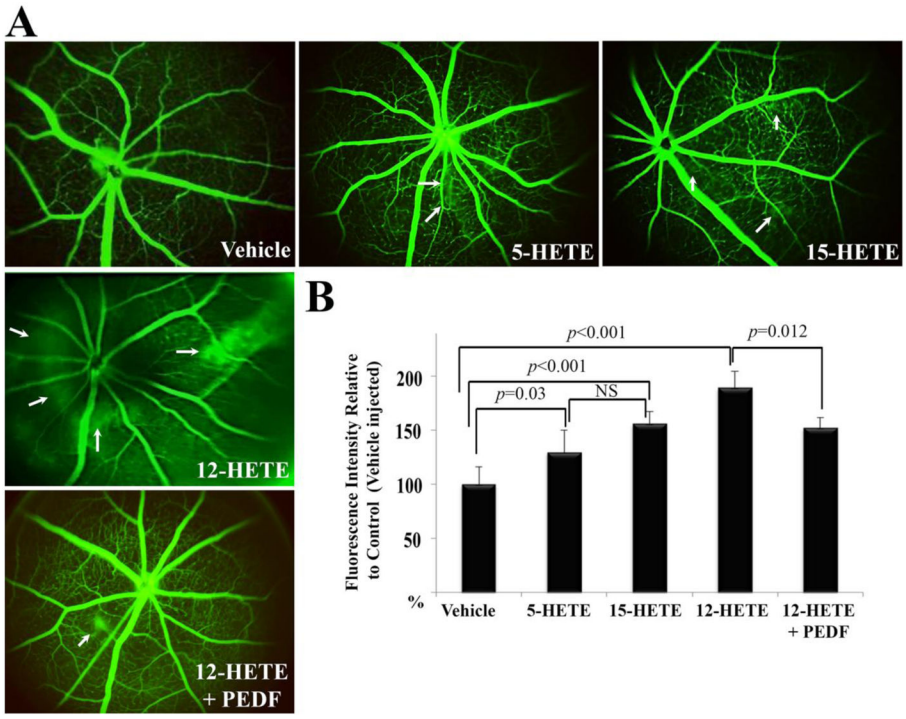

Figure 1. Anti-permeability effect of PEDF on HETEs-induced retinal vascular leakage assessed by fluorescein angiography

A) Fluorescein angiography (FA) of normal C57BL/6J mice injected intravitreally with vehicle (control), 5-HETE, 15-HETE, or 12-HETE with/without PEDF. FA pictures were obtained one week post-injection to evaluate the changes of retinal vascular leakage. Data are representative of 4–6 mice studied in each group. **B)** Quantification of fluorescein intensity per mouse retina among studied groups relative to control (vehicle injected).

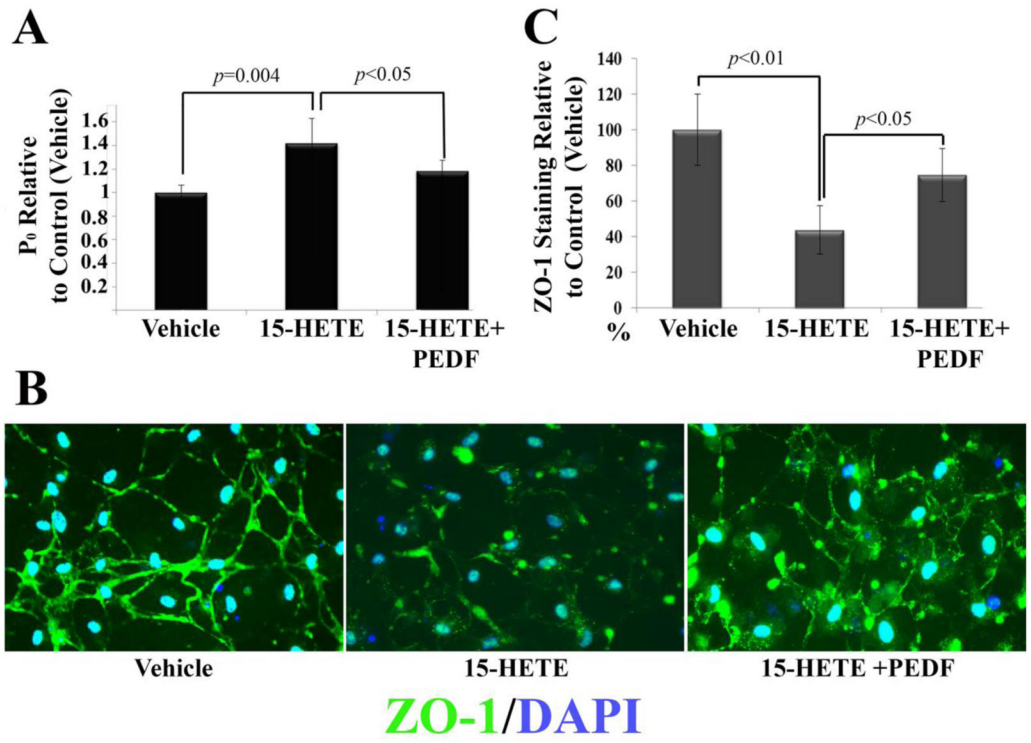

Figure 2. Effect of PEDF on 15-HETE-induced increase in permeability and alteration in ZO-1 expression in human retinal endothelial cells (HRECs)

A) Fold change in diffusive flux (Po) of 70-kDa-dextran evaluated after 24 hours of 15-HETE treatment in absence or presence of PEDF (100 nM), relative to control group (vehicle). **B)** Immunofluorescence of ZO-1 (green) in HRECs treated by 15-HETE in presence or absence of PEDF. **C)** Quantification of ZO-1 immunofluorescence staining in 10 fields/slide normalized as percentage of control group (vehicle). Data shown are the mean ± SD of 3 independent experiments. Results show a significant decrease in ZO-1 expression by 15-HETE-treatment that was restored by PEDF-pretreatment. The Blue staining (DAPI) is a nuclear marker.

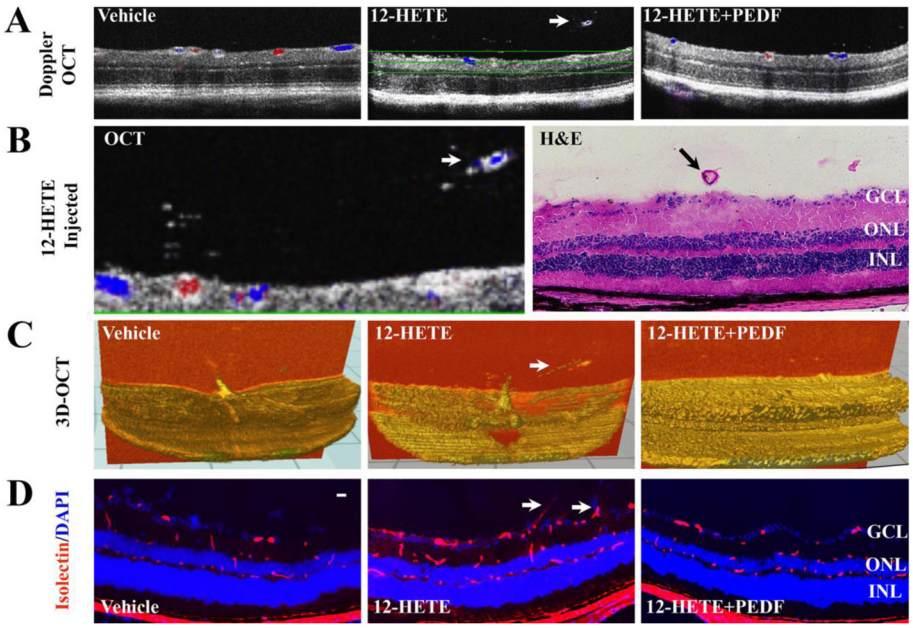

Figure 3. Anti-angiogenic effect of PEDF on 12-HETE-induced retinal neovascularization

Normal C57BL/6J mice were injected intravitreally with vehicle or 12-HETE with/without PEDF. **A)** Optical coherence tomography (OCT) B-scan reported a pre-retinal neovascularization (arrow) in mouse eye receiving 12-HETE as confirmed by Color Doppler imaging that enabled vascular imaging in false-colors (red–blue). The red or blue color corresponds to blood flow in the direction towards or away from the incident OCT beam. Meanwhile, these new vessels were not observed in the examined PEDF-administered mice. **B)** Enlarged OCT picture for the boxed areas in Figure 3A supplemented with hematoxylin and eosin (H&E) staining for the areas that show pre-retinal neovascularization in mouse eye receiving 12-HETE. **C)** Three dimensional OCT (3D-OCT) imaging of the retina. The vehicle-injected eye shows clear tomographic in the vitreous while 12-HETE-injected eye has aberrant structures in vitreous corresponding to the presumptive vitreal blood vessels observed in the B-scan (arrow). These new vessels were not observed in PEDF-administered group. **D)** Retinal sections stained with isolectin-B4 (endothelial cell marker) to label retinal vasculature from mice injected intravitreally with vehicle, or 12-HETE with/without PEDF. Arrows point to pre-retinal neovascularization in 12-HETE-injected mice. Nuclei were counter-stained with DAPI (blue). Scale bar = 10 μM, data are representative of 4–6 mice studied in each group.). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

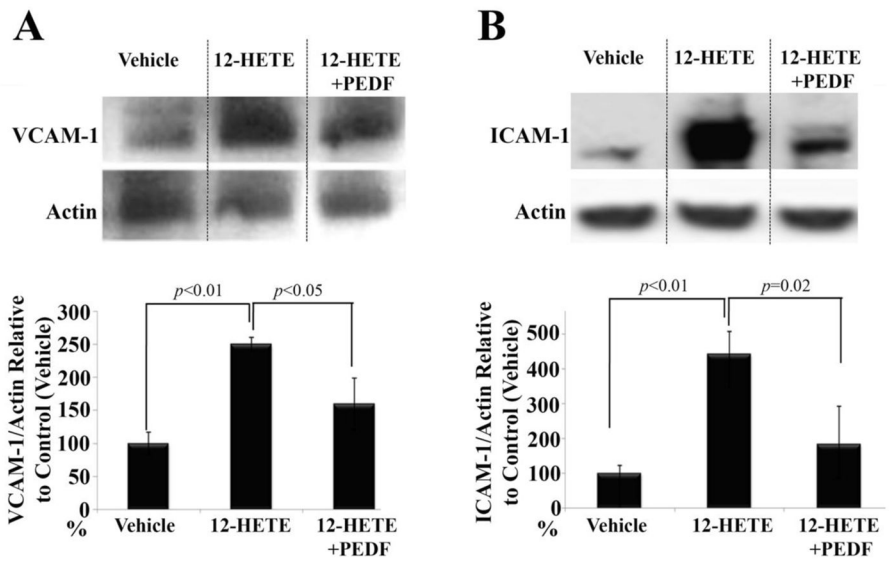

Figure 4. Anti-inflammatory effect of PEDF on 12-HETE-induced retinal inflammation evaluated by Western blot analysis for VCAM-1 and ICAM-1

Normal C57BL/6J mice were injected intravitreally with vehicle control or 12-HETE with/without PEDF. The animals were sacrificed one week later, and retinas were dissected and prepared for Western blot analysis. **A and B)** Western blot and densitometric analysis of retinal expression of VCAM-1 and ICAM-1, respectively. VCAM-1 or ICAM-1 intensity relative to the actin for groups injected with 12-HETE with/without PEDF were quantified as percentage of vehicle injected group. Data shown are representative of 4–6 mice studied in each group.

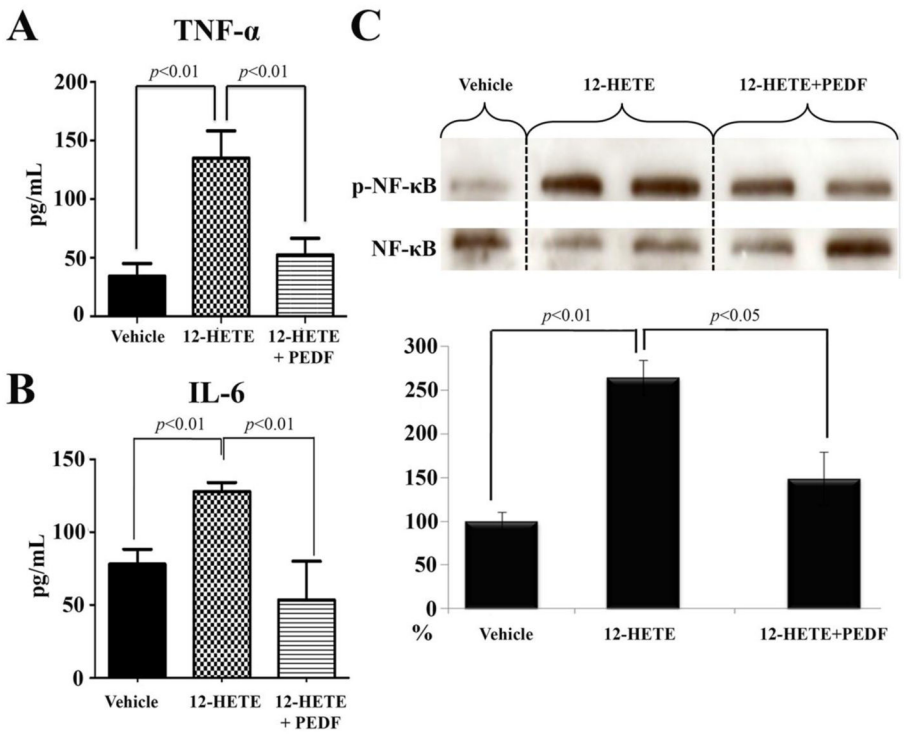

Figure 5. Identification of PEDF anti-inflammation signaling in retinal Müller cells

Levels of inflammatory cytokines, (**A**) Tumor necrosis factor (TNF)-α, and **(B)** Interleukin (IL)-6, were determined in the conditioned media of retinal Müller cells treated with 12-HETE (0.1μM, 24 hours), in the presence or absence of pigment epithelium-derived factor (PEDF, 100 nM). TNF-α as well as IL-6 levels were determined by enzyme-linked immunosorbent assay (ELISA). Data shown are the mean ± SD of three experiments**. C)** Western blot analysis of Phospho-NF-κB p65 in extracts from Müller cells treated 24 hours with vehicle (control), 12-HETE (0.1μM) in presence or absence of pigment epithelium-derived factor (PEDF, 100 nM), using Phospho-NF-κB p65 (Ser536) antibody (top) or NF-κB p65 antibody (bottom). Ratios of the Phospho-NF-κB p65 intensity relative to the NF-κB p65 for groups treated with 12-HETE with/without PEDF were compared densitometrically as percent of vehicle-treated group (control). Data shown are the mean ± SD of three experiments.

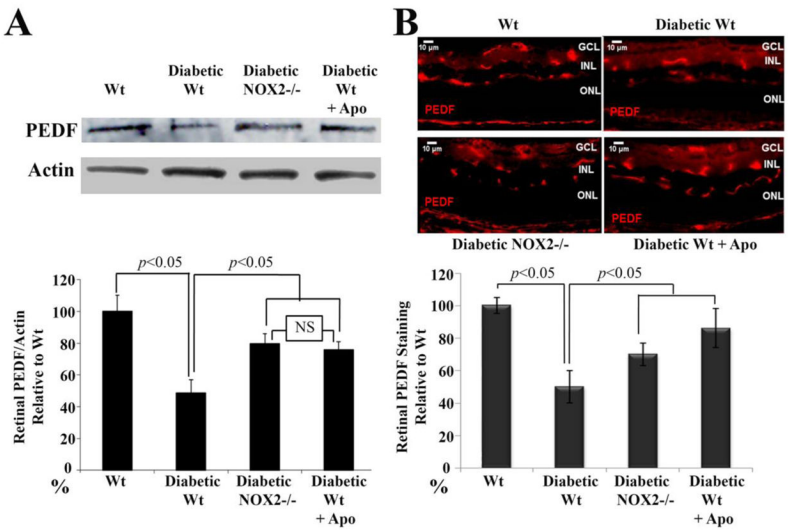

Figure 6. NADPH oxidase inhibition or NOX2 ablation restored diabetes-induced retinal pigment epithelium-derived factor (PEDF) suppression

Using streptozotocin, diabetes was induced in 8 weeks old NOX2−/− mice and age-matched wild-type (Wt) littermates. One group of Wt diabetic mice was treated with a NADPH oxidase inhibitor, Apocynin (Apo) (10 mg/kg) in drinking water for the duration of the study, 5-weeks. **A)** Western blot analysis of PEDF in retinal samples from Wt (normal), diabetic Wt, diabetic NOX2 $^{-/-}$, and apocynin-treated diabetic WT mice (Diabetic Wt + Apo). PEDF intensity relative to the actin for all diabetic groups are presented as percentage of Wt. (n = 4 to 6). **B)** Representative photomicrographs of PEDF distribution in the retinal sections from Wt, diabetic Wt, diabetic NOX2$^{-/-}$, and Diabetic Wt + Apo. Quantification of retinal PEDF immunofluorescence in these micrographs as a percent of Wt (normal) was carried in 10 adjacent locations along the vertical meridian within 4 mm of the optic disk (10 fields/retina section) (n = 4 to 6). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

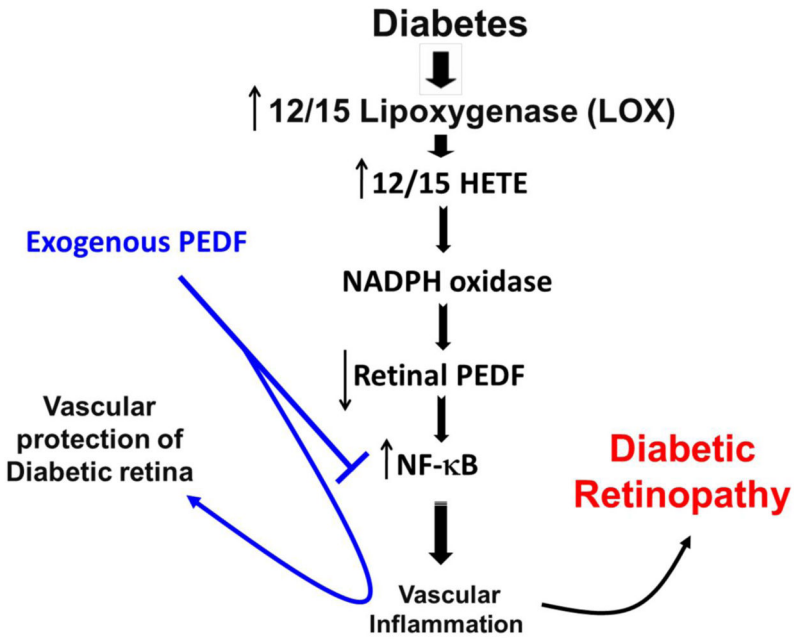

Figure 7. Proposed signaling pathways for the novel mechanism in treating diabetic retinopathy

Diabetes-induced 12/15-LOX activation leads to 12/15-HETEs formation that activates NADPH oxidase resulted in PEDF suppression, increased inflammation, and eventually vascular abnormalities (hallmarks of diabetic retinopathy).