

# Pathogenic role of diabetes-induced PPAR- $\alpha$ down-regulation in microvascular dysfunction

Yang Hu<sup>a,1,2</sup>, Ying Chen<sup>a,b,1</sup>, Lexi Ding<sup>a,c</sup>, Xuemin He<sup>a</sup>, Yusuke Takahashi<sup>b</sup>, Yang Gao<sup>a,d</sup>, Wei Shen<sup>a,e</sup>, Rui Cheng<sup>a</sup>, Qian Chen<sup>a</sup>, Xiaoping Qi<sup>f</sup>, Michael E. Boulton<sup>f</sup>, and Jian-xing Ma<sup>a,b,2</sup>

<sup>a</sup>Department of Physiology and <sup>b</sup>Harold Hamm Diabetes Center, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104; <sup>c</sup>Department of Ophthalmology, Xiangya Hospital, Central South University, Changsha 410008, China; <sup>d</sup>State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou 510060, China; <sup>e</sup>College of Life Science, Jilin University, Changchun, Jilin 130012, China; and <sup>f</sup>Department of Ophthalmology, Indiana University School of Medicine, Indianapolis, IN 46202

Edited by Jeremy Nathans, Johns Hopkins University, Baltimore, MD, and approved August 2, 2013 (received for review April 16, 2013)

Two independent clinical studies have reported that fenofibrate, a peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) agonist, has robust therapeutic effects on microvascular complications of diabetes, including diabetic retinopathy (DR) in type 2 diabetic patients. However, the expression and function of PPAR $\alpha$  in the retina are unclear. Here, we demonstrated that PPAR $\alpha$  is expressed in multiple cell types in the retina. In both type 1 and type 2 diabetes models, expression of PPAR $\alpha$ , but not PPAR $\beta/\delta$  or PPAR $\gamma$ , was significantly down-regulated in the retina. Furthermore, high-glucose medium was sufficient to down-regulate PPAR $\alpha$  expression in cultured retinal cells. To further investigate the role of PPAR $\alpha$  in DR, diabetes was induced in PPAR $\alpha$  knockout (KO) mice and wild-type (WT) mice. Diabetic PPAR $\alpha$  KO mice developed more severe DR, as shown by retinal vascular leakage, leukostasis, pericyte loss, capillary degeneration, and over-expression of inflammatory factors, compared with diabetic WT mice. In addition, overexpression of PPAR $\alpha$  in the retina of diabetic rats significantly alleviated diabetes-induced retinal vascular leakage and retinal inflammation. Furthermore, PPAR $\alpha$  overexpression inhibited endothelial cell migration and proliferation. These findings revealed that diabetes-induced down-regulation of PPAR $\alpha$  plays an important role in DR. Up-regulation or activation of PPAR $\alpha$  may represent a novel therapeutic strategy for DR.

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear hormone receptors that function as transcription factors regulating the expression of a number of genes involving lipid metabolism and insulin resistance (1). There are three PPAR isotypes—PPAR $\alpha$ , PPAR $\beta/\delta$ , and PPAR $\gamma$ —that play important roles in the regulation of cell differentiation, proliferation, development, and metabolism (carbohydrate, lipid, protein) (2, 3). PPAR $\alpha$  is also crucial in the regulation of inflammation and angiogenesis (4–6) and is expressed in the liver, kidney, heart, muscle, adipose tissue, and other organs with significant fatty-acid catabolism (7, 8). PPAR $\alpha$  is primarily activated through binding of ligands (9), which include lipids and synthetic fibrates drugs, such as fenofibrate (10, 11).

Diabetic microvascular complications include diabetic nephropathy, neuropathy, and retinopathy, which can occur in both type 1 and type 2 diabetes mellitus (12–15). Recently, fenofibrate, a specific PPAR $\alpha$  agonist, has displayed surprising and robust efficacy in arresting the progression of microvascular complications in type 2 diabetes in the FIELD and ACCORD studies (16, 17). Further, PPAR $\alpha$  knock-out (KO) mice with diabetes developed more severe nephropathy, compared with diabetic wild-type (WT) mice (18). Moreover, our recent study showed that fenofibrate had therapeutic effects on diabetic retinopathy (DR) via a PPAR $\alpha$ -dependent mechanism (19). Also, PPAR $\alpha$  ligands inhibited endothelial cell proliferation and migration (20) and reduced angiogenesis in a porcine model (6). However, the function of PPAR $\alpha$  in the retina and its role in DR have not been clearly understood.

The present study determined the expression of PPAR $\alpha$  in the retina and demonstrated down-regulation of PPAR $\alpha$  in the retina

of diabetes models. Further, we also induced diabetes in PPAR $\alpha$  KO mice that displayed more severe retinopathy than diabetic WT mice. Moreover, we also demonstrated that overexpression of PPAR $\alpha$  has therapeutic effects on DR. Our findings reveal the function of PPAR $\alpha$  in the retina and may extend a novel therapeutic strategy of clinical relevance.

## Results

**PPAR $\alpha$  Is Expressed in Multiple Cells in Human and Rat Retinas.** To determine whether PPAR $\alpha$  is expressed in the retina, we first performed immunostaining of PPAR $\alpha$  in human retinal sections. Immunostaining detected PPAR $\alpha$  in the retinal pigment epithelium (RPE), outer nuclear layer, inner nuclear layer, and ganglion cell layer (Fig. 1 *A* and *E*). Further, we compared PPAR $\alpha$  levels in the retinas of nondiabetic human donors (Fig. 1 *A* and *E*) with those of type 2 diabetic human donors with nonproliferative DR (Fig. 1 *B* and *F*). PPAR $\alpha$  levels in the retina were substantially lower in the human donors with DR, compared with those in nondiabetic human donors.

To define the cell types expressing PPAR $\alpha$  in the retina, we double stained the retinal sections using antibodies for PPAR $\alpha$  and for glial fibrillary acidic protein (GFAP), a glial cell marker, in normal (Fig. 1 *C* and *G*) and diabetic rats (Fig. 1 *D* and *H*). The result demonstrated the colocalization of PPAR $\alpha$  and GFAP in the ganglion cell layer, suggesting the expression of PPAR $\alpha$  in glial cells, including Müller cells. Further, PPAR $\alpha$  is also expressed in the inner retina in mice, similar to that in rats (Fig. S1). Under the same

## Significance

This study investigated the expression and function of peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) in the retina and its role in diabetic retinopathy. In both type 1 and type 2 diabetes models, expression of PPAR $\alpha$  was significantly down-regulated in the retina. PPAR $\alpha$  knockout exacerbated diabetes-induced retinal vascular leakage and retinal inflammation, while over-expression of PPAR $\alpha$  in the retina of diabetic rats significantly alleviated diabetic retinopathy. This study reveals that PPAR $\alpha$  has an anti-inflammatory function in the retina. These findings also suggest that diabetes-induced down-regulation of PPAR $\alpha$  plays an important role in diabetic retinopathy and represents a novel therapeutic target for diabetic retinopathy.

Author contributions: Y.H., Y.C., and J.-x.M. designed research; Y.H., Y.C., L.D., X.H., Y.T., Y.G., W.S., R.C., and Q.C. performed research; X.Q. and M.E.B. contributed new reagents/analytic tools; Y.H., Y.C., and L.D. analyzed data; and Y.H. and J.-x.M. wrote the paper.

The authors declare no conflict of interest.

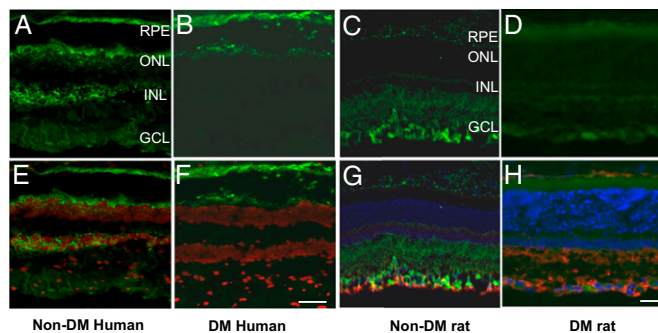
This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

<sup>1</sup>Y.H. and Y.C. contributed equally to this work.

<sup>2</sup>To whom correspondence may be addressed. E-mail: jian-xing-ma@ouhsc.edu or yang-hu@ouhsc.edu.

This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1307211110/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1307211110/-DCSupplemental).



**Fig. 1.** Expression of PPAR $\alpha$  in the human and rat retinas. (A, B, E, and F) Immunostaining of PPAR $\alpha$  in human retinas. PPAR $\alpha$  was immunostained (green) in the retina section from six nondiabetic human donors (A) and six type 2 diabetic donors with nonproliferative retinopathy (B). The nuclei were counterstained with DAPI (red) and merged with PPAR $\alpha$  staining (green) (E and F). (C, D, G, and H) Immunostaining of PPAR $\alpha$  in rat retinas. Retinal sections from nondiabetic rats (16 wk old) (C and G) and age-matched rats with 8 wk of STZ-induced diabetes (D and H) were double stained with an anti-PPAR $\alpha$  antibody and anti-GFAP antibody. The nuclei were counterstained with DAPI. (G and H) Merged signals of PPAR $\alpha$  (green), GFAP (red), and DAPI (blue). RPE, retinal pigment epithelium; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. (Scale bar: 50  $\mu$ m.)

staining conditions, diabetic rat retina showed less intense PPAR $\alpha$  staining, compared with that in nondiabetic rat retina. Taken together, these results demonstrate that PPAR $\alpha$  is expressed in the retinas, and its levels are reduced in the retinas with DR.

**STZ-Induced Diabetic Rats, Akita Mice, and *db/db* Mice Have Down-Regulated PPAR $\alpha$  Expression but Unchanged PPAR $\beta$  and PPAR $\gamma$  Expression in the Retina.** We next investigated whether PPAR $\alpha$  expression is changed in the retina under diabetic conditions in animal models. Western blot analysis showed that protein levels of PPAR $\alpha$  were significantly lower in the retina of STZ-induced diabetic rats, compared with those in nondiabetic rats, whereas PPAR $\beta$  and PPAR $\gamma$  levels showed no significant differences (Fig. 2A and D). Similar results were observed in the retina of Akita mice, a genetic type 1 diabetic model, compared with their WT control mice (Fig. 2B and E). Further, PPAR $\alpha$  levels were also significantly decreased in the retina of *db/db* mice, a type 2 diabetic model, compared with their WT controls (Fig. 2C and F). Similar to that in STZ-diabetic rats, PPAR $\beta$  and PPAR $\gamma$  expression was not significantly changed in the retinas of Akita or *db/db* mice. Taken together, these results indicated that, among the PPAR family members, PPAR $\alpha$  is selectively down-regulated in the retinas of both type 1 and type 2 diabetes models.

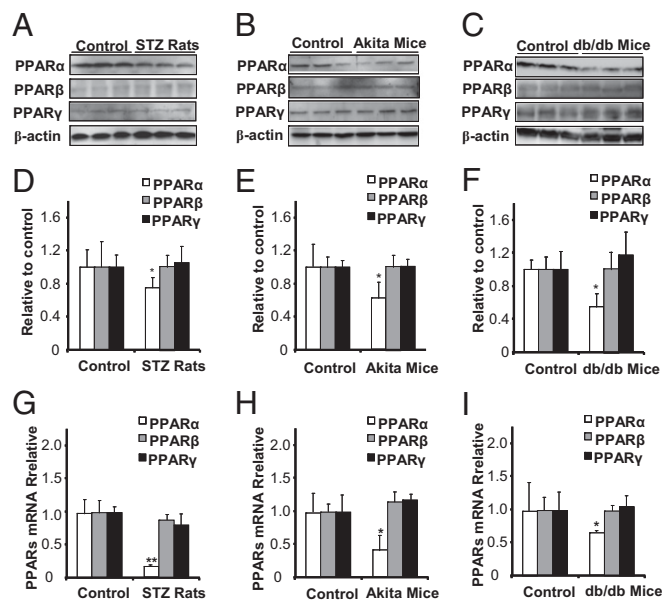
**PPAR $\alpha$  mRNA Levels Are Decreased in the Retinas of STZ-Diabetic Rats, Akita Mice, and *db/db* Mice.** To determine whether the decreases in retinal PPAR $\alpha$  levels in diabetes occur at the gene expression level, PPAR $\alpha$ , PPAR $\beta$ , and PPAR $\gamma$  mRNA levels were measured using real-time RT-PCR in the retinas of STZ-diabetic rats, Akita mice, *db/db* mice, and their respective nondiabetic controls. PPAR $\alpha$  mRNA levels were significantly decreased in the retina of STZ-diabetic rats compared with normal rats whereas PPAR $\beta$  and PPAR $\gamma$  mRNA levels were unchanged (Fig. 2G). Similar results were observed in the retinas of Akita mice (Fig. 2H) and *db/db* mice (Fig. 2I). These results demonstrated that PPAR $\alpha$  mRNA expression is down-regulated in the retinas in both type 1 and type 2 diabetic animal models, consistent with the observation of the protein levels.

**PPAR $\alpha$  Expression Is Down-Regulated by High Glucose in Retinal Cells.** To determine which type of retinal cells has impaired expression of PPAR $\alpha$  in diabetes, we mimicked the diabetic condition using high-glucose medium in cultured retinal cells. Expression of

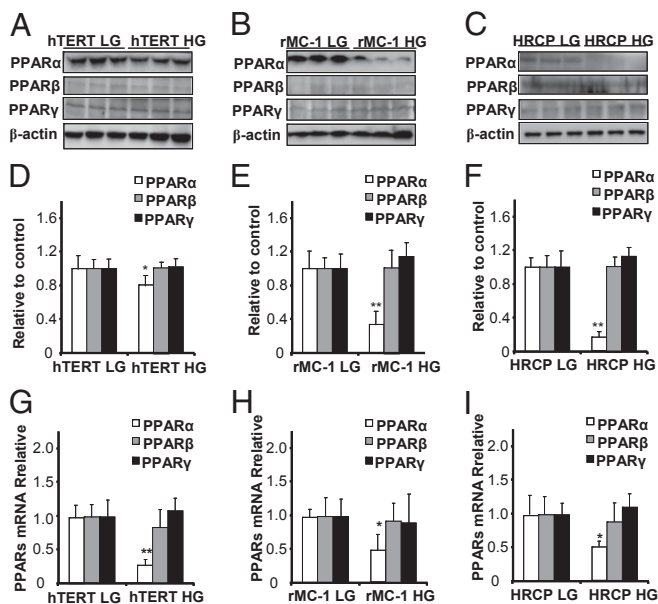
PPAR $\alpha$  was significantly down-regulated in hTERT RPE cells treated with high glucose (30 mM glucose) for 72 h, compared with low glucose control (5 mM glucose and 25 mM mannitol) whereas PPAR $\beta$  and PPAR $\gamma$  expression levels showed no significant differences (Fig. 3A and D). Similar results were observed in high glucose-treated rMC-1, a cell line derived from rat Müller cells (Fig. 3B and E) and primary human retinal capillary pericytes (HRCP) (Fig. 3C and F). Taken together, these results indicated that high glucose is a diabetic stressor that down-regulates PPAR $\alpha$  expression in multiple retinal cells.

**PPAR $\alpha$  mRNA Levels Are Decreased by High Glucose in Retinal Cells.** To confirm the down-regulation of PPAR $\alpha$  mRNA expression by high glucose, PPAR $\alpha$ , PPAR $\beta$ , and PPAR $\gamma$  mRNA levels were measured by real-time RT-PCR. PPAR $\alpha$  mRNA levels were significantly decreased in high glucose-treated hTERT RPE (Fig. 3G), rMC-1 cells (Fig. 3H), and HRCP (Fig. 3I), compared with their respective controls. However, PPAR $\beta$  and PPAR $\gamma$  mRNA levels showed no significant changes between these groups (Fig. 3G–I).

**Diabetic PPAR $\alpha$  KO Mice Show More Severe Retinal Vessel Impairment and Higher Vascular Leakage Compared with Diabetic WT Mice.** To test the hypothesis that down-regulated PPAR $\alpha$  expression may exacerbate DR, we induced diabetes in PPAR $\alpha$  KO mice and age- and genetic background-matched WT mice. Retina trypsin digestion was performed in nondiabetic WT mice (Fig. 4A), nondiabetic PPAR $\alpha$  KO mice (Fig. 4B), STZ-induced diabetic WT



**Fig. 2.** Down-regulation of PPAR $\alpha$  expression in the retinas of STZ-diabetic rats, Akita mice, and *db/db* mice. (A and D) The same amount (50  $\mu$ g) of retinal proteins from rats with 8 wk of STZ-induced diabetes and age-matched nondiabetic rats was used for Western blot analysis of PPAR $\alpha$ , PPAR $\beta$ , and PPAR $\gamma$  (A), semiquantified by densitometry and normalized by  $\beta$ -actin levels (D). (B and E) The same amount (50  $\mu$ g) of retinal proteins from Akita mice (age of 16 wk) and WT controls was used for Western blot analysis of PPAR $\alpha$ , PPAR $\beta$ , and PPAR $\gamma$  (B), semiquantified by densitometry and normalized by  $\beta$ -actin levels (E). (C and F) The same amount (50  $\mu$ g) of retinal proteins from *db/db* mice (age of 12 wk) and WT controls was used for Western blot analysis of PPAR $\alpha$ , PPAR $\beta$ , and PPAR $\gamma$  (C) and normalized by  $\beta$ -actin levels (F). (G–I) Real-time RT-PCR was performed to measure mRNA levels of PPAR $\alpha$ , PPAR $\beta$ , and PPAR $\gamma$  in the retinas of STZ-induced diabetic rats (G), Akita mice (H), and *db/db* mice (I) and their respective nondiabetic controls at the ages indicated above. All mRNA levels were expressed as fold of respective control (mean  $\pm$  SD,  $n = 4$ , \* $P < 0.05$ , \*\* $P < 0.01$ , compared with control group).



**Fig. 3.** Down-regulation of PPAR $\alpha$  expression by high glucose in retinal cells. hTERT RPE cells, rMC-1 cells, and HRCP were exposed to 30 mM D-glucose for 72 h, with 5 mM D-glucose plus 25 mM L-glucose as control. (A–F) Total cell lysates were used for Western blot analysis of PPAR $\alpha$ , PPAR $\beta$ , and PPAR $\gamma$  in hTERT RPE cells (A), rMC-1 cells (B), and HRCP (C). The results were semiquantified by densitometry and normalized by  $\beta$ -actin levels (D–F). (G–I) Real-time RT-PCR was performed to measure mRNA levels of PPAR $\alpha$ , PPAR $\beta$ , and PPAR $\gamma$  in hTERT RPE cells (G), rMC-1 cells (H), and HRCP (I). All values are fold of respective control (mean  $\pm$  SD,  $n = 4$ , \* $P < 0.05$ , \*\* $P < 0.01$ ).

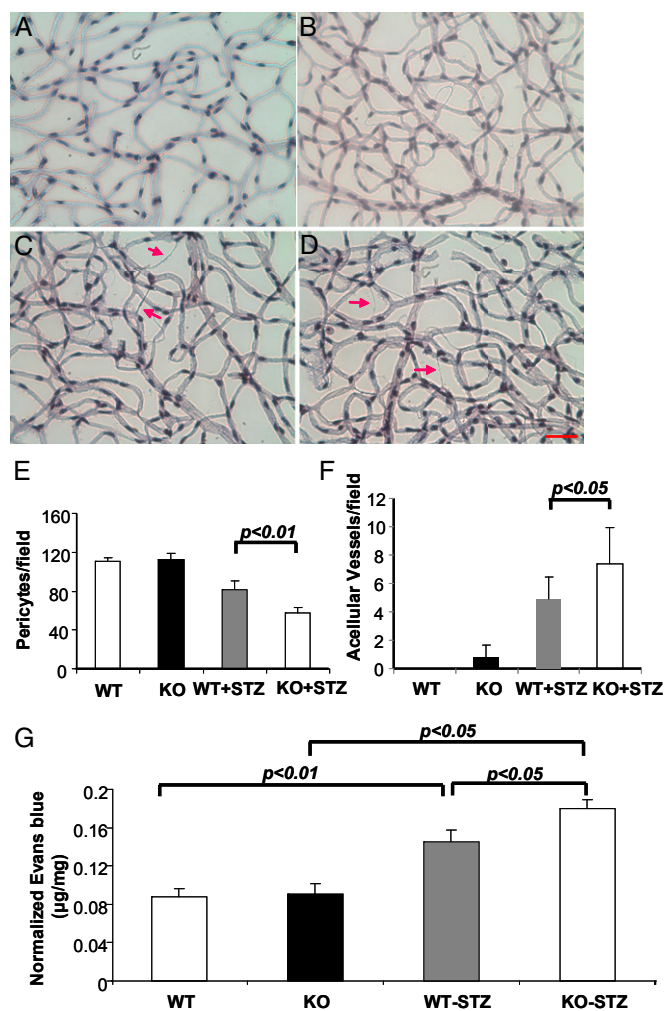
mice (Fig. 4C), and STZ-induced diabetic PPAR $\alpha$  KO mice (Fig. 4D) at 8 wk after the onset of diabetes. Pericytes and acellular capillaries were counted in a double-blind manner, which showed that, whereas there was no significant difference in numbers of pericytes between nondiabetic WT mice and nondiabetic PPAR $\alpha$  KO mice, diabetic PPAR $\alpha$  KO mice had significantly lower numbers of pericytes, compared with diabetic WT mice, with similar hyperglycemia levels and the same duration of diabetes (Fig. 4E), suggesting more severe diabetes-induced pericytes loss. Further, numbers of acellular capillaries were significantly higher in diabetic PPAR $\alpha$  KO mice, compared with diabetic WT mice, suggesting aggravated capillary degeneration (Fig. 4F).

To determine severity of the diabetes-induced retinal vascular leakage, a retinal vascular permeability assay was performed in the same four groups (Fig. 4G). Retinal vascular permeability showed no significant differences between nondiabetic WT mice and PPAR $\alpha$  KO mice; both diabetic WT mice and diabetic PPAR $\alpha$  KO mice had significantly increased retina vascular leakage compared with respective nondiabetic controls. Diabetic PPAR $\alpha$  KO mice had significantly higher retina vascular leakage than diabetic WT mice. Taken together, these results indicated that diabetic PPAR $\alpha$  KO mice developed more severe vascular impairment compared with diabetic WT mice.

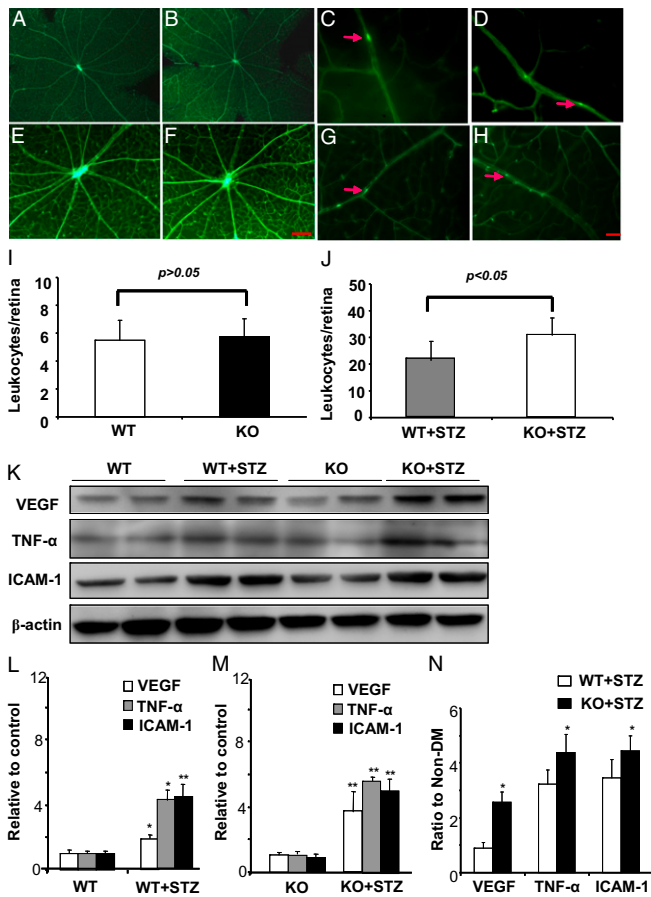
**Diabetic PPAR $\alpha$  KO Mice Develop More Severe Retina Inflammation Compared with Diabetic WT Mice.** To determine the difference of retinal inflammation in PPAR $\alpha$  KO mice and WT mice under normal/diabetic conditions, a retinal leukostasis assay was performed in nondiabetic WT mice (Fig. 5A and C), nondiabetic PPAR $\alpha$  KO mice (Fig. 5B and D), STZ-diabetic WT mice (Fig. 5E and G), and STZ-diabetic PPAR $\alpha$  KO mice (Fig. 5F and H). Quantification of adherent leukocytes in flat-mounted retinas showed no significant difference between nondiabetic WT mice and nondiabetic PPAR $\alpha$  KO mice (Fig. 5I); in contrast, diabetic PPAR $\alpha$  KO mice had significantly more adherent leukocytes per

retina than diabetic WT mice (Fig. 5J). Further, retinal levels of inflammatory factors including VEGF, TNF- $\alpha$ , and ICAM-1 were compared by Western blot analysis (Fig. 5K) and ELISA (Fig. S2). Both diabetic WT mice and diabetic PPAR $\alpha$  KO mice displayed elevated retinal levels of VEGF, TNF- $\alpha$ , and ICAM-1, compared with their nondiabetic controls (Fig. 5L and M and Fig. S2). Diabetic PPAR $\alpha$  KO mice showed higher inductions of VEGF, TNF- $\alpha$ , and ICAM-1 expression by diabetes, compared with diabetic WT mice (Fig. 5N and Fig. S2). Taken together, these results demonstrated that diabetic PPAR $\alpha$  KO mice had more severe diabetes-induced retinal inflammation, compared with diabetic WT mice.

**PPAR $\alpha$  Overexpression Reduces Vascular Leakage and Retina Inflammation in STZ-Diabetic Rats.** To investigate whether overexpression of PPAR $\alpha$  in the retina may rescue the retinal vascular defects in DR, we overexpressed PPAR $\alpha$  in the retina of diabetic rats. Adenovirus-expressing PPAR $\alpha$  (Ad-PPAR $\alpha$ ) was injected intravitreally into nondiabetic and STZ-induced diabetic



**Fig. 4.** PPAR $\alpha$  knockout exacerbates diabetic microvascular damage by diabetes. Diabetes was induced in PPAR $\alpha$  KO mice and WT control by STZ injections. At 12 wk after diabetes onset, retina trypsin digestion was performed in age-matched nondiabetic WT mice (A), nondiabetic PPAR $\alpha$  KO mice (KO) (B), diabetic WT mice (WT-STZ) (C), and diabetic PPAR $\alpha$  KO mice (KO+STZ) (D). (Scale bar: 25  $\mu$ m). Red arrows indicate acellular capillaries. (E) Pericytes were quantified in five random fields per retina and averaged. (F) Acellular capillaries were quantified in five random fields and averaged. Retina vascular leakage was measured in the same groups by permeability assay (G). All values are mean  $\pm$  SD ( $n = 4$ ).



**Fig. 5.** PPAR $\alpha$  knockout exacerbates retinal inflammation induced by diabetes. Diabetes was induced in PPAR $\alpha$  KO mice and WT mice by STZ injections. (A–H) A retina leukostasis assay was performed 12 wk after the onset of diabetes in age-matched nondiabetic WT mice (A and C), nondiabetic PPAR $\alpha$  KO mice (B and D), diabetic WT mice (E and G), and diabetic PPAR $\alpha$  KO mice (F and H). [Scale bars: 100  $\mu$ m (A, B, E, and F) and 25  $\mu$ m (C, D, G, and H).] (I and J) Adherent leukocytes per retina were quantified and compared between nondiabetic WT and PPAR $\alpha$  KO mice (I) and between diabetic WT and diabetic PPAR $\alpha$  KO mice (J) (mean  $\pm$  SD,  $n = 4$ ). (K–N) The same amount (50  $\mu$ g) of retina protein from these four groups was used for Western blot analysis of VEGF, TNF- $\alpha$ , and ICAM-1 (K). These protein levels were semi-quantified with densitometry, normalized by  $\beta$ -actin levels, and compared between nondiabetic and diabetic WT mice (L) (mean  $\pm$  SD,  $n = 4$ , \* $P < 0.05$ , \*\* $P < 0.01$ ) and between nondiabetic and diabetic PPAR $\alpha$  KO mice (M) (mean  $\pm$  SD,  $n = 4$ , \*\* $P < 0.01$ ). The increased folds of VEGF, TNF- $\alpha$ , and ICAM-1 levels induced by diabetes in WT mice were compared with those in PPAR $\alpha$  KO (N) (mean  $\pm$  SD,  $n = 4$ , \* $P < 0.05$ , compared with diabetic WT group).

rats at 8 wk after the onset of diabetes; Ad-GFP was injected as control. A retinal vascular permeability assay was performed in normal rats, untreated STZ-diabetic rats, STZ-diabetic rats with an intravitreal injection of Ad-GFP (control adenovirus), and STZ-diabetic rats injected with Ad-PPAR $\alpha$  at 4 wk after the injection. The results showed that the intravitreal injection of Ad-PPAR $\alpha$  significantly reduced vascular leakage, compared with Ad-GFP injection and untreated diabetic groups (Fig. 6A). Further, to determine the retina inflammation change, a retinal leukostasis assay was performed in normal rats (Fig. 6C and G), untreated diabetic rats (Fig. 6D and H), diabetic rats with Ad-GFP injection (Fig. 6E and I), and diabetic rats with Ad-PPAR $\alpha$  injection (Fig. 6F and J). Numbers of adherent leukocytes per retina were significantly decreased in the Ad-PPAR $\alpha$ -treated diabetic rats, compared with the Ad-GFP-treated diabetic rats (Fig. 6B). Moreover, expression levels of VEGF, TNF- $\alpha$ , and ICAM-1 in the retina were measured by Western blot analysis

and ELISA. The quantified data showed that Ad-PPAR $\alpha$  treatment significantly down-regulated the expression of VEGF, TNF- $\alpha$ , and ICAM-1, compared with the Ad-GFP group (Fig. 6K and L and Fig. S3). Taken together, these results showed that PPAR $\alpha$  overexpression in the retina alleviated vascular leakage and retinal inflammation in diabetic rats.

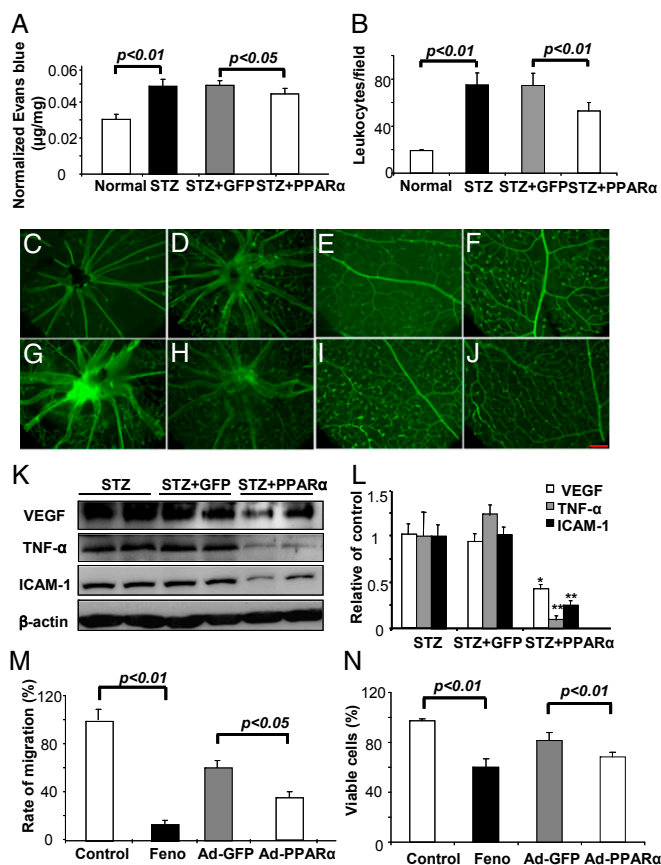
**PPAR $\alpha$  Overexpression Inhibits Migration and Proliferation of Human Retinal Capillary Endothelial Cell.** Next, we investigated the direct effects of PPAR $\alpha$  on endothelial cell migration and proliferation, important steps in angiogenesis. An in vitro scratch-wound healing assay was performed in primary retinal capillary endothelial cells (HRCECs) to measure cell migration. HRCECs were treated with fenofibrate (positive control group) and with DMSO as the vehicle control and were infected with Ad-PPAR $\alpha$ , with Ad-GFP as the control virus (Fig. S4). The quantified data showed that fenofibrate and Ad-PPAR $\alpha$  both significantly inhibited HRCEC migration, compared with DMSO and Ad-GFP, respectively (Fig. 6M). Further, HRCEC growth was measured using trypan blue staining and quantified in the same four groups. Fenofibrate and PPAR $\alpha$  overexpression both significantly decreased viable HRCEC numbers compared with DMSO and Ad-GFP (Fig. 6N). Taken together, these results showed that PPAR $\alpha$  overexpression also had an antiangiogenic effect by directly inhibiting HRCEC migration and proliferation.

## Discussion

PPAR $\alpha$  is an important transcription factor known to regulate lipid metabolism (1). Its function in the retina has not been investigated. The results presented here demonstrated that PPAR $\alpha$ , but not PPAR $\beta$  or PPAR $\gamma$ , is down-regulated in the retinas of both type 1 and type 2 diabetic models and that high glucose is a direct cause of the PPAR $\alpha$  down-regulation. The present study also showed that PPAR $\alpha$  KO exacerbates diabetes-induced vascular impairment, vascular leakage, and inflammation in the retina. Furthermore, PPAR $\alpha$  overexpression can rescue vascular leakage and inflammation in the retina of diabetic rats and inhibit endothelial cell migration and proliferation. These observations suggest that the diabetes-induced down-regulation of PPAR $\alpha$  plays an important role in DR and may represent a new therapeutic target for the treatment of diabetic microvascular complications.

PPAR $\alpha$ , PPAR $\beta$ , and PPAR $\gamma$  have been shown to have key roles in regulation of lipid metabolism through regulating related gene expression (21–23); however, these PPAR family members show tissue-specific distribution (24, 25) and have different roles in signaling modulation in microvascular (26–28) and macrovascular cells (29). Our results demonstrated that, unlike PPAR $\alpha$ , expression of PPAR $\beta$  and PPAR $\gamma$  has no significant changes in the retina in both type 1 and type 2 diabetic models, suggesting that PPAR $\alpha$  down-regulation by diabetes is selective and that PPAR $\alpha$  rather than PPAR $\beta$  and PPAR $\gamma$  is implicated in DR. This assumption is supported by the clinical findings that agonist of PPAR $\alpha$ , but not PPAR $\gamma$  agonist, has beneficial effects on DR (16).

To define the direct cause of PPAR $\alpha$  down-regulation under diabetic conditions, we used high-glucose media in cultured retinal cells. The results showed that high glucose alone is sufficient to cause PPAR $\alpha$  down-regulation in multiple cell types. Previously, PPAR $\alpha$  expression was found to be down-regulated by hypoxia inducible factor-1 (HIF-1) under hypoxia condition in intestinal epithelial cells (30). To investigate whether HIF-1 mediates down-regulation of PPAR $\alpha$  induced by high glucose, we applied hypoxia in vitro and in vivo. However, our results showed that PPAR $\alpha$  expression levels were not significantly altered by CoCl $_2$ -induced hypoxia in hTERT RPE cells and rat Müller cells at the time points analyzed although HIF-1 was activated (Fig. S5A and B). Moreover, PPAR $\alpha$  expression was not down-regulated in the retina of oxygen-induced retinopathy (OIR) mice (Fig. S5C) and OIR rats (Fig. S5D), an ischemia-induced retinal neovascularization (NV) model with activation of



**Fig. 6.** PPAR $\alpha$  overexpression ameliorates vascular leakage, leukostasis, and overexpression of angiogenic and inflammatory factors in the retinas of diabetic rats and inhibits endothelial cell migration and proliferation. Diabetic rats with 8 wk of STZ-induced diabetes received an intravitreal injection of Ad-PPAR $\alpha$  or Ad-GFP (control). (A) Four weeks after the injection, retinal vascular leakage was measured by permeability assay and normalized by total retinal protein concentration (mean  $\pm$  SD,  $n = 4$ ). (B–J) Retina leukostasis assay was performed in age-matched nondiabetic rats (C and E), untreated diabetic rats (D and F), Ad-GFP-treated diabetic rats (G and I), and Ad-PPAR $\alpha$ -treated diabetic rats (H and J). Adherent leukocytes per retina were quantified and compared (B) (mean  $\pm$  SD,  $n = 4$ ). [Scale bars: 100  $\mu$ m (A, B, E, and F) and 25  $\mu$ m (C, D, G, and H).] (K and L) The same amount (50  $\mu$ g) of retina proteins was used for Western blot analysis of VEGF, TNF- $\alpha$ , and ICAM-1 (K). These protein levels were semiquantified with densitometry and normalized by  $\beta$ -actin levels (L) (mean  $\pm$  SD,  $n = 4$ , \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the Ad-GFP treatment group). (M and N) HRCECs were exposed to 25  $\mu$ M fenofibrate, with DMSO as control or infected with Ad-PPAR $\alpha$  at multiplicity of infection (MOI) of 20, with Ad-GFP as control, for 24 h and then subjected to in vitro scratch-wound healing assay with images captured at 0 and 8 h after the scratch using phase-contrast microscope. The rate of migration was measured and expressed as % of the vehicle control (M) (mean  $\pm$  SD,  $n = 6$ ). HRCECs were treated similarly as described above for 24 h and then changed to normal culture medium for 48 h. Viable cells were quantified by trypan blue exclusion assay and expressed as % of vehicle control (N) (mean  $\pm$  SD,  $n = 4$ ).

HIF-1. These results demonstrate that PPAR $\alpha$  is down-regulated by chronic diabetes stressors but not by acute ischemia. Furthermore, PPAR $\alpha$  KO mice with OIR did not develop more severe retinal NV, compared with OIR WT mice (Fig. S6). Taken together, these observations suggest that acute hypoxia alone is not sufficient to lead to down-regulation of PPAR $\alpha$  under diabetic conditions. The mechanism and signaling pathways responsible for the PPAR $\alpha$  down-regulation in DR remain to be elucidated in the future.

Chronic inflammation is believed to play a key role in DR (31). Inflammation has been shown to contribute to endothelium impairment, vascular leakage, pericyte loss, and increased capillary degeneration, leading to increased acellular vessels in DR (32). Although under normal conditions PPAR $\alpha$  KO mice did not show detectable vascular phenotypes in the retina at the ages analyzed, PPAR $\alpha$  KO significantly exacerbated leukostasis and overexpression of inflammatory factors induced by diabetes, suggesting more severe diabetes-induced retinal inflammation in the absence of PPAR $\alpha$ . Consistently, PPAR $\alpha$  KO mice with diabetes showed increased retinal vascular leakage, more severe pericyte loss, and increased acellular vessels, a result of capillary degeneration induced by retinal inflammation in DR. Taken together, these observations suggest that PPAR $\alpha$  has an anti-inflammatory activity under diabetic conditions, which is responsible for its beneficial effects on DR.

Our immunohistochemical analysis in the retina and Western blot analysis in cultured retinal cells both demonstrated that PPAR $\alpha$  is expressed in multiple retinal cell types. PPAR $\alpha$  overexpression inhibited endothelial cell proliferation and migration. PPAR $\alpha$  up-regulation also suppressed expression of ICAM-1, an adhesion molecule responsible for leukocyte adherence. These results support a direct effect of PPAR $\alpha$  on vascular cells. Our results also showed that PPAR $\alpha$  suppressed expression of inflammatory factors such as TNF- $\alpha$  and VEGF in other retinal cell types, such as Müller cells and RPE cells, which are major producers of inflammatory cytokines under diabetic conditions. It is likely that diabetes stressors down-regulate PPAR $\alpha$  expression in these retinal cells, leading to increased expression and secretion of inflammatory cytokines (33–35) whereas PPAR $\alpha$  overexpression suppresses overexpression of inflammatory factors in these cells. Thus, the pathological role of PPAR $\alpha$  down-regulation in diabetic microvascular complications may be through both direct effects on vascular cells and indirect effects on vascular homeostasis, involving secretion of inflammatory cytokines from nonvascular cells. To investigate the molecular mechanism or signaling pathway by which the PPAR $\alpha$  regulates inflammation factors, we investigated the interactions of PPAR $\alpha$  with the NF- $\kappa$ B signaling pathway. The results showed that PPAR $\alpha$  overexpression significantly increased I $\kappa$ B $\alpha$  levels, while decreasing phosphorylated NF- $\kappa$ B levels in high glucose-treated retinal cells (Fig. S7 A and B). Similarly, intravitreal injection of Ad-PPAR $\alpha$  also elevated I $\kappa$ B $\alpha$  levels and decreased phosphorylated NF- $\kappa$ B levels in the retina of STZ-induced diabetic rats (Fig. S7 C and D). These observations are consistent with previous studies using PPAR $\alpha$  agonist (36). Taken together, these findings suggest that the anti-inflammatory activity of PPAR $\alpha$  is, at least in part, through inhibition of NF- $\kappa$ B signaling under diabetic stress.

PPAR $\alpha$  activity can be enhanced through two different mechanisms: increase of PPAR $\alpha$  activity (primarily via ligand binding) (37) and up-regulation of its expression. There are endogenous ligands, such as intracellular fatty acids, and synthetic ligands, such as fibrates, that are clinically used for hyperlipidemia treatment (16, 38). Recently, two independent, perspective clinical studies reported that fenofibrate, a PPAR $\alpha$  agonist, has therapeutic effects on diabetic microvascular complications (16, 17). Our recent study using diabetic animal models showed that the fenofibrate effect on DR is PPAR $\alpha$ -dependent (19). We and other groups have reported that fenofibrate has anti-inflammatory effects (4, 5, 19), consistent with the anti-inflammatory activity of PPAR $\alpha$  observed in this study. It is likely that fenofibrate induces PPAR $\alpha$  activity, which compensates for the down-regulation of PPAR $\alpha$  under diabetes conditions, leading to amelioration of retinal inflammation, a major pathogenic feature of DR. These observations suggest that PPAR $\alpha$  is a promising drug target for the treatment of retinal inflammation and vascular dysfunctions in diabetic microvascular complications.

In summary, this study suggests that diabetes-induced PPAR $\alpha$  down-regulation represents a unique pathogenic mechanism for

diabetic microvascular complications. Up-regulation or activation of PPAR $\alpha$  may become a new therapeutic strategy for DR.

## Materials and Methods

**Animals.** Care, use, and treatment of experimental animals were in strict agreement with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Male C57BL/6J mice, PPAR $\alpha$  KO mice, Akita mice, *db/db* mice, and C57BLKS/J mice (The Jackson Laboratory) and female Brown Norway rats (Charles River) were used in this study.

**Induction of Diabetes in Rats.** Diabetes was induced in female Brown Norway rats (8 wk old) by an i.p. injection of STZ (55 mg/kg in 10 mM citrate buffer, pH 4.5) as described previously (19).

**Immunohistochemistry.** Human donor eyes were obtained from the National Diseases Research Interchange with full ethical approval for use in research. Diabetic eyes were categorized and stained according to a standardized protocol (39). Rat and mouse eyes were dissected and sectioned and stained as described previously (19).

**Western Blot Analysis.** The retinas of each mouse/rat were dissected, combined, and homogenized. The equal amount (50  $\mu$ g) of total protein from each sample was used for Western blot analysis as described previously (40).

1. Michalik L, et al. (2006) International Union of Pharmacology. LXI. Peroxisome proliferator-activated receptors. *Pharmacol Rev* 58(4):726–741.
2. Berger J, Moller DE (2002) The mechanisms of action of PPARs. *Annu Rev Med* 53: 409–435.
3. Filip-Giubotaru F, Foia L, Manciuc C, Grigore C (2011) PPARs: Structure, mechanisms of action and control. Note I]. *Rev Med Chir Soc Med Nat Iasi* 115(2):477–484.
4. Cuzzocrea S, et al. (2006) The role of the peroxisome proliferator-activated receptor-alpha (PPAR-alpha) in the regulation of acute inflammation. *J Leukoc Biol* 79(5): 999–1010.
5. Duval C, Fruchart JC, Staels B (2004) PPAR alpha, fibrates, lipid metabolism and inflammation. *Arch Mal Coeur Vaiss* 97(6):665–672.
6. Kasai T, Miyauchi K, Yokoyama T, Aihara K, Daida H (2006) Efficacy of peroxisome proliferative activated receptor (PPAR)-alpha ligands, fenofibrate, on intimal hyperplasia and constrictive remodeling after coronary angioplasty in porcine models. *Atherosclerosis* 188(2):274–280.
7. Issemann I, Green S (1990) Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* 347(6294):645–650.
8. Dreyer C, et al. (1992) Control of the peroxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors. *Cell* 68(5):879–887.
9. Miyachi H, Uchiki H (2003) Analysis of the critical structural determinant(s) of species-selective peroxisome proliferator-activated receptor alpha (PPAR alpha)-activation by phenylpropanoic acid-type PPAR alpha agonists. *Bioorg Med Chem Lett* 13(19): 3145–3149.
10. Liu J, et al. (2011) PPAR- $\alpha$  agonist fenofibrate upregulates tetrahydrobiopterin level through increasing the expression of guanosine 5'-triphosphate cyclohydrolase-I in human umbilical vein endothelial cells. *PPAR Res* 2011:523520.
11. Castillero E, et al. (2011) Fenofibrate, a PPARalpha agonist, decreases atrogenes and myostatin expression and improves arthritis-induced skeletal muscle atrophy. *Am J Physiol Endocrinol Metab* 300(5):E790–E799.
12. Hanssen KF (1997) Blood glucose control and microvascular and macrovascular complications in diabetes. *Diabetes* 46(Suppl 2):S101–S103.
13. Maji D (2004) Prevention of microvascular and macrovascular complications in diabetes mellitus. *J Indian Med Assoc* 102(8):426–, 428, 430 passim.
14. Hiukka A, Maranghi M, Matikainen N, Taskinen MR (2010) PPARalpha: An emerging therapeutic target in diabetic microvascular damage. *Nat Rev Endocrinol* 6(8): 454–463.
15. Tandon N, Ali MK, Narayan KM (2012) Pharmacologic prevention of microvascular and macrovascular complications in diabetes mellitus: Implications of the results of recent clinical trials in type 2 diabetes. *Am J Cardiovasc Drugs* 12(1):7–22.
16. Keech AC, et al.; FIELD study investigators (2007) Effect of fenofibrate on the need for laser treatment for diabetic retinopathy (FIELD study): A randomised controlled trial. *Lancet* 370(9600):1687–1697.
17. Ginsberg HN, et al.; ACCORD Study Group (2010) Effects of combination lipid therapy in type 2 diabetes mellitus. *N Engl J Med* 362(17):1563–1574.
18. Park CW, et al. (2006) Accelerated diabetic nephropathy in mice lacking the peroxisome proliferator-activated receptor alpha. *Diabetes* 55(4):885–893.
19. Chen Y, et al. (2013) Therapeutic effects of PPAR $\alpha$  agonists on diabetic retinopathy in type 1 diabetes models. *Diabetes* 62(1):261–272.
20. Goetze S, et al. (2002) PPAR activators inhibit endothelial cell migration by targeting Akt. *Biochem Biophys Res Commun* 293(5):1431–1437.
21. Barak Y, Sadovsky Y, Shalom-Barak T (2008) PPAR signaling in placental development and function. *PPAR Res* 2008:142082.

**Quantitative Real-Time Reverse Transcription-PCR.** Total RNA was isolated from the retina using TRIzol according to the manufacturer's protocol (Invitrogen). The RNA was used for reverse-transcription (RT) and amplified by quantitative real-time PCR as described previously (40).

**Retinal Vascular Permeability Assay.** Retinal vascular permeability was quantified using the Evans blue-albumin leakage method as described previously (19).

**Retina Trypsin Digestion Assay.** Trypsin digestion of the retina was performed following the method of Cogan and Kuwabara (41) with modifications (42).

**Retina Leukostasis Assay.** The leukostasis assay was performed by staining adherent leukocytes in the vasculature in flat-mounted retina as described previously (19).

**Statistical Analysis.** All of the values in the results were expressed as mean  $\pm$  SD. Statistical analyses were performed using the Student t test.  $P < 0.05$  was considered statistically significant.

**ACKNOWLEDGMENTS.** This study was supported by National Institutes of Health Grants EY018659, EY012231, EY019309, EY018358, and P20GM104934, and by American Diabetes Association Grant 7-11JF-10.

22. Takeyama K, Kodera Y, Suzawa M, Kato S (2000) [Peroxisome proliferator-activated receptor(PPAR)—structure, function, tissue distribution, gene expression]. *Nihon Rinsho* 58(2):357–363.
23. Kawada T (1998) [Lipid metabolism related nuclear receptor—the structure, function, expression and classification of peroxisome proliferation-activated receptor (PPAR)]. *Nihon Rinsho* 56(7):1722–1728.
24. Wang F, et al. (2008) Tissue-specific expression of PPAR mRNAs in diabetic rats and divergent effects of cilostazol. *Can J Physiol Pharmacol* 86(7):465–471.
25. Hegarty BD, Furler SM, Oakes ND, Kraegen EW, Cooney GJ (2004) Peroxisome proliferator-activated receptor (PPAR) activation induces tissue-specific effects on fatty acid uptake and metabolism in vivo—a study using the novel PPARalpha/gamma agonist tesaglitazar. *Endocrinology* 145(7):3158–3164.
26. Araujo CV, et al. (2012) PPAR gamma activation protects the brain against microvascular dysfunction in sepsis. *Microvasc Res* 84(2):218–221.
27. Madonna R, et al. (2011) Omega-3 fatty acids attenuate constitutive and insulin-induced CD36 expression through a suppression of PPAR  $\alpha/\gamma$  activity in microvascular endothelial cells. *Thromb Haemost* 106(3):500–510.
28. Biscetti F, et al. (2008) Selective activation of peroxisome proliferator-activated receptor (PPAR)alpha and PPAR gamma induces neoangiogenesis through a vascular endothelial growth factor-dependent mechanism. *Diabetes* 57(5):1394–1404.
29. Hsueh WA, Jackson S, Law RE (2001) Control of vascular cell proliferation and migration by PPAR-gamma: A new approach to the macrovascular complications of diabetes. *Diabetes Care* 24(2):392–397.
30. Narayana S, Colgan SP (2001) Hypoxia-inducible factor 1-mediated inhibition of peroxisome proliferator-activated receptor alpha expression during hypoxia. *J Immunol* 166(12):7543–7548.
31. Fujita T, et al. (2013) Complement-mediated chronic inflammation is associated with diabetic microvascular complication. *Diabetes Metab Res Rev* 29(3):220–226.
32. Tang J, Kern TS (2011) Inflammation in diabetic retinopathy. *Prog Retin Eye Res* 30(5): 343–358.
33. Shi G, et al. (2008) Control of chemokine gradients by the retinal pigment epithelium. *Invest Ophthalmol Vis Sci* 49(10):4620–4630.
34. Eichler W, Yafai Y, Wiedemann P, Reichenbach A (2004) Angiogenesis-related factors derived from retinal glial (Müller) cells in hypoxia. *Neuroreport* 15(10):1633–1637.
35. Holtkamp GM, Kijlstra A, Peek R, de Vos AF (2001) Retinal pigment epithelium-immune system interactions: Cytokine production and cytokine-induced changes. *Prog Retin Eye Res* 20(1):29–48.
36. Okayasu T, Tomizawa A, Suzuki K, Manaka K, Hattori Y (2008) PPARalpha activators upregulate eNOS activity and inhibit cytokine-induced NF-kappaB activation through AMP-activated protein kinase activation. *Life Sci* 82(15-16):884–891.
37. Hertz R, Bar-Tana J (1998) Peroxisome proliferator-activated receptor (PPAR) alpha activation and its consequences in humans. *Toxicol Lett* 102-103:85–90.
38. Varet J, et al. (2003) Fenofibrate inhibits angiogenesis in vitro and in vivo. *Cell Mol Life Sci* 60(4):810–819.
39. Chen Y, et al. (2009) Activation of the Wnt pathway plays a pathogenic role in diabetic retinopathy in humans and animal models. *Am J Pathol* 175(6):2676–2685.
40. Hu Y, et al. (2013) Pathogenic role of the Wnt signaling pathway activation in laser-induced choroidal neovascularization. *Invest Ophthalmol Vis Sci* 54(1):141–154.
41. Cogan DG, Kuwabara T (1984) Comparison of retinal and cerebral vasculature in trypsin digest preparations. *Br J Ophthalmol* 68(1):10–12.
42. Michael JC, De Venecia G (1995) Retinal trypsin digest study of cystoid macular edema associated with peripheral choroidal melanoma. *Am J Ophthalmol* 119(2):152–156.