Effects of Peroxisome Proliferator-Activated Receptor γ and Its Ligand on Blood–Retinal Barrier in a Streptozotocin-Induced Diabetic Model

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Purpose. To clarify whether endogenous peroxisome proliferator-activated receptor γ (PPARγ) and its ligand, rosiglitazone, affect retinal leukostasis and the associated vascular leakage using an experimental diabetic model.

Methods. Diabetes was induced in heterozygous PPARγ$^{+/-}$ mice and Brown Norway rats with an intraperitoneal streptozotocin (STZ) injection. Retinal leukostasis and leakage, quantified by concanavalin A (Con A) lectin perfusion labeling combined with a fluorophotometric dextran leakage assay, were investigated at 120 days in diabetic PPARγ$^{+/-}$ and wild-type mice and at 21 days in diabetic rats receiving rosiglitazone or the vehicle. The retinal protein expression levels of vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF)-α, and the intercellular adhesion molecule (ICAM)-1 were investigated by means of the ELISA assay.

Results. In the diabetic PPARγ$^{+/-}$ mice, retinal leukostasis and leakage were greater than in the diabetic wild-type mice. In addition retinal leukostasis and leakage were suppressed by treatment with rosiglitazone in experimental diabetic rats. ELISA analysis revealed that the upregulated ICAM-1 expression in the diabetic rat retina was reduced by rosiglitazone treatment.

Conclusions. An endogenous pathway involving PPARγ provides protection against retinal leukostasis and retinal leakage in diabetes and treatment with PPARγ specific ligands inhibits retinal leukostasis and retinal leakage in diabetic rats. (*Invest Ophthalmol Vis Sci.* 2006;47:4547–4552) DOI:10.1167/iovs.05-1432

Diabetic retinopathy is a leading cause of acquired visual impairment in developed countries[1] and is frequently complicated by macular edema. Macular edema can appear at any time during the course of diabetic retinopathy and is a major cause of vision loss in diabetic patients.[2] It is a direct consequence of diabetic blood–retinal barrier breakdown.[3] Much effort has been directed toward establishing effective treatments; however, there is no satisfactory pharmacological treatment for this diabetic complication.[2]

The retinal vessels have a barrier consisting of the tight junction of the retinal pigment epithelium and the retinal vascular endothelium. Each barrier exhibits increased permeability in experimental diabetes models[4] and human diabetic retinopathy.[5] Endothelial cell damage and capillary nonperfusion are aggravated by enhanced retinal vascular permeability.[6] In recent studies, inflammation has been linked to vascular leakage in diabetic retinopathy, at least in part.[7,8] Biological support for the role of inflammation in early diabetes is that the adhesion of leukocytes to the retinal vasculature (leukostasis) has been observed in both humans[9] and rats.[10] Increased adhesion of leukocytes to the retinal vasculature promotes vascular leakage. Blockage of the bioactivity of adhesion molecules, such as the intercellular adhesion molecule (ICAM)-1, leads to decreased retinal leukocyte adhesion and reduced vascular leakage.[11]

PPARγ is a member of a ligand-activated nuclear receptor superfamily and plays a critical role in a variety of biological processes, including adipogenesis, glucose metabolism, angiogenesis, and inflammation.[12] Synthetic ligands of PPARγ (i.e., thiazolidine derivatives, such as rosiglitazone and pioglitazone), are used as oral antihyperglycemic agents for the therapy of non–insulin-dependent diabetes mellitus. In addition, recent studies have shown that PPARγ ligands modulate the production of inflammatory mediators. In fact, it has been reported that PPARγ ligands, such as rosiglitazone and pioglitazone, suppress inflammatory diseases such as adjuvant-induced arthritis.[13] Considering the close link between inflammation and diabetes, PPARγ ligand therapy may also improve diabetic retinopathy. However, thus far the role of PPARγ ligands and their receptor systems have not been studied in the diabetic retina.

In this study, we investigated the effects of a synthetic PPARγ ligand, rosiglitazone, in an experimental diabetic model. In addition, we used heterozygous PPARγ-deficient mice to determine whether endogenous PPARγ plays a role. This investigation provides strong evidence to support the theory that PPARγ activity plays an central role in the pathogenesis of diabetic retinopathy and introduces the novel possibility that the therapeutic targeting of PPARγ may be beneficial in preventing the disease.

MATERIALS AND METHODS

Animals

C57BL/6 mice weighing between 20 and 25 g were obtained from CLEA Japan (Tokyo, Japan). Mice lacking one allele of PPARγ (heterozygous PPARγ-deficient mice [PPARγ$^{+/-}$]) were essentially as has been previously described.[14] As homozygous PPARγ-deficient embryos (−/−) have been reported to have died of placental dysfunction,[14] only heterozygous (PPARγ$^{+/-}$) mice were used in this study.

Male Brown Norway (BN) rats weighing between 100 and 200 g were obtained from CLEA Japan. All experiments were conducted in accordance with the Animal Care and Use Committee guidelines and

the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Experimental Diabetes

After a 24-hour fast, diabetes was induced with a single 60-mg/kg intraperitoneal injection of streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO) in 10 mM citrate buffer (pH 4.5). Animals that served as the nondiabetic control received an equivalent amount of citrate buffer alone. The mice and rats were bred in an air-conditioned room with a 12-hour light–dark cycle until they were used in the experiments. They were fed with standard laboratory chow and allowed free access to 4% glucose water. Seven days later, mice and rats with blood glucose levels over 250 mg/dL were deemed diabetic. Thereafter, the animals were fed with standard laboratory chow and distilled water. Before the retinal leukostasis and leakage assays, blood glucose levels were measured again to confirm diabetic status. A Retinal leukostasis quantification and a retinal leakage assay were performed 21 and 120 days after the STZ-injection in mice and rats, respectively.

Administration of Rosiglitazone to Experimental Diabetic Rats

Rosiglitazone (3 mg/kg), a PPARγ-specific agonist (the kind gift of Glaxo Smith Kline; West Sussex, UK) was administered orally to rats once daily, from 24 hours after the injection of the STZ. The vehicle alone (distilled water) was administered as a control. The dose was chosen based on a previous study.[15]

Quantification of Retinal Leukostasis

The retinal vasculature and adherent leukocytes were imaged with fluorescein-isothiocyanate (FITC) or rhodamine-coupled concanavalin A (Con A) lectin (40 μg/mL in PBS [pH 7.4], 5 mg/kg body weight [BW]; Vector Laboratories, Burlingame, CA), as has been described previously.[6] Animals were deeply anesthetized with intramuscular xylazine hydrochloride and ketamine hydrochloride. The chest cavity was opened, and a 14-gauge perfusion cannula was introduced into the aorta. After achieving drainage from the right atrium, the animals were perfused with 500 mL/kg body weight (BW) of PBS. Perfusion with Con A was then performed to label adherent leukocytes and vascular endothelial cells, followed by the removal of residual unbound lectin with a PBS perfusion. The retinas were removed and flatmounted in a mounting medium for fluorescence studies (Vector Laboratories). Flatmounts were examined by fluorescence microscopy (model DP50; Olympus, Tokyo, Japan), and the total number of adherent leukocytes per retina was determined in a masked manner.

Retinal Leakage Assay

After deep anesthesia with intramuscular xylazine hydrochloride and ketamine hydrochloride, FITC-conjugated dextran (4.4 kDa, 50 mg/mL in PBS, 50 mg/kg BW; Sigma-Aldrich) was injected intravenously. After 10 minutes, the chest cavity was opened, and a 14-gauge perfusion cannula was introduced into the aorta. A blood sample was collected immediately before perfusion. After drainage from the right atrium was completed, each animal was perfused with PBS (500 mL/kg BW) to clear the remaining intravascular dextran. The blood sample was centrifuged at 7000 rpm for 20 minutes at 4°C, and the supernatant was diluted at 1:1000. After perfusion, the retinas were removed, weighed,

and homogenized to extract the FITC-dextran in 0.4 mL of PBS. The extract was processed through a 30,000 molecular weight filter (Ultrafree-MC; Millipore, Bedford, MA) at 7000 rpm for 90 minutes at 4°C. The fluorescence in each 300-μL sample was measured (excitation 485 nm, emission 538 nm) using a spectrofluorometer (ARVO SX; Perkin Elmer, Wellesley, MA) with PBS as a blank. Corrections were made by subtracting the autofluorescence of retinal tissue from animals not injected with the FITC-dextran. The amount of FITC-dextran in each retina was calculated from a standard curve of FITC-dextran in PBS. For normalization, the retinal FITC-dextran amount was divided by the retinal weight and by the concentration of FTTC-dextran in the plasma. Retinal leakage was calculated using the following equation[16,17]:

$$\text{Retinal leakage} = \frac{\text{Retinal FITC} - \text{dextran } (\mu g)/\text{retinal weight } (g)}{\text{Plasma FITC} - \text{dextran concentration } (\mu g/\mu L)}$$
$$\cdot \text{circulation time (h)}$$

Plasma Lipid Measurements

Mice were fasted overnight, and blood was collected through retroorbital veins with mice under isoflurane anesthesia. Plasma total cholesterol, HDL cholesterol, and triglyceride levels were measured by enzymatic assays, with a kit used according to the manufacturer's protocol (Takara Bio, Tokyo, Japan).

Enzyme-Linked Immunosorbent Assay

The retina was carefully isolated and placed into 150 μL of RIPA buffer (20 mM Tris-HCl [pH 7.4], SDS 0.1%, Triton X-100 1%, and sodium deoxycholate 1%) supplemented with a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and sonicated. The lysate was centrifuged at 14,000 rpm for 15 minutes at 4°C and the protein levels, vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF)-α, and ICAM-1 in the supernatant were determined with an ELISA assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Absorbance at 450 nm was measured using a microplate reader (model 3550; Bio-Rad, Hercules, CA). The protein levels were normalized by the total protein content, as determined by a bicinchoninic acid (BCA) kit (Bio-Rad).

Immunohistochemistry

Eyes of the animals were immediately enucleated, immersed in 4% paraformaldehyde for 12 hours, transferred into 70% ethanol, and processed for paraffin embedding. Once embedded, 4.0-μm sections of tissue were prepared for immunostaining. For immunohistochemistry, slides were deparaffinized and incubated in blocking solution (PBS containing 0.1% BSA and 2% calf serum) for 30 minutes, followed by an overnight incubation with mouse anti-ICAM-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100 in the blocking solution. Negative control slides were made by omitting the primary antibody from the reaction. A standard immunoperoxidase procedure was performed with a kit (Histofine; Nichirey, Tokyo, Japan) and an alkaline phosphatase substrate (Vector Red; Vector Laboratories), which reacts with peroxidase to give a red reaction product.

Statistical Analyses

All results are expressed as the mean $\pm$ SD. The values were processed for statistical analyses using the *t*-test. $P < 0.05$ was considered statistically significant.

TABLE 1. Blood Glucose Levels of Nondiabetic Control (Rat and Wild-Type Mice), 3-Week Diabetic Rats, and 120-Day Diabetic Mice

	Nondiabetic Rats	Diabetic Rats		Nondiabetic Wild-Type Mice	Diabetic Mice	
		Vehicle	Rosiglitazone		Wild-Type	PPARg$^{+/-}$
n	4	6	12	9	6	6
Blood glucose (mg/mL)	114 $\pm$ 27	483 $\pm$ 129	515 $\pm$ 145	117 $\pm$ 38	289 $\pm$ 30	327 $\pm$ 63

Data represent mean glucose levels as measured twice in rats (days 7 and 21) and three times in mice (days 7 and 14 and before death).

TABLE 2. Plasma Lipid Measurements and White Blood Cell Counts

	DM(−)	DM(−)	DM(+)	DM(+)
Mice				
Genotype	Wild-type	PPAR$^{+/-}$	Wild-type	PPAR$^{+/-}$
n	10	10	5	3
TG	100 ± 22	117 ± 20	127 ± 86.2	121 ± 28.3
NEFA	0.879 ± 0.262	0.854 ± 0.164	1.39 ± 0.776	1.325 ± 0.276
T-cho	73.2 ± 9.76	72.3 ± 7.61	68.2 ± 25	73.3 ± 19.2
Hb(g/dL)	ND	ND	11.8 ± 2.12	13.6 ± 3.47
RBC (×10,000/mm^3)	ND	ND	757 ± 180	854 ± 238
WBC (cells/μL)	ND	ND	2550 ± 1910	2400 ± 361
Rats				
Rosiglitazone treatment	−	+	−	+
n	4	6	5	6
TG	63.5 ± 23.3	74.3 ± 21.2	102 ± 18.5	71.68 ± 28.6
T-cho	42.9 ± 3.35	43.8 ± 7.19	47.6 ± 7.87	35.42 ± 9.69
NEFA	0.818 ± 0.344	0.752 ± 0.14	0.693 ± 0.136	0.522 ± 0.202
Hb(g/dL)	14.5 ± 1.88	14.3 ± 1.16	16 ± 0.692	13.8 ± 1.08
RBC (×10,000/mm^3)	700 ± 78.3	677 ± 48.3	794 ± 57.5	690.4 ± 34.6
WBC (cells/μL)	4430 ± 3020	4780 ± 2740	6720 ± 2030	7080 ± 3510

DM, diabetes mellitus.

RESULTS

Induction of Diabetes and Blood Glucose Levels

Table 1 represents blood glucose levels as measured twice in the rats (days 7 and 21) and three times in the mice (days 7 and 14 and before death). In the mice, the blood glucose level was upregulated by STZ-treatment both in the wild-type and PPARγ$^{+/-}$ mice. The blood glucose levels of the diabetic PPARγ$^{+/-}$ mice were not different from those of the diabetic wild-type mice (Table 1). Similarly, in the rats, diabetes was induced by STZ-treatment. The blood glucose levels from the rosiglitazone-treated rats were no different from those without rosiglitazone treatment (Table 1). The results of plasma lipid measurements and white blood cell counts are shown in Table 2. Overall, there was no difference in the plasma lipid in the wild-type and PPAR$^{+/-}$ mice. The plasma lipid levels from the nondiabetic mice treated with rosiglitazone were not different from those of the nondiabetic rats without treatment. In the diabetic rats, triglyceride, total cholesterol and nonesterified fatty acid (NEFA) levels were higher than in the nondiabetic rats and decreased after rosiglitazone treatment. In the diabetic rats, the number of white blood cells was higher than in the nondiabetic rats, and rosiglitazone treatment did not affect the white blood cell counts.

Effect of PPARγ Deficiency on Retinal Leukostasis and Retinal Leakage in Mice

To investigate the effects of endogenous PPARγ, leukostasis and retinal leakage, were measured in PPAR$^{+/-}$ mice at 120 days after STZ treatment. Increased retinal leukostasis was observed after STZ treatment in mice, similar to previous studies.[18] In the diabetic PPARγ$^{+/-}$ mice, the retinal leukostasis was significantly upregulated (2.1 times the levels in the diabetic wild-type mice). Similarly, the STZ-treatment caused an increase in retinal leakage that was 1.9 times greater in the diabetic PPARγ$^{+/-}$ mice than in the diabetic wild-type mice (Fig. 1).

Effects of Rosiglitazone on Retinal Leukostasis and Retinal Leakage in Rats

The results led us to examine the effects of the administration of the PPARγ agonist rosiglitazone on leukostasis and leakage as analyzed at 21 days after the STZ injection. As in the mice, retinal leukostasis was induced in the rats by STZ treatment. Retinal leukostasis decreased 0.6-fold with rosiglitazone treatment, and retinal leakage increased by 250% with STZ-treatment. As expected, retinal leakage decreased 0.6-fold in the diabetic rats with rosiglitazone treatment (Fig. 2).
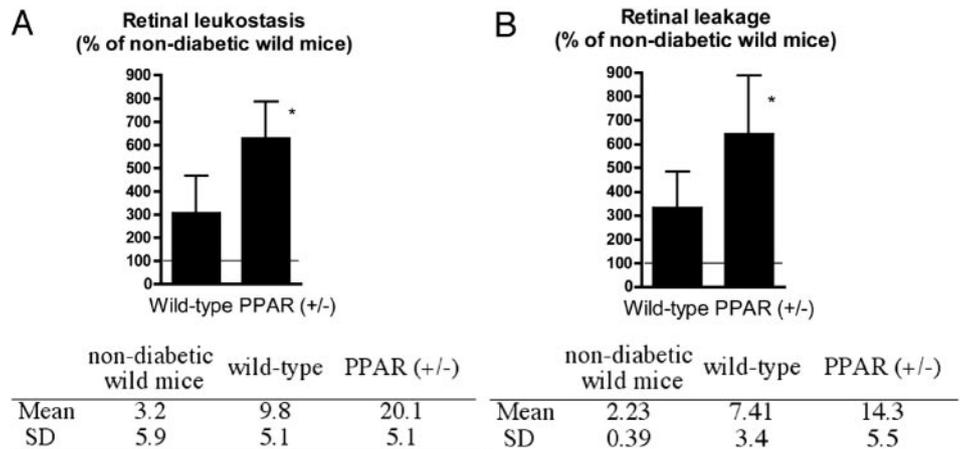

FIGURE 1. Increase of retinal leukostasis and leakage in PPARγ$^{+/-}$ mice in diabetes. (**A, B**) Retinal leukostasis and leakage was 205.1% (**A**) and 191.0% (**B**) greater in PPARγ$^{+/-}$ mice than in wild-type mice. Graphs show the average; error bars, SD ($n = 6$ in each group). *$P < 0.05$ versus wild-type mice.
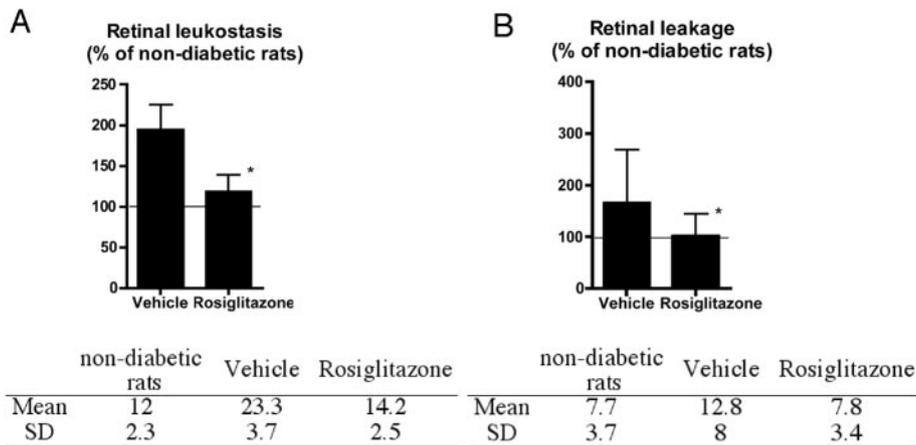
A Retinal leukostasis (% of non-diabetic wild mice)

	non-diabetic wild mice	wild-type	PPAR (+/-)
Mean	3.2	9.8	20.1
SD	5.9	5.1	5.1

B Retinal leakage (% of non-diabetic wild mice)

	non-diabetic wild mice	wild-type	PPAR (+/-)
Mean	2.23	7.41	14.3
SD	0.39	3.4	5.5

FIGURE 2. Suppression of diabetic retinal leukostasis and leakage by administration of rosiglitazone in rats. (**A**, **B**) Compared with the vehicle, rosiglitazone treatment resulted in (**A**) 60.9% suppression of retinal leukostasis and (**B**) 60.8% suppression of retinal leakage. Graphs show the average; error bars, SD ($n = 6$ in each group). *$P < 0.05$ versus non-treated control.

Expression Level of ICAM-1 in the Rosiglitazone-Treated Rats

To examine the underlying mechanism, the effects of rosiglitazone treatment on the expression of proinflammatory cytokines (i.e., VEGF and TNF-α) and an adhesion molecule, ICAM-1, were examined in the retina using ELISA, which demonstrated that expression levels of VEGF and TNF-α protein were not affected by the rosiglitazone-treated rats (data not shown). However, in the retina of the rosiglitazone-treated rats, the upregulated ICAM-1 expression was significantly suppressed by rosiglitazone treatment (Fig. 3). Immunohistochemical analysis revealed ICAM-1 was expressed in the endothelial cells, similar to a previous study.[19] In the retina of the diabetic rats, the ICAM-1 was expressed at higher levels than in the nondiabetic rats and the expression of ICAM-1 was lower in the diabetic rat retina treated with rosiglitazone (Fig. 4).

Discussion

In this study, the decreased expression of the endogenous PPARγ in mice led to aggravation of retinal leukostasis and retinal leakage in diabetic mice, suggesting that PPARγ plays an intrinsic role in diabetic retinopathy. Furthermore, an inhibitory effect of the PPARγ agonist rosiglitazone was shown on both retinal leukostasis and retinal leakage in experimental diabetic rats. Together, these findings support the theory that the PPARγ signaling pathway inhibits diabetes-induced retinal leukostasis and leakage. This is the first report showing the involvement of PPARγ and its ligand on retinal leukostasis and leakage in vivo, suggesting that PPARγ ligand is related to diabetic retinopathy.

Previous studies have shown that VEGF, a vasopermeability factor, and inflammatory cytokines, such as ICAM-1, increase leukostasis and vascular permeability and play major roles in the progression of diabetic retinopathy.[20,21] ICAM-1 and leukocyte integrin CD18 are upregulated during diabetic retinopathy, and VEGF drives the upregulation of retinal ICAM-1.[20,21] CD18$^{-/-}$ and ICAM-1$^{-/-}$ mice demonstrate significantly fewer adherent leukocytes in the retinal vasculature after induction of diabetes with STZ.[22] In the present experiments, neither retinal VEGF nor TNF-α levels, which were upregulated in the STZ-induced diabetic rat, were significantly affected by administration of rosiglitazone. However, PPARγ ligand suppressed ICAM-1 expression, similar to observations in a murine model of intestinal ischemia–reperfusion injury[23] and in human umbilical vein endothelial cells in vitro.[24] In addition, PPARγ has an anti-inflammatory effect mediated through the inhibition of NFκ-B activation.[25] As the expression level of ICAM-1 is controlled by NFκ-B,[26] we suspect one possible mechanism by which PPARγ controls retinal leukostasis and retinal leakage is mediated by NFκ-B.

Elevated serum lipid levels, and protein levels of inflammatory cytokines, chemokines, and adhesion molecules are related to the severity of diabetic retinopathy, suggesting that systemic factors including inflammation influence the severity of retinopathy.[27–33] PPARγ is expressed by macrophages and other cell types that influence inflammation.[34–37] Although further studies are necessary, it is tempting to speculate that
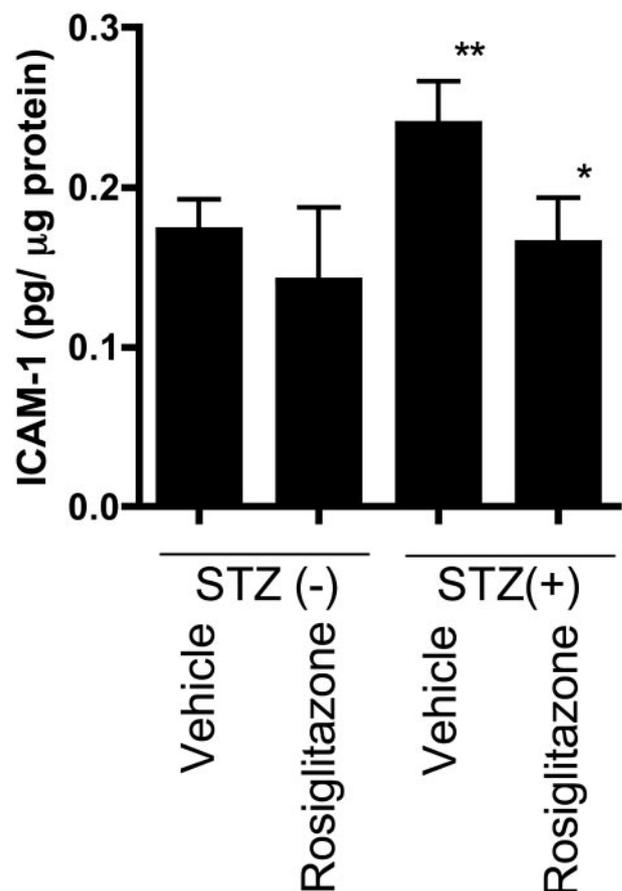

FIGURE 3. Effects of rosiglitazone on the expression of ICAM-1 protein in diabetic rat retinas. Graphs show the average; error bars, SD ($n = 6$ in each group). **$P < 0.05$ versus nontreated control; *$P < 0.05$ versus nondiabetic control.

FIGURE 4. Immunohistochemical analysis of ICAM-1 expression in diabetic rat retinas. (**A**) Nondiabetic rat; (**B**) nondiabetic rat treated with rosiglitazone; (**C**) diabetic rat; and (**D**) diabetic rat treated with rosiglitazone. Normal retina expressed detectable levels of ICAM-1 protein in the inner plexiform layer. Note that in the retina of the diabetic rats, the ICAM-1 was expressed at a higher level than in the nondiabetic rats, and the expression of ICAM-1 was lower in the diabetic rat retina treated with rosiglitazone.
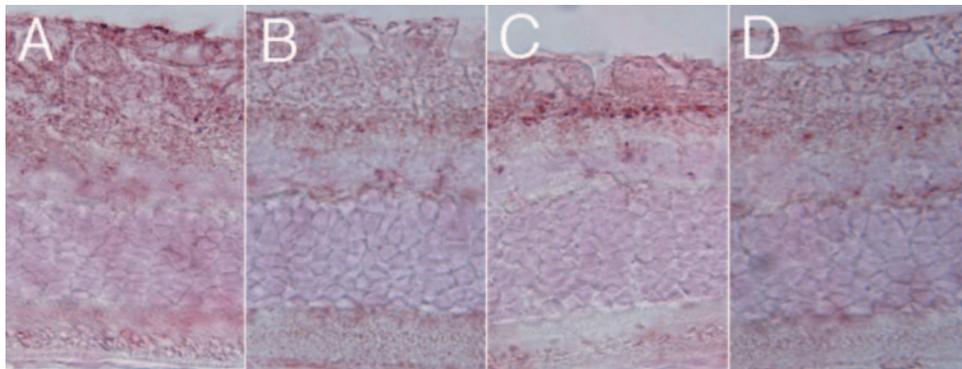
the action of PPARγ is mediated through systemic effects, possibly by modulating macrophages and other cells modulating immune reaction.

A study has shown that the administration of nonsteroidal anti-inflammatory drugs, such as aspirin, the cyclooxygenase-2 inhibitor meloxicam, or the TNF-α inhibitor eternacept, resulted in a 38% to 52% suppression of retinal leukostasis in experimental diabetic rat venules.[8] An intravitreal injection of angiostatin, a proteolytic fragment of plasminogen, decreased retinal leakage to approximately 70%.[26] Others have demonstrated that with an intraperitoneal injection of anti-ICAM-1 mAb, retinal leukostasis and leakage decreased to 48.5% and 85.6%, respectively.[19] In the present study, the administration of rosiglitazone resulted in a 60.9% suppression of retinal leukostasis and a 60.8% suppression of retinal leakage. Considering these results, it can be suggested that rosiglitazone may have a more potent suppressive effect of leukostasis and leakage than other anti-inflammatory drugs and ICAM-1 inhibitors.

In summary, In the present study an endogenous pathway involving PPARγ provided potent protection against retinal leukostasis and retinal leakage in diabetes, and treatment with a PPARγ-specific ligand inhibited retinal leukostasis and retinal leakage in diabetic rats. Given these findings, therapy with PPARγ ligands may inhibit retinal leukostasis and retinal leakage in diabetes.

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