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

Akiko Higuchi; Koji Ohashi; Rei Shibata; Saki Sono-Romanelli; Kenneth Walsh; Noriyuki Ouchi

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 given by gavage once a day from postnatal day 7 to postnatal day 17. Systemic treatment of 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) \( \alpha \) expression in ischemic retina in WT mice but not in APN-KO mice. Furthermore, pioglitazone increased plasma APN levels in TNF-\( \alpha \)-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-\( \alpha \) production. (Arterioscler Thromb Vasc Biol. 2010;30:46-53.)

Key Words: pioglitazone ■ adiponectin ■ neovascularization ■ ischemia ■ angiogenesis
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 macrovascular complications, including atherosclerosis and ischaemic 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, Massachusetts. To produce a clinically relevant ischemic retinopathy model, Vision Research, and were approved by the Institutional Animal Care and Use Committee at Boston University, Boston, Massachusetts.

Results

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, Massachusetts.

Measurement of Neovascularization and Vascular Obliteration

The degree of neovascularization and vascular obliteration was assessed by well-established methods. Eyes were removed from mice on postnatal day 17, and fixed in 4% paraformaldehyde. The retinas were dissected and stained with fluorescence-activated cell sorting (FACS) and imaged using a fluorescence microscope. Retinal segments were merged to generate a whole retinal image using Adobe Photoshop. Central nonperfused regions without capillaries and an optic disc were defined as vascular obliteration. 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 areas. Investigators were masked to the mouse treatment.

Determination of Messenger RNA Levels

Total RNA was isolated from the retinas of mice using a commercial kit (Qiagen, Valencia, CA), and complementary DNA was produced using the ThermoScript RT-PCR System (Invitrogen, Carlsbad, Calif.). Quantitative real-time polymerase chain reaction was performed using iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, Calif.) using SYBR Green I as a double-stranded DNA specific dye, as described previously. Primers for mouse TNF-α were purchased from Qiagen. Primers were as follows: 5′-CTGTAACGATGAACCCCTGGAG-3′ and 5′-TTGGTGGCTGCTA-3′ for mouse VEGF, 5′-ATTCTCCGT-TTGTGGCTGCTA-3′ and 5′-CAATCCCCCTCCTGCAAATC-3′ for mouse PPARα, 5′-GCCGGCATGCGTTTGG-3′ and 5′-TGGAG-ATTACCTTTCAGTGCAA-3′ for mouse CD36, 5′-TACCTG-GAAAGACAGCTGCT-3′ and 5′-AATCCCATTTACGCTGATG-3′ for mouse fatty acid binding protein 4, and 5′-GGAGGAAA-TCGTGCGTGAC-3′ and 5′-CAATATGATGACCTGGCCCTG-3′ for mouse β-actin.

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. The 4×10⁷ plaque-forming units of Ad-APN or Ad-βgal were injected into the jugular vein of APN-KO mice on postnatal day 10.

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, Gibo-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: phosphorylated 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 t 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 ischemic retina in WT (vehicle, n = 8; PIO, n = 8) and APN-KO (vehicle, n = 9; PIO, n = 9) mice on postnatal day 14. The TNF-α mRNA levels were determined by quantitative real-time polymerase chain reaction analysis and expressed relative to β-actin mRNA levels. B, The TNF-α protein levels in ischemic retina in WT (vehicle, n = 6; PIO, n = 6) and APN-KO (vehicle, n = 6; PIO, n = 6) mice on postnatal day 14. Data are given as mean ± SEM.

Figure 2. Involvement of adiponectin (APN) induction in suppression of pathological vessel formation by pioglitazone (PIO).
A, Upregulation of APN by PIO during ischemic retinopathy. Pioglitazone (n = 10) or vehicle (n = 8) was given to wild-type (WT) mice by gavage from postnatal day 7 to postnatal day 17. Plasma APN levels were determined by enzyme-linked immunosorbent assay. *P < 0.05 and **P < 0.01 vs vehicle-treated mice on postnatal day 7 and ***P < 0.01 vs vehicle-treated mice. B, Administration of PIO has no effects on pathological retinal neovascularization in APN-knockout (KO) mice. The APN-KO mice were subjected to ischemia-induced retinopathy. Pioglitazone (n = 14) or vehicle (n = 13) was given to APN-KO mice from postnatal day 7 to postnatal day 17. Adenoviral vectors expressing adiponectin (Ad-APN, n = 9) or β-galactosidase (Ad-βgal, n = 8) were delivered intravenously to APN-KO mice on postnatal day 10. Neovascular areas were measured on postnatal day 17. C, Effect of rosiglitazone on pathological neovascular formation in ischemic retina in WT and APN-KO mice. The WT and APN-KO mice were treated with rosiglitazone (ROS) or vehicle from postnatal day 7 to postnatal day 17 (n = 8 in each group). Results are presented as mean ± SEM. NS indicates not significant.
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 reports. 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 PPARα and AMPK activation in vivo. Similarly, APN is reported to increase PPARα expression and enhance AMPK activity in various tissues, including liver and muscles. 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).

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=9) and TNF-α-KO (vehicle, n=14; PIO, n=10) mice from postnatal day 7 to postnatal day 14. Plasma adiponectin levels on postnatal day 14 were determined by enzyme-linked immunosorbent assay.

### Figure 4. Role of tumor necrosis factor (TNF) α deficiency in pioglitazone (PIO)-mediated retinal protection. A, Plasma adiponectin levels of wild-type (WT) and TNF-α–knockout (KO) mice after treatment with PIO or vehicle. The WT and TNF-α-KO mice in a background of C57BL/6/129 were subjected to ischemia-induced retinopathy. Pioglitazone or vehicle was given to WT (vehicle, n=8; PIO, n=9) and TNF-α-KO (vehicle, n=14; PIO, n=10) 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. 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. Herein, we show that pioglitazone suppresses TNF-α expression in
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 in 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 (i.e., Erp1-Lα, 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 studies 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. However, a recent study has shown that TZDs are associated with a moderately increased risk of diabetic macular edema. Thiazolidinedione treatment also decreases in circulating VEGF levels in diabetic subjects, which might exacerbate pathological retinal neovascularization.

Thus, additional studies will be required to assess the safety and efficacy of TZDs for the treatment of diabetic retinal vessel disorders.

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.

Sources of Funding
This study was supported by grants HL77774, HL86785, AG15052, and HL81587 from the National Institutes of Health (Dr Walsh); and by a grant from the American Heart Associations (Dr Ouchi).

Disclosures
None.

References


Thiazolidinediones Reduce Pathological Neovascularization in Ischemic Retina Via an Adiponectin-Dependent Mechanism
Akiko Higuchi, Koji Ohashi, Rei Shibata, Saki Sono-Romanelli, Kenneth Walsh and Noriyuki Ouchi

Arterioscler Thromb Vasc Biol. 2010;30:46-53; originally published online November 12, 2009; doi: 10.1161/ATVBAHA.109.198465
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/30/1/46

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/