Thiazolidinediones Reduce Pathological Neovascularization in Ischemic Retina Via an Adiponectin-Dependent Mechanism

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**Background**—The insulin-sensitizing agents referred to as thiazolidinediones (TZDs) possess antiatherogenic and anti-inflammatory actions that contribute to protection against diabetic macrovascular complications. However, little is known about the effects of TZDs on retinal microvascular disorders.

**Objective**—To investigate whether TZDs modulate retinal vessel formation in a mouse model of oxygen-induced retinopathy.

**Methods and Results**—Neonatal mice were subjected to ischemia-induced retinopathy to produce pathological neovascular tuft formation. Pioglitazone, 10 mg/kg per day, rosiglitazone, 10 mg/kg per day, or vehicle was administered by gavage once a day from postnatal day 7 to postnatal day 17. Systemic treatment with wild-type (WT) mice with TZDs led to a significant decrease in pathological retinal neovascularization during ischemia compared with vehicle treatment, which was accompanied by increased plasma levels of the fat-derived hormone adiponectin (APN). In contrast to WT mice, TZDs had no effects on ischemia-induced pathological retinal vessel formation in APN-knockout (KO) mice. Pioglitazone reduced tumor necrosis factor (TNF) α expression in ischemic retina in WT mice but not in APN-KO mice. Furthermore, pioglitazone increased plasma APN levels in TNF-α-KO mice but did not affect ischemia-induced pathological retinal neovascularization in this strain.

**Conclusion**—These data show that TZDs attenuate pathological retinal microvessel formation through APN-mediated modulation of TNF-α production. (Arterioscler Thromb Vasc Biol. 2010;30:46-53.)

**Key Words:** pioglitazone ■ adiponectin ■ neovascularization ■ ischemia ■ angiogenesis

Thiazolidinediones (TZDs) and peroxisome proliferator–activated receptor (PPAR) γ agonists (pioglitazone and rosiglitazone) have well-established antihyperglycemic effects in type 2 diabetes mellitus; these effects are mediated by a reduction in insulin resistance.1–3 The administration of TZDs inhibits the development of atherosclerotic lesion formation in animal models of atherosclerosis4,5 and suppresses neointimal thickening in response to injury in rabbits and rats.6,7 Thiazolidinediones are also reported to protect the heart from myocardial ischemia-reperfusion injury.8 These actions are believed to be mediated through the ability of TZDs to affect vascular function and modulate inflammation.1–3 Although these data suggest that TZDs exert favorable effects on diabetic macrovascular complications, the epidemiological findings on TZD treatment and cardiovascular diseases are complex. The Prospective Pioglitazone Clinical Trial in Macrovascular Events study and a meta-analysis reported that pioglitazone therapy has a beneficial impact on cardiovascular outcomes in diabetic populations.9,10 A meta-analysis revealed that rosiglitazone is associated with adverse cardiovascular events in diabetic patients,11 but the more recent RECORD study did not find that rosiglitazone is linked to increased cardiovascular risk.12

Ischemic retinopathies, including retinopathy of prematurity and diabetic retinopathy, are the main cause of blindness.13,14 Retinal ischemia causes vascular injury and dysfunction, leading to pathological blood vessel formation in the retina.14 Furthermore, hyperglycemia can contribute to the development of diabetic retinopathy by enhancing vascular permeability, inflammation, and endothelial cell damage.13 These abnormal neovascular changes are associated with the overproduction of several growth factors and cytokines, including vascular endothelial growth factor (VEGF) and tumor necrosis factor (TNF) α.13,14 Thus, inhibition of pathological retinal vessel formation is a logical strategy for preventing retinal injury and preserving visual acuity.15

Adiponectin (APN) is an anti-inflammatory adipose-derived cytokine that is down-regulated in patients with obesity-linked diseases, including type 2 diabetes mellitus.16,17 More evidence indicates that TZD treatment increases plasma APN levels in healthy, obese, and type 2 diabetic subjects.18,19 Thiazolidinedione treatment also increases APN levels in diabetic db/db,Albany St, W611, Boston, MA 02118. E-mail nouchi@bu.edu

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ob/ob, and wild-type (WT) mice. A number of experimental studies demonstrate that APN plays a protective role in the development of insulin resistance and diabetic microvascular complications, including atherosclerosis and ischemic heart disease. Of significance, APN-knockout (KO) mice are refractory to the beneficial effects of TZDs on insulin resistance. These findings suggest that the ability of TZDs to ameliorate insulin sensitivity is mediated, at least in part, by the ability of these drugs to up-regulate APN expression.

A recent clinical study showed that rosiglitazone treatment is associated with the delayed onset of proliferative diabetic retinopathy. In experimental models, it has been shown that intravitreal injection of TZDs suppresses pathological vessel formation in the ischemic retina, and that TZDs suppress retinal leukostasis and vascular leakage. However, the molecular mechanisms of the action of TZDs in retinal vessel disease remain unclear.

Recently, we have shown that APN protects against pathological microvessel formation in the retina in a mouse model of ischemic retinopathy. Thus, we hypothesized that TZDs protect against retinal vessel disorders through their ability to increase circulating APN levels. Herein, we investigated the effects of TZDs on ischemia-induced retinal vessel formation in a mouse model of oxygen-induced retinopathy and assessed the participation of APN and TNF-α in this process with loss-of-function genetic manipulations.

**Methods**

**Mouse Model of Ischemia-Induced Retinopathy**

Adiponectin-KO and littermate WT mice in a C57BL/6 background, and TNF-α-KO and littermate WT mice in a C57BL/6/129 background (The Jackson Laboratory, Bar Harbor, Maine), were used. Both sexes were used. All animal studies were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Institutional Animal Care and Use Committee at Boston University, Boston, Mass. Both sexes were used. All animal studies were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Institutional Animal Care and Use Committee at Boston University, Boston, Mass.

**Retinal Vessel Disease**

To examine the effects of TZDs on ischemia-induced retinal vessel formation in a mouse model of oxygen-induced retinopathy and to assess the participation of APN and TNF-α in this process with loss-of-function genetic manipulations, we used a mouse model of ischemic retinopathy.

**Determination of Messenger RNA Levels**

The extent of vascular obliteration and neovascular tuft formation was quantified by comparing the number of pixels in the affected areas with the total number of pixels in the retina. The physiological normal vascularization was calculated by subtracting the neovascular and vascular obliteration areas from total retinal area.

**Adenovirus-Mediated Gene Transfer**

Adenovirus vectors containing the gene for β-galactosidase (Ad-βgal) and full-length mouse APN (Ad-APN) were prepared as described previously. 

**Western Blot Analysis**

Retinal tissues were obtained on postnatal day 14 and homogenized in RIPA buffer (Cell Signaling, Danvers, Mass.) with protease inhibitor cocktail (Roche, Gipf-Oberfrick, Switzerland). Tissue lysates were separated with denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The membranes were immunoblotted with the indicated antibodies followed by the secondary antibody conjugated with horseradish peroxidase. An ECL Western Blotting Detection kit (Amersham Pharmacia, Piscataway, NJ) was used for detection. Antibodies used in this study were as follows: phosphor-AMP-activated protein kinase (AMPK) (Thr172) antibody (Cell Signaling), pan-α-AMPK antibody (Cell Signaling), and β-actin antibody (Sigma). Quantitative analysis of relative phosphorylation levels was performed by using the ImageJ program. Immunoblots were normalized to total loaded protein.

**Statistical Analysis**

All data are presented as the means ± SEM. Differences were analyzed by the Student unpaired r test for 2 groups or by ANOVA for multiple comparisons. P < 0.05 was accepted as statistically significant.
Results
Systemic Delivery of Pioglitazone Inhibits Ischemia-Driven Pathological Retinal Neovascularization in WT Mice

A previous study analyzed the effects of TZDs on pathological retinal vascularization after intravitreal delivery. To test the impact of systemic pioglitazone administration, neonatal WT mice in a C57BL/6 background were exposed to hyperoxia (75% oxygen) for 5 days from postnatal day 7 to postnatal day 12 and returned to room air at postnatal day 12 to induce pathological neovascular tufts. Pioglitazone or vehicle was given to mice by gavage from postnatal day 7 to postnatal day 17. Retinas were stained with fluoresceinated isolectin B4 to detect vascularization on postnatal day 17. Figure 1A shows representative photographs of retinal whole mounts, stained with fluorescein-labeled isolectin B4. Quantitative analysis of neovascular tufts/total retinal area revealed that pioglitazone significantly reduced pathological retinal neovascular formation in this model (Figure 1B). Pioglitazone treatment also reduced vascular obliteration/total retinal area compared with vehicle treatment (Figure 1C). Pioglitazone increased areas of physiologically normal vascularization in ischemic retina (77.5% ± 0.7%) compared with vehicle (71.2% ± 0.8%). Thus, systemic administration of pioglitazone is protective in the context of ischemia-induced pathological retinal neovascularization. In contrast, under normoxic conditions, WT mice treated with pioglitazone or vehicle showed no central avascular area and no peripheral pathological vascular tuft formation (data not shown).

Contribution of APN to the Suppressive Action of Pioglitazone on Pathological Neovascularization

Recently, we found that APN is protective against the development of ischemic retinopathy. Because TZDs have been shown to increase APN levels in mice and humans, we hypothesized that the beneficial actions of pioglitazone on pathological neovascularization in response to ischemia might be the result of the induction of APN. To test this hypothesis, we first measured plasma APN levels during oxygen-induced retinopathy in the presence of pioglitazone or vehicle. Pioglitazone treatment significantly increased plasma APN levels on postnatal days 12, 14, and 17 compared with vehicle (Figure 2A). Plasma APN levels were also slightly elevated on postnatal days 14 and 17 in vehicle-treated WT mice (Figure 2A). Plasma glucose levels on postnatal day 17 did not significantly differ between vehicle- and pioglitazone-treated WT mice (glucose levels, 95 ± 6 mg/dL in vehicle-treated mice and 91 ± 8 mg/dL in pioglitazone-treated mice).

To test whether APN mediates the protective actions of pioglitazone on ischemic retinopathy, we investigated the effect of pioglitazone on pathological vessel formation in APN-KO mice in a C57BL/6 background. Compared with WT mice, APN-KO mice treated with vehicle exhibited an increased pathological neovascular area (17.2% ± 0.6%), consistent with our previous observations. Pioglitazone treatment had no effect on retinal neovascular areas in APN-KO on postnatal day 17 compared with vehicle treatment (Figure 2B). In contrast, systemic delivery of adenoviral vectors expressing Ad-APN attenuated retinal neovascular area in APN-KO on postnatal day 17 compared with Ad-βgal treatment (Figure 2B). Although APN levels were undetectable in Ad-βgal–treated APN-KO, Ad-APN–treated APN-KO had APN levels of 11.4 ± 2.1 μg/mL on postnatal day 17, which is similar to levels in WT mice. These data suggested that the inhibitory effect of pioglitazone on ischemia-induced neovascularization is dependent on its ability to up-regulate APN production.

We also evaluated the extent of pathological tuft formation in ischemic retina in WT and APN-KO mice after gavage.
administration of rosiglitazone, another TZD. Treatment of WT mice with rosiglitazone resulted in a significant increase in plasma APN levels on postnatal day 14 relative to vehicle treatment, which is comparable to the levels in pioglitazone-treated WT mice (APN levels, 13.7 ± 1.0 μg/mL in WT/vehicle and 28.1 ± 1.0 μg/mL in WT/rosiglitazone). Neovascular tuft formation in the retinas of WT mice was attenuated by rosiglitazone treatment (Figure 2C). In contrast, rosiglitazone did not affect the areas of retinal neovascular formation in APN-KO mice (Figure 2C).

Pioglitazone Reduces TNF-α Expression in Ischemic Retina
To elucidate the mechanism by which pioglitazone protects against abnormal retinal neovascularization, we analyzed the expression of VEGF and TNF-α in ischemic retina in vehicle- and pioglitazone-treated WT mice on postnatal day 14 by quantitative real-time polymerase chain reaction methods. Vascular endothelial growth factor messenger RNA (mRNA) levels did not significantly differ between vehicle- and pioglitazone-treated WT mice (fold changes in transcripts in pioglitazone vs vehicle, 0.92 ± 0.10). In contrast, pioglitazone treatment significantly reduced retinal TNF-α mRNA levels in WT mice compared with vehicle treatment (Figure 3A). To test the involvement of APN in the suppression of retinal TNF-α expression by pioglitazone, we assessed retinal TNF-α mRNA levels in the presence of pioglitazone or vehicle. Adiponectin-KO mice treated with pioglitazone showed a significant increase in retinal TNF-α mRNA levels compared with vehicle-treated WT mice. In contrast to the inhibition of TNF-α levels in WT mice by pioglitazone, the administration of pioglitazone to APN-KO mice did not lead to a significant reduction of TNF-α mRNA levels in the ischemic retina (Figure 3A).
To corroborate the TNF-α transcript data, TNF-α protein levels were quantified by enzyme-linked immunosorbent assay. Treatment of WT mice with pioglitazone significantly reduced TNF-α protein expression in ischemic retina (Figure 3B). In contrast, APN-KO mice exhibited increased levels of TNF-α production in the ischemic retina, and pioglitazone treatment had little or no effect on these elevated levels of TNF-α protein (Figure 3B).

Role of TNF-α Deficiency in the Suppression of Pathological Neovascularization by Pioglitazone

To investigate the role of TNF-α in retinal protection by pioglitazone, we assessed the impact of pioglitazone on abnormal retinal neovascularization under conditions of TNF-α deficiency. Tumor necrosis factor α–KO and WT mice in a background of C57BL/6/129 were subjected to ischemia-induced retinopathy. Pioglitazone or vehicle was administered to WT (vehicle, n = 8; PIO, n = 10) and TNF-α–KO (vehicle, n = 14; PIO, n = 13) mice from postnatal day 7 to postnatal day 14. Plasma adiponectin levels on postnatal day 14 were determined by enzyme-linked immunosorbent assay. B, Effect of PIO on pathological retinal neovascularization in TNF-α–KO mice. Pioglitazone or vehicle was administered to WT (vehicle, n = 14; PIO, n = 13) and TNF-α–KO (vehicle, n = 17; PIO, n = 17) mice from postnatal day 7 to postnatal day 17. Data are given as mean±SEM (n = 13–20).

Discussion

Clinical and experimental studies have shown that TZDs suppress pathological vessel formation in the retina.26–28 In the present study, we analyzed the effects of systemic TZD (pioglitazone and rosiglitazone) administration on pathological microvessel formation in the retina in a mouse model of oxygen-induced retinopathy. The TZD-mediated suppression of pathological neovascularization was accompanied by elevations in plasma APN levels, and mice lacking APN were refractory to the therapeutic actions of TZDs in ischemic retina. Recently, we reported that the protective effects of APN on pathological retinal vessel growth are mainly the result of suppression of TNF-α production.29 Herein, we show that pioglitazone suppresses TNF-α expression in strains compared with vehicle treatment (APN levels, 11.2±0.4 μg/mL in WT [C57BL/6/129]/vehicle, 19.7±2.1 μg/mL in WT [C57BL/6/129]/pioglitazone, 7.9±1.2 μg/mL in TNF-α–KO/vehicle, and 18.1±1.2 μg/mL in TNF–α–KO/pioglitazone). Paradoxically, APN levels were significantly lower in TNF–α–KO mice than in WT mice. Tumor necrosis factor α–KO mice treated with vehicle exhibited decreased retinal neovascular area compared with vehicle-treated WT mice, in agreement with previous reports20,31 (Figure 4B). Treatment of WT mice with pioglitazone resulted in a significant decrease in retinal neovascular areas on postnatal day 17 (41%), but pioglitazone treatment had little or no effect on neovascular tuft formation in retina in TNF–α–KO mice (17% reduction).

APN Mediates PPARα Induction and AMPK Activation Caused by Pioglitazone

Pioglitazone has been shown to stimulate PPARα33 and AMPK activity in vivo.20,21 Similarly, APN is reported to increase PPARα expression and enhance AMPK activity in various tissues, including liver and muscles.34 Therefore, we tested whether pioglitazone modulates PPARα expression and AMPK signaling during ischemic retinopathy in WT and APN-KO mice. Quantitative real-time–polymerase chain reaction analysis revealed that PPARα mRNA levels were lower in the retinas of APN-KO mice than in those of WT mice during vehicle treatment (Figure 5A). Treatment of WT mice with pioglitazone led to a significant increase in PPARα transcript levels in ischemic retinas, whereas no effects of pioglitazone were observed in APN-KO mice (Figure 5A). Western blot analysis demonstrated that pioglitazone enhanced phosphorylation of AMPK in ischemic retinas in WT mice, but not in APN-KO mice (Figure 5B). The levels of retinal AMPK phosphorylation tended to be lower in APN-KO mice than in WT mice after vehicle treatment, but the difference was not statistically significant. AMPK protein levels did not significantly change among four different groups. Finally, we examined the effect of pioglitazone on mRNA levels of PPARγ-regulated genes, CD36 and fatty acid binding protein 4, and aP2 in retinas of WT and APN-KO mice. No significant differences were observed in CD36 and aP2 mRNA levels between the two strains, and pioglitazone up-regulated CD36 and aP2 transcript levels in ischemic retinas to similar extents in WT and APN-KO mice compared with vehicle (Figure 5C).
ischemic retinas of WT but not APN-KO mice. We also show that pioglitazone enhances plasma APN levels in TNF-α-KO mice; however, pioglitazone was ineffective at suppressing the lower levels of pathological retinal neovascularization in this strain. Collectively, these data suggest that APN up-regulation mediates the beneficial actions of pioglitazone on ischemic retinopathy in vivo, and that the suppression of TNF-α regulation mediates the beneficial actions of pioglitazone on this strain. Collectively, these data suggest that APN up-
mice; however, pioglitazone was ineffective at suppressing

to exacerbation of diet-induced insulin resistance, which leads to improvement of tissue injury.

Thiazolidinediones have been shown to protect against pathological inflammation and tissue injury through suppression of proinflammatory genes, including TNF-α. Inhibition of TNF-α largely contributes to the effects of TZD on inflammatory responses in adipose tissue by analysis of mice lacking TNF-α function. These findings are consistent with our observations showing that the retinal protection by pioglitazone is largely dependent on the suppression of TNF-α by APN. Thus, it is tempting to speculate that the therapeutic actions of TZDs in these different systems result in large part from the abilities of these drugs to influence the APN–TNF-α regulatory axis.

Several in vitro studies show that APN suppresses agonist-stimulated TNF-α production in multiple cell types. Adiponectin has been shown to block the increase in TNF-α expression in response to lipopolysaccharide in adipocytes and cardiac cells. Adiponectin also reduces TNF-α production after lipopolysaccharide stimulation in macrophages through its ability to suppress nuclear factor κB activity. Furthermore, APN stimulates apoptotic body clearance by macrophages, leading to a reduction of TNF-α levels. Because TNF-α in the ischemic retina has shown to be produced mainly by macrophage and macrophage–like microglia, the resolution of retinal vascular injury by APN may be largely attributed to suppression of TNF-α in retinal macrophages.

In our study, treatment of neonatal WT mice with TZDs, 10 mg/kg per day, resulted in an increase in APN level (approximately 2-fold increase), which is comparable with the APN induction in adult obese mice after TZD treatment. Thiazolidinediones up-regulate APN expression in adipocytes through a number of mechanisms that are stimulated by PPARγ. Thiazolidinediones stimulate APN gene transcript in cultured adipocytes and in adipose tissues of obese mice in a PPARγ-dependent mechanism. Thiazolidinediones are also reported to promote secretion of APN from adipocytes. Recent studies have shown that TZDs promote the induction of endoplasmic reticulum chaperones (ie, Erp1-L, Erp44, and DsbA-like protein) that are crucial for APN secretion.

It has been demonstrated that TZD treatment promotes AMPK activation in the liver of obese mice in an APN-dependent manner. Consistent with these findings, we report that pioglitazone treatment leads to AMPK activation in the ischemic retina of WT mice but not in APN-KO mice. Likewise, we found that pioglitazone induced PPARα expression in WT but not APN-KO mice. Thus, APN up-regulation by adipocytes mediates the ability of pioglitazone to control a broad regulatory network in the retina that can contribute to the resolution of vascular dysfunction and inflammation. However, the causal relationships between TNF-α expression and activation of PPARα or AMPK have not been established, and the role of these signaling proteins in ischemic retinopathy is not known. Finally, in contrast to AMPK and PPARα, the genetic ablation of APN did not affect the pioglitazone-induced expression of other PPARγ-responsive
genes in retina, including CD36 and aP2. Collectively, these data indicate that TZDs exert both direct and indirect actions on the retina. However, our study indicates that it is the indirect action of TZDs, via increased APN expression by adipocytes, that mediates their beneficial actions on the ischemic retina.

Previous experimental studies27,28 and our current data suggest that TZD therapy may be therapeutically beneficial for diseases of retinal inflammation and vascular dysfunction. In this context, rosiglitazone therapy is shown to delay the onset of diabetic proliferative retinopathy and to reduce loss of visual acuity.26 However, a recent study29 has shown that TZDs are associated with a moderately increased risk of diabetic macular edema. Thiazolidinedione treatment also leads to increases in circulating VEGF levels in diabetic retinopathy.29

In conclusion, our study shows that systemic administration of pioglitazone protects against ischemic retinal injury by negatively regulating TNF-α expression. The ability of pioglitazone to suppress TNF-α expression and pathological neovascularization in ischemic retina in vivo is mediated through the up-regulation of the anti-inflammatory adipokine APN. Thus, pioglitazone treatment, or other approaches that stimulate APN production, should be investigated further to assess its potential usefulness for the treatment of ischemia-induced retinal microvascular disease.

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**Disclosures**

None.

**References**


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