Response of Experimental Retinal Neovascularization to Thiazolidinediones

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Objective: To determine the effect of thiazolidinediones (TZDs) on experimental retinal neovascularization.

Methods: The ability of the TZDs troglitazone and rosiglitazone to inhibit retinal endothelial cell (REC) proliferation, migration, tube formation, and signaling was determined in response to vascular endothelial growth factor (VEGF). In vivo studies were performed using the oxygen-induced ischemia model of retinal neovascularization. Neonatal mice were treated with intravitreous injection of 0.5 µL of troglitazone (100 µmol/L) or rosiglitazone maleate (100 µmol/L), or vehicle, and retinal neovascularization was assayed qualitatively and quantitatively by means of angiography and histological examination.

Results: Expression of the TZD receptor, peroxisome proliferator-activated receptor γ, was confirmed in RECs by means of Western immunoblotting. Rosiglitazone and troglitazone inhibited VEGF-induced migration (P<.05), proliferation (P<.05), and tube formation (P<.01) by RECs in vitro beginning at 10 µmol/L. Rosiglitazone and troglitazone inhibited phosphorylation of extracellular signal-regulated mitogen-activated protein kinase 1 in RECs. Intravitreous injection of rosiglitazone or troglitazone inhibited development of retinal neovascularization (P<.01) but did not significantly inhibit VEGF overexpression in the ganglion cell layer of the ischemic retina.

Conclusion: The TZDs inhibit experimental retinal neovascularization with an effect that is primarily downstream of VEGF expression.

Clinical Relevance: The TZDs are widely prescribed and should be evaluated for their potential to inhibit the progression of diabetic retinopathy.


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Diabetic retinopathy is a major cause of blindness in the United States.1 Almost all patients in whom diabetes begins before 30 years of age show some evidence of retinopathy 20 years later, which is not surprising, given the finding that the risk for retinopathy is directly related to the degree and duration of hyperglycemia.2 In those patients in whom diabetes develops after 30 years of age, retinopathy is less frequent but may be the first manifestation of the disease.3 Proliferative diabetic retinopathy (PDR) is the most sight-threatening form of diabetic retinopathy and is characterized by retinal neovascularization. The growth of new vessels on the surface of the retina in PDR can be correlated with the presence of retinal ischemia and increased local expression of vascular endothelial growth factor (VEGF).4,9 Retinal endothelial cells (RECs) have been shown to express VEGF receptors, and endothelial cells respond to VEGF by activation of multiple signaling molecules, including phospholipase C γ, phosphatidylinositol 3-kinase, protein kinase C, and the extracellular signal-regulated mitogen-activated protein kinase (ERK-MAPK) pathway.10-13

A major effort has been made to develop novel therapeutic agents to inhibit retinal neovascularization. Experimental oxygen-induced retinopathy is commonly used in these studies as a model for PDR.14,15 In this model, VEGF is upregulated in the inner retina after neonatal mice exposed to hyperoxic conditions are returned to room air, resulting in the development of consistent retinal neovascularization. Several approaches have met with success in this and other ischemic model systems, including those that inhibit VEGF by means of soluble receptor or antibody16,17 and those that inhibit VEGF receptor activation18 or its downstream signaling19; however, none of these have been proven efficacious in humans with PDR.

We considered the possibility that a class of drugs currently in use for the primary treatment of diabetes may also inhibit neovascular complications of diabetes independent of their effects on blood glucose level, thus potentially decreasing the risk for PDR for these patients. Thiazolidinediones (TZDs), including trogli-
tazone, rosiglitazone maleate, and pioglitazone hydrochloride, constitute a novel class of drugs that can be used to improve insulin resistance in type 2 diabetes. Two members of this class (rosiglitazone and pioglitazone) are currently approved for clinical use. The TZDs are synthetic high-affinity ligands for the nuclear peroxisome proliferator-activated receptor γ (PPAR-γ). Expression of PPAR-γ is most abundant in adipose tissue, where it promotes adipocyte differentiation and regulates the expression of genes involved in fatty acid metabolism; however, recent studies have shown a more widespread distribution of PPAR-γ, suggesting that it may affect other tissues. Recently, we and others have demonstrated the expression of PPAR-γ on endothelial cells and have shown antiangiogenic effects in corneal and choroidal experimental systems.

In the present study, we show that RECs express PPAR-γ protein and that TZDs inhibit angiogenic responses to VEGF in vitro. We then show that 2 TZDs inhibit the development of retinal neovascularization in the oxygen-induced retinopathy model in mice. To further define the mechanism of this effect, we examined the effect of TZDs on expression of VEGF in vivo and VEGF-induced ERK-MAPK phosphorylation in vitro.

RESULTS

EXPRESSION AND SUBCELLULAR LOCALIZATION OF PPAR-γ IN RECs

To detect the expression of PPAR-γ protein in bovine RECs, we performed Western immunoblotting of nuclear
preretinal angiogenesis and normal retinal vascular channels. Immunohistochemical analysis for VEGF (sc-507; Santa Cruz Biotechnology) was used to detect its overexpression in the hypoxic retina. Immunoperoxidase detection was performed by the avidin-biotin complex method with 3-aminobenzidine as the red chromogen. Negative controls included omission of primary antibody and use of an irrelevant primary antibody of the same isotype.

**p44/p42 ERK-MAPK PHOSPHORYLATION IN RECs**

The RECs were seeded into 6 multiwell plates in endothelial growth medium (Clonetics, Walkerville, Md). Confluent RECs were starved in Dulbecco’s Modified Eagle Medium (DMEM; Cellgro, Herndon, Va) containing 0.1% bovine serum albumin (Sigma-Aldrich Corp) for 8 hours. The RECs, untreated or treated with 25 ng/mL recombinant human VEGF 165 (R & D Systems Inc, Minneapolis, Minn) for 5 minutes, were washed once in cold phosphate-buffered saline solution and lysed in Laemmli buffer (50 mMmolar/L Tris [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol; BioRad, Hercules, Calif) containing protease inhibitors. Cell lysates were heated to 95°C for 2 minutes, and equal volumes of lysate were subjected to 10% SDS–polyacrylamide gel electrophoresis (PAGE). The blots were incubated with antiphosphospecific ERK1/ERK2 (p44/p42) antibody (New England Biolabs, Beverly, Mass), followed by incubation with horseradish peroxidase–conjugated secondary antibody (Amersham, Piscataway, NJ). Visualization was performed using the Amersham enhanced chemiluminescence detection system. Lane-loading differences were normalized by reblotting with non–phosphorylation-specific anti-ERK1 antibody (Santa Cruz Biotechnology).

The RECs were then pretreated with vehicle, rosiglitazone maleate (10 µmol/L), or troglitazone (1-20 µmol/L) for 15 minutes before exposure to vehicle alone or VEGF 165 (25 ng/mL) for 5 minutes. Cell lysates were subjected to SDS-PAGE, and the evaluation of p44/p42 ERK-MAPK phosphorylation was performed as already described.

**STATISTICAL EVALUATION**

Experimental and control groups were compared using independent sample t tests. Statistical significance was defined as α < .05.

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**Figure 1.** Retinal endothelial cells (RECs) express peroxisome proliferator-activated receptor-γ1 (PPAR-γ1) protein. By means of Western immunoblotting, 25 µg of nuclear extract or cytosolic protein isolated from bovine RECs (BRECs) was assayed using a rabbit polyclonal antibody against human PPAR-γ1 (Santa Cruz Biotechnology, Santa Cruz, Calif). Specificity of the antibody is demonstrated by its detection of full-length in vitro translated PPAR-γ1 protein. Image of blot was digitally acquired but not enhanced.

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**Figure 2.** Effect of thiazolidinediones on retinal endothelial cell (REC) thymidine incorporation, migration, and tube formation in response to vascular endothelial growth factor (VEGF). **A.** The proliferation of RECs was induced with VEGF (10 ng/mL), and measured by means of tritiated thymidine incorporation. Rosiglitazone maleate and troglitazone each inhibited VEGF-induced thymidine incorporation in a dose-dependent manner beginning at concentrations of 10 µmol/L (P < .05). B. Chemotactic migration of RECs to VEGF (10 ng/mL) was measured in a modified Boyden chamber. Rosiglitazone and troglitazone each inhibited VEGF-induced REC migration in a dose-dependent manner beginning at concentrations of 10 µmol/L (P < .05). C. Tube formation of RECs was induced by adding VEGF (10 ng/mL) to collagen gels. Rosiglitazone and troglitazone prominently inhibited tube formation at concentrations of 10 µmol/L (P < .01) and greater. For each experiment, the maximal effect was obtained for VEGF-stimulated RECs, and this result was assigned a value of 100; all subsequent results are presented as an index relative to this number. Error bars represent SD.

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**IN VITRO EFFECTS OF TZDs ON RECs**

Proliferation of RECs was induced using VEGF (10 ng/mL). Rosiglitazone and troglitazone each significantly inhibited VEGF-induced thymidine incorporation, beginning at concentrations of 10 µmol/L (P < .05) and becoming more prominent at concentrations of 20 µmol/L (P < .005) (Figure 2A). Both TZDs were equally...
effective at this dose range. Viability, as measured by means of trypan blue exclusion, was greater than 95% at all drug concentrations tested.

Rosiglitazone and troglitazone inhibited the chemotactic migration of RECs to VEGF in a modified Boyden chamber in a dose-dependent manner beginning at 10 µmol/L (P < .05) (Figure 2B). Viability was maintained at greater than 95% at all drug concentrations used in this experiment.

Dramatic inhibition of VEGF-induced tube formation by RECs in collagen gels was seen in the presence of rosiglitazone or troglitazone at a concentration of 10 µmol/L (P < .01) (Figure 2C). At a concentration of 20 µmol/L, essentially no tube formation was found. No morphologic evidence of cell death was observed at any of the TZD drug concentrations tested.

**EFFECT OF TZDs ON RETINAL NEOVASCULARIZATION**

To evaluate the antiangiogenic effect of TZDs on oxygen-induced retinopathy, retinas were examined by means of fluorescein-dextran injection angiography. Retinas of the DMSO-injected eyes of animals at postnatal day 17 (5 days of relative retinal hypoxia) contained prominent neovascular tufts extending into the vitreous body at the junction between the perfused and the nonperfused tissue (Figure 3A-B). In contrast, in the retinas from experimental animals treated with 0.5 µL of troglitazone (100 µmol/L) or rosiglitazone maleate (100 µmol/L) and examined on postnatal day 17, the amount of neovascular tissue was markedly reduced, despite the presence of comparable pericentral regions of nonperfusion (Figure 3C). The capillary network and nonperfused areas were well formed, but there were no tufts of neovascularization extending into the vitreous (original magnification ×100). Images were digitally acquired but not enhanced.
retinal hypoxia) contained multiple neovascular tufts extending into the vitreous (Figure 4A). These tufts originated from retinal vessels, forming clusters of immature endothelial cells (Figure 4B). Intravitreal injection of troglitazone (Figure 4C-D) or rosiglitazone reduced the histologically evident retinal neovascularization in all 8 animals compared with the DMSO-treated experimental controls (Figure 5). The mean (±SD) number of endothelial nuclei anterior to the inner limiting membrane was significantly reduced from 474±93 in control eyes (DMSO-injected) with oxygen-induced retinopathy to 267±51 in troglitazone-treated eyes (P<.01) and 299±61 in rosiglitazone-treated eyes (P<.01).

IMMUNOHISTOCHEMICAL ANALYSIS FOR VEGF AND vWF

Consistent expression of VEGF was seen in all hypoxic retinas, regardless of the agents injected into the vitreous (Figure 6). In DMSO-injected eyes, VEGF was expressed mainly in the ganglion cell layer and to a lesser extent in the inner plexiform layer of the retinas, just beneath the prominent retinal neovascularization (Figure 6A), and adjacent to the intraretinal neovascularization (Figure 6C). Although retinal neovascularization was significantly reduced in troglitazone- or rosiglitazone-injected eyes, VEGF was also prominently expressed in the ganglion cell layer of these TZD-treated retinas (Figure 6B).

VEGF STIMULATES ERK-MAPK PHOSPHORYLATION IN RECs

After 5 minutes of stimulation, VEGF induced p44/p42 ERK-MAPK phosphorylation (Figure 7). Troglitazone and rosiglitazone (10 μmol/L) inhibited VEGF-induced ERK-MAPK phosphorylation by greater than 50% for...
Insulin resistance plays a crucial role in the pathogenesis of type 2 diabetes; however, much of the morbidity of this disease results from its complications, including microangiopathy and cardiovascular disease. The TZDs constitute a class of pharmacologic compounds that enhance insulin action through activation of the PPAR-γ receptor. These drugs, when used alone or added to traditional oral hypoglycemic agents, result in better control of glycemic markers and amelioration of hyperinsulinemia. Recently, there has been considerable interest in the additional benefits of these drugs for other metabolic abnormalities in diabetes, including improvements in lipid profile and blood pressure control. Moreover, it has recently been suggested that TZDs independently delay certain diabetic complications through direct activation of PPAR-γ in vascular cells. Troglitazone inhibits endothelial cell activation and platelet aggregation and delays development of atherosclerosis in animal models. Beneficial effects on other diabetic complications have also been reported. The TZDs may inhibit microalbuminuria in patients with incipient diabetic nephropathy and may protect against nephropathy in Zucker fatty rats. Inhibitory effects of TZDs on diabetic nephropathy in streptozocin-induced diabetic rats have also been described.

In the present report, we provide evidence that TZDs may reduce the complications of diabetic retinopathy. Diabetic retinopathy encompasses the following 5 basic abnormalities: formation of retinal capillary microaneurysms, development of excessive vascular permeability, vascular occlusion, proliferation of new blood vessels, and contraction of accompanying fibrous tissue on the surface of the retina. Proliferative diabetic retinopathy is particularly devastating and is characterized by the growth of new vessels on the surface of the retina. Despite these improvements, it remains a very common cause of blindness that would be treated more appropriately by prevention.

Neovascularization is a multistep process that includes degradation of basement membrane and proliferation, migration, and tube formation by endothelial cells. The neovascularization process is stimulated by a variety of growth factors and cytokines; however, VEGF has been shown to be central to this process in PDR. It has been reported previously that endothelial cells from several sources, including the umbilical vein, aorta, and choroid, express the TZD receptor PPAR-γ; nevertheless, it is important to confirm this finding in the specific endothelial populations being studied, since endothelial cells are heterogeneous in their phenotype and function. We show herein that RECs strongly express PPAR-γ1 protein. The PPAR-γ gene produces 2 messenger RNA species by means of alternative promoter use, each containing different 5’ exons that are spliced onto common downstream exons. The PPAR-γ2 protein differs from PPAR-γ1 by the presence of 30

p44 ERK1. Dose-response analysis demonstrated that 20-mmol/L troglitazone inhibited ERK-MAPK phosphorylation even more prominently. Troglitazone also eliminated the weak basal p42 ERK-MAPK phosphorylation without affecting cellular shape or attachment. In all experiments, blots of total ERK-MAPK revealed that total ERK-MAPK protein concentration remained unchanged.

Figure 6. Immunohistochemical analysis for vascular endothelial growth factor (VEGF) and the endothelium-specific marker von Willebrand factor (vWF) in mice with oxygen-induced retinopathy. A, From dimethyl sulfoxide–injected (control) animals with oxygen-induced retinopathy, increased VEGF expression was found in the ganglion cell layer (GCL) and inner plexiform layer (IPL) of the retina in the region of the prominent retinal neovascularization (arrows) (original magnification ×200). B, From troglitazone–injected eyes, increased VEGF expression was found in the GCL, although retinal neovascularization is mild (arrow) (original magnification ×200). C, From control animals with oxygen-induced retinopathy, endothelial cell labeling with vWF shows that neovascularization is located not only on the surface of the retina but also in the inner retina (arrows), adjacent to where VEGF is prominently expressed (original magnification ×20). Images were digitally acquired but not enhanced (for all). Immunohistochemical stain using 3-amino 9-ethylcarbazole as the red chromogen and hematoxylin as a blue nuclear counterstain.
Thiazolidinedione inhibits vascular endothelial growth factor (VEGF)–induced extracellular signal-regulated mitogen-activated protein kinase (ERK-MAPK) phosphorylation in retinal endothelial cells (RECs). Quiescent RECs, untreated or treated with VEGF at a concentration of 25 ng/mL (5 minutes), were lysed, and the lysates were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The membrane was probed with anti–phosphospecific PPAR-γ ligands because it also contains an α-tocopherol moiety, which could have effects due to its antioxidant activity or its ability to inhibit protein kinase C. Both TZDs studied in this report inhibited the phosphorylation, migration, and tube formation of RECs in response to VEGF. There was no apparent difference in the sensitivity of RECs to troglitazone and rosiglitazone, providing strong support for the notion that both drugs have effects on RECs by binding to PPAR-γ.

Previous in vivo studies on the antiangiogenic effects of PPAR-γ ligands have been limited to a VEGF-containing corneal pocket assay and a laser injury model of choroidal neovascularization. The present study uses a much more pathologically relevant model in which the neovascularization is stimulated by increased VEGF expression in the inner retina. Although the model lacks other metabolic abnormalities found in diabetes, it isolates the VEGF-driven process to the inner retina and allows for more precise examination of the mechanism within a clinically relevant microenvironment. By use of qualitative and quantitative analyses, we demonstrated a decrease in the number of microvascular tufts that were induced on the retinal surface, suggesting that the TZDs are inhibiting an early aspect of neovascularization. Again, it was important to demonstrate similar degrees of inhibition of in vivo angiogenesis by 2 PPAR-γ ligands, implying that the drugs are acting similarly through PPAR-γ activation. Although rosiglitazone binds to PPAR-γ with higher avidity than to troglitazone, the TZDs were equally potent in inhibiting VEGF-mediated growth and REC migration. These observations are similar to our previous findings for their antiproliferative and antimigration activity in vascular smooth muscle cells. When VEGF expression was examined immunohistochemically, we found that approximately equal amounts of VEGF were expressed in the ganglion cell layer of positive control animals with neovascularization as were found in animals in which retinal neovascularization was inhibited by TZDs, supporting the contention that the TZDs do not interfere with VEGF expression in the region where neovascularization occurs. This contention is further supported by in vitro data demonstrating that hypoxia-induced VEGF secretion by viable retinal pericytes and astrocytes is not significantly reduced by treatment of cells with troglitazone (results not shown).

Previous studies in vascular smooth muscle cells show that PPAR-γ ligands may be acting, at least in part, downstream of growth factor receptor tyrosine kinase activation through inhibition of the ERK-MAPK pathway. Although in those studies it was suggested that the effect may be downstream of ERK1/2 activation, we demonstrate here in RECs the significant inhibition of VEGF-induced ERK1/2 phosphorylation by a TZD. Troglitazone has also been shown to inhibit angiotensin II–induced ERK-MAPK activity in vascular smooth muscle cells. Since the ERK-MAPK pathway plays an important role in cellular proliferation and migration, inhibition of this pathway could explain the effects that we found in RECs in vitro.

The TZDs may also have more widespread effects that might improve their effectiveness as inhibitors of neovascularization in PDR. Although VEGF is thought to be the primary effector of PDR, neovascularization occurs in a complex environment of multiple growth factors. The ERK-MAPK pathway is known to be a common pathway of activation by multiple growth factors, and PPAR-γ ligands have been shown to inhibit migration mediated by multiple chemoattractants in vascular smooth muscle cells, including insulin-like growth factor I and platelet-derived growth factor; therefore, effects of other growth factors in endothelial cells may be inhibited. Other possible mechanisms by which PPAR-γ ligands may inhibit
retinal vascular dysfunction include inhibition of macrophage activation and inflammation, decrease in VEGF receptor expression, decrease in matrix metalloproteinase 9 secretion, decrease in platelet aggregation, increase in plasminogen activator inhibitor 1 expression, induction of endothelial cell apoptosis, and suppression of endothelin 1 secretion from endothelial cells.

The results of the present study further support the idea that TZDs may have beneficial effects on the diabetic patient beyond those of improving insulin resistance by reducing or delaying the onset of complications such as PDR. The common effect of both agents studied herein suggests that the effect on experimental retinal neovascularization is mediated through PPAR-γ, and our results suggest that much of the effect appears to be mediated downstream of VEGF expression, possibly through inhibition of ERK1 in the ERK-MAPK pathway. Carefully designed clinical studies should be initiated to determine whether diabetic patients currently being treated with PPAR-γ ligands demonstrate inhibition in the development or progression of PDR.

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From the Archives of the ARCHIVES

A look at the past . . .

Dr George E. Deschweinitz of Philadelphia, read (by invitation) a paper on ocular headaches. Eye strain due to refractive error, heterophoria and accommodative dysfunction, is responsible, in whole or in part, for about 60 per cent of functional headaches; small error of refraction are often more potent in this respect than the larger ones; all types of refractive error and muscle imbalance are capable of causing headache, simple and compound astigmatism and hyperphoria being the most potent refractive defects in this list.