Pioglitazone normalizes insulin signaling in the diabetic rat retina through reduction in tumor necrosis factor α and suppressor of cytokine signaling 3

Youde Jiang¹, Shalini Thakran¹, Rajini Bheemreddy¹, Eun-Ah Ye¹, Hui He³, Robert J. Walker¹,⁴, Jena J. Steinle¹,²,³

¹Department of Ophthalmology, ²Department of Anatomy & Neurobiology, ³Department of Pharmaceutical Sciences, University of Tennessee Health Science Center, Memphis, TN and ⁴Department of Biology, Philander University, Little Rock, AR

Corresponding author: Jena J. Steinle, Associate Professor, Hamilton Eye Institute, Department of Ophthalmology, 930 Madison Ave, Suite 768A, Memphis, TN, 38163; Phone: (901) 448-1910; FAX: (901) 448-5028; email: jsteinl1@uthsc.edu

Running Title: Pioglitazone improves insulin signaling

Keywords: TNFα; SOCS3; apoptosis; insulin signaling; type 2 diabetes

Capsule

Background: Tumor necrosis factor alpha (TNFα) impairs insulin signaling in the retina.
Results: Pioglitazone reduced TNFα and SOCS3-activated insulin resistance pathways in retinal cells, as well as in lysates from whole rat retina.
Conclusion: PPARγ regulates insulin signaling in retina
Significance: Increased understanding of retinal insulin signaling may lead to new therapies for type 2 diabetes.

Abstract

Dysfunctional insulin signaling is a key component of type 2 diabetes. Little is understood of the effects of systemic diabetes on retinal insulin signaling. A number of agents are used to treat patients with type 2 diabetes to normalize glucose levels and improve insulin signaling; however, little has been done to investigate the effects of these agents on retinal insulin signal transduction. We hypothesized that pioglitazone, a peroxisome proliferator-activated receptor gamma (PPARγ) agonist, would normalize retinal insulin signal transduction through reduced tumor necrosis factor alpha (TNFα) and suppressor of cytokine signaling 3 (SOCS3) activities in whole retina and retinal endothelial (REC) and Müller cells. To test this hypothesis, we used the BBZDR/Wor type 2 diabetic rat model, as well as REC and Müller cells cultured in normoglycemia and hyperglycemic conditions, to investigate the effects of pioglitazone on TNFα, SOCS3, and downstream insulin signal transduction proteins. We also evaluated pioglitazone’s effects on retinal function using electroretinogram (ERG) and markers of apoptosis. Data demonstrate that two months of pioglitazone significantly increased ERG amplitudes in type 2 diabetic obese rats, which was associated with improved insulin receptor activation. These changes occurred in both REC
and Müller cells treated with pioglitazone, suggesting that these two cell types are key to insulin resistance in the retina. Taken together, these data provide evidence of impaired insulin signaling in type 2 diabetes rats, which was improved by increasing PPARγ activity. Further work into PPARγ actions in the retina may provide improved treatment options.

Introduction

Rates of type 2 diabetes are expected to continually increase due to increased obesity worldwide (1). In order to best treat these patients, we need to understand both systemic and organ-specific changes related to diabetes and the mechanisms by which therapeutics offer potential treatment. Since diabetic retinopathy is a debilitating complication of both type 1 and type 2 diabetes, it is particularly important to understand how treatments for controlling systemic glucose might affect retinal function. Peroxisome proliferator-activated receptor gamma (PPARγ) agonists, such as rosiglitazone or pioglitazone, have been commonly prescribed for systemic glucose control, yet their effects on retinal function have yet to be established. Systemic effects of these drugs show promise for treatment of diabetes based on their ability to increase insulin sensitivity, modify lipid profiles, decrease blood pressure, and decrease inflammatory mediators (2-4).

Cell-specific effects of the PPARγ agonist pioglitazone have been reported in adipocytes indicating that treatment eliminates TNFα-induced insulin resistance (5). It has also been shown that pioglitazone increases IRS-1 levels in muscle and liver lysates from streptozotocin-treated rats or rats fed a high sucrose diet (6). An important but unresolved question is whether PPARγ agonists alter interactions between TNFα in two retinal cell types, REC and Müller cells, known to be pivotal in development of diabetic retinopathy.

We hypothesize that pioglitazone inhibition of TNFα actions may be key in its potential protective effects in diabetic retinopathy, since TNFα has been linked to insulin resistance in other tissues. Results from our lab and others have indicated that diabetes increases TNFα levels, which in turn triggers apoptosis of retinal cells and leads to diabetic retinopathy (7,8). Use of TNFα receptor knockout mice to investigate whether diabetes-induced increase in TNFα in the retina is key to diabetic retinopathy showed that loss of TNFα protected the retina against the effects of high glucose on formation of degenerate capillaries and pericyte ghosts, two key markers of vascular damage in early diabetic retinopathy (7). Also, we have recently shown that TNFα can induce phosphorylation of IRS-1 on serine 307 to block Akt activity, leading to apoptosis in both REC (9) and Müller cells (10). Increased understanding of pioglitazone actions on TNFα may provide novel insight into mechanisms by which TNFα can induce insulin resistance in retinal cells.

In addition to directly regulating insulin resistance through phosphorylation of IRS-1, TNFα also increases SOCS1/SOCS3 levels (11), which in turn blocks IRS-1, resulting in increased REC apoptosis (9). SOCS3 is also reported to inhibit insulin signaling by other potential mechanisms as well, including increased phosphorylation of insulin receptor on tyrosine 960 (IR Tyr960), which inhibits the interaction between insulin receptor and IRS-1 (12). We recently have shown that SOCS3 can also directly regulate TNFα (Jiang et al in submission), suggesting a cross talk between TNFα and SOCS3 pathways that may induce insulin resistance. Of particular interest is the report that PPARγ agonists have been reported to decrease both SOCS3 and TNFα in hepatic tissues in rats fed a high fat diet (13).

The goal of this study was to investigate whether pioglitazone could restore insulin signal transduction through reduction of TNFα/SOCS3 pathways in REC and Müller cells in vitro, and in a type 2 diabetic rat model, BBZDR/Wor, in vivo. The data demonstrate that pioglitazone normalized glucose blood levels in obese diabetic rats and improved retinal function measured by electroretinogram amplitudes. Additionally, pioglitazone reduced TNFα and SOCS3-activated insulin resistance pathways, including phosphorylation of IRS-1 on serine 307 and insulin receptor on tyrosine 960, in REC and Müller cells, as well as in lysates from...
whole rat retina. This reduction in TNFα/SOC3 was associated with reduced apoptotic proteins, and increased levels of Akt and Bcl-xL, two key anti-apoptotic proteins.

Methods

Animals. BBZDR/Wor lean (10 male) and obese rats (10 male) were purchased from Biomedical Research Models (Worcester, MA). Glucose measurements were taken weekly and once obese rats reached a glucose of >250mg/dl, an initial electroretinogram (ERG) was taken prior to treatment initiation. Five lean and five obese rats were placed into treatment groups and received a daily intraperitoneal injection of 25mg/kg pioglitazone for 2 months. After 2 months of treatment, animals in all four groups (lean, lean+pio, obese, obese+pio) underwent an additional ERG prior to sacrifice.

The BBZDR/Wor rat model of type 2 diabetes has been used for studies of autonomic neuropathy (14,15), myogenic tone of the ophthalmic artery (16), and vascular damage repair using endothelial cell progenitors (17). The BBZDR/Wor rat model of type 2 diabetes has been used for studies of autonomic neuropathy (14,15), myogenic tone of the ophthalmic artery (16), and vascular damage repair using endothelial cell progenitors (17). The BBZDR/Wor rat model of type 2 diabetes has been used for studies of autonomic neuropathy (14,15), myogenic tone of the ophthalmic artery (16), and vascular damage repair using endothelial cell progenitors (17). The BBZDR/Wor rat was produced using the BBZDP/Wor (BB rat with an Iddm2 type 1 genetic locus) and mating with lean BBZ/Wor rats (BB rat with Zucker diabetic gene) to remove the Iddm2 locus. The obese male BBZDR/Wor rat spontaneously develops type 2 diabetes at 10 weeks of age (100%) when fed standard rat chow (18,19). The obese BBZDR/Wor rat is lymphopenic, hyperinsulinemic with peripheral insulin-resistance and develops spontaneous autoimmune noninsulin dependent diabetes mellitus at an age of 70 days (18). Results in the oxygen-induced retinopathy model and the STZ model using endothelial cell progenitor cells suggest that the BBZDR/Wor rat is a good model of type 2 diabetic retinal changes (17).

ERG. Prior to sacrifice for protein analyses, animals were subjected to ERG analyses to evaluate the changes in the electrical activity of the retina as we have previously described (20,21). Briefly, rats were dark-adapted overnight. ERG responses were recorded from both eyes together using platinum wire corneal electrodes, forehead reference electrode, and ground electrode in the tail. Pupils were fully dilated using 1% tropicamide solution (Alcon). Methylcellulose (Celluvise; Allergan, Irvine, CA) drops were applied as well to maintain a good electrical connection and body temperature was maintained at 37°C by a water-based heating pad. All ERG experiments were approved by the University of Tennessee Institutional Animal Care and Use Committee on Protocol #1992. ERG waveforms were recorded with a bandwidth of 0.3-100Hz and samples at 2kHz by a digital acquisition system and were analysed a custom-built program (MatLab). Statistical analysis was applied to the means ±SD amplitudes of the a- and b- wave of each treatment group. Comparisons were made of ERG amplitudes, but implicit times were not measured.

Retinal endothelial cells. Primary human retinal endothelial cells (REC) were obtained from Cell Systems Corporation (CSC, Kirkland, Washington). Cells were grown in M131 medium containing microvascular growth supplements, 10ug/mL gentamycin, and 0.25ug/mL amphotericin B (Invitrogen, Carlsbad, CA). Before the experiments, cells were transferred to high glucose (25 mM) medium or maintained in normal glucose (5 mM) medium and grown to 80% confluence. Primary cells (passage 2-4) were used. Cells were quiesced by incubating in high or normal glucose medium without growth factors for 18-24 hours and then treated with 25uM pioglitazone for 24 hours.

Müller cells. Rat retinal Müller cells (rMC-1, courtesy of Vijay Sarthy, Northwestern University) were grown in 5mM or 25mM glucose DMEM medium (HyClone Laboratories, Logan, UT) supplemented with 10%FBS and antibiotics. Cells were cultured to 80% confluence (2-4days), and then cells were starved for 18-24 hours by reduction to 2% FBS in the growth medium to eliminate any residual growth factors in the serum. We chose to reduce serum to 2% rather than remove it completely, in order to eliminate activation of apoptotic pathways. We have used this method...
in the past for measurements of TNFα and insulin pathways (22). In preliminary studies, we initiated work in Müller cells using 25μM pioglitazone because this was effective in increasing PPARγ activity in REC experiments; however this dose did not significantly increase PPARγ activity in Müller cells. Therefore, we increased the dose to 50μM pioglitazone in Müller cells and found significantly increased PPARγ at this dose (Figure 7), so 50μM pioglitazone was used for all further work.

PPARγ antagonist. Both REC and Müller cells were treated with T0070907 prior to pioglitazone treatment to verify that pioglitazone actions on the cells were specific to PPARγ actions. T0070907 is a highly selective PPARγ antagonist. Cells were treated at 50nM (23) for 30 minutes prior to pioglitazone administration, as described above. The dose used to inhibit PPARγ activity in our system is much lower than reported in other studies (ranging from 1-50μM).

Western blotting. After appropriate treatments and rinsing with cold phosphate-buffered saline, whole retinal lysates or cell lysates were collected in lysis buffer containing protease and phosphatase inhibitors and scraped into the tubes. Protein extracts were prepared by sonication. Equal amounts of protein from the cell or tissue extracts were separated on the pre-cast tris-glycine gel (Invitrogen, Carlsbad, CA), blotted onto a nitrocellulose membrane. After blocking in TBST (10mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and 5% (w/v) BSA, the membrane was treated with appropriate primary antibodies followed by incubation with secondary antibodies labeled with horseradish peroxidase. Antigen-antibody complexes were detected by chemoluminescence reagent kit (Thermo Scientific). Primary antibodies used were SOCS3, phosphorylated IRS-1 (Ser 307), total IRS-1, phosphorylated Akt (Serine 473), Akt, Cytochrome C, Bax, Bcl-xL, phosphorylated insulin receptor (tyrosine 1150/1151), and insulin receptor (all purchased from Cell Signaling, Danvers, MA). Insulin receptor phosphorylated on tyrosine 960 was purchased from Cell Applications (San Diego, CA). PPARγ antibody was purchased from R&D Systems (Minneapolis, MN). β actin was purchased from Santa Cruz (Santa Cruz, CA).

PPARγ transcription factor assay. PPARγ activity was determined with the ELISA based PPARγ transcription factor assay kit (Abcam, Cambridge, MA) following the manufacturer’s protocol. In brief, 100μg of nuclear extract from retinal endothelial cells and Müller cells was added to each well of 96 well plate pre-coated with a specific double stranded DNA (dsDNA) sequence containing the peroxisome proliferator response element (PPRE). The wells were incubated overnight at 4°C without agitation. Specific primary antibody directed against PPARγ was added followed by HRP conjugated secondary antibody. PPARγ was detected with sensitive colorometric readout at 450nm after addition of developing and stop solution.

ELISA analyses. A TNFα ELISA (Fisher Scientific, Pittsburgh, PA) and IGFBP-3 ELISA (R&D systems, Minneapolis MN) were used according to manufacturer’s instructions. Fifty ug of protein was used to insure equal protein amounts in all wells. A cleaved caspase 3 ELISA was purchased from Cell Signaling (Danvers, MA) with equal protein loaded, so optical density measurements can be used for analyses.

Statistics. For all experiments, excluding ERG studies, data are presented as means±SEM. Five animals were used in each group for all in vivo experiments. At least 4 separate dishes of each treatment were used for all cell culture experiments. Data was analyzed by a Kruskal-Wallis test, followed by a post-hoc Dunn’s test. A representative blot is shown for Western blot results.

Results
Pioglitazone treatment improved the glucose levels of obese rats, with no change in
intraocular pressure or body weight. Within 1 week of daily treatment with 25mg/kg pioglitazone, the BBZDR/Wor obese rats had glucose levels similar to lean animals, which were maintained throughout the remainder of the 2-month study (Table 1). Treatment of lean and obese rats with pioglitazone did not alter intraocular pressure levels. Pioglitazone increased the weight of the obese animals by 36% (Table 1). We measured intraocular pressure to insure that pioglitazone did not produce deleterious effects in the eye. This suggests that while pioglitazone did not decrease the obesity of the rats, it was still highly effective in lowering blood glucose levels.

Pioglitazone treatment significantly increased PPARγ activity in whole retinal lysates of BBZDR/Wor obese rats. To insure that pioglitazone was working through expected pathways in the retina, we measured PPARγ levels in the retina of all animals. We had to use a Western blot for this assay, as we only had whole retinal lysates from the animals and could not isolate the nuclear extract as we did for cell culture work. PPARγ levels were decreased in retinas from untreated type 2 diabetic BBZDR/Wor obese rats, but were significantly increased in retinas from both lean and type 2 obese rats treated with pioglitazone (Figure 1), demonstrating that systemic pioglitazone reaches the retina to elicit its effects through activation of the PPARγ pathway.

Pioglitazone treatment protected against the loss of ERG amplitudes in type 2 diabetes obese rats within 2 months. While the ultimate goal of these studies was to evaluate whether pioglitazone could restore normal insulin signaling in type 2 diabetic BBZDR/Wor obese rats, we also wanted to evaluate whether effects of pioglitazone were linked to improved retinal function. To test this, we performed ERG analyses on both lean and type 2 diabetic obese rats prior to initiation of pioglitazone treatment and just prior to sacrifice after 2 months of treatment. Figure 2 demonstrates that diabetic animals had reduced A-wave, B-wave and oscillatory potentials prior to treatment. Treatment with pioglitazone significantly improved all wave amplitudes, but did not restore amplitudes to the same level observed in lean animals (Figure 2). This suggests that pioglitazone did improve retinal function, likely through improved glucose tolerance.

Pioglitazone significantly reduced TNFα levels in the retinas of type 2 diabetic obese rats, leading to reduced IRS-1ser307 phosphorylation. Studies in adipocytes (24) and myeloid progenitor cells (25) suggest that TNFα induces insulin resistance through phosphorylation of IRS-1 on serine 307. We wanted to determine whether pioglitazone could inhibit this insulin resistance pathway. Figure 3 demonstrates that pioglitazone given to type 2 diabetic BBZDR/Wor obese rats significantly decreased TNFα levels, leading to decreased IRS-1ser307 phosphorylation, which should improve insulin signal transduction. This data provides in vivo evidence of PPARγ regulation of TNFα in the retina in a type 2 diabetic rat model.

SOCS3 and IRtyr960 were decreased significantly after pioglitazone treatment in the retinas of type 2 diabetic obese rats. In addition to TNFα enhancement of phosphorylation of IRS-1ser307 and resulting inhibition of insulin signaling, increased TNFα levels can also activate SOCS3 (11) to promote insulin resistance (26). SOCS3 can inhibit the insulin pathway through phosphorylation of the insulin receptor on tyrosine 960, which blocks the interaction between insulin receptor and IRS-1 (26). Since pioglitazone was able to significantly reduce TNFα levels, we also wanted to investigate whether SOCS3 and IRtyr960 levels were attenuated with pioglitazone treatment. Our results show that pioglitazone significantly decreased SOCS3 levels in the diabetic rats, which led to a decrease in the phosphorylation of IRtyr960 (Figure 4). This suggests that pioglitazone restores insulin signal transduction likely through inhibition of both IRS-1ser307 and IRtyr960.

Pioglitazone significantly increased IGFBP-3 protein levels in the retinas of type 2 obese BBZDR/Wor rats, likely associated with increased insulin receptor activity. While
Pioglitazone clearly decreased TNFα and SOCS3 actions, we also wanted to measure whether pioglitazone could increase insulin receptor phosphorylation. Additionally, since we recently showed that IGFBP-3 could induce insulin receptor phosphorylation in the retina of diabetic rats, we measured IGFBP-3 levels (27). Figure 5 demonstrates that obese animals have reduced IGFBP-3 levels, which are restored with pioglitazone treatment. This is the first demonstration that pioglitazone can regulate IGFBP-3 levels in diabetic rats. Additionally, pioglitazone significantly restored insulin receptor phosphorylation in the type 2 diabetic rats (Figure 5B). These results show that pioglitazone likely works in multiple pathways to restore normal glucose levels, both through inhibition of TNFα and SOCS3 inhibitory pathways, as well as restoration of insulin activating pathways.

Apoptotic markers were decreased after pioglitazone treatment in the retinas of type 2 diabetic obese rats. The key outcome of normal insulin signal transduction is increased phosphorylation of Akt and inhibition of apoptosis. Data in Figure 6 demonstrates that type 2 diabetic rats have increased apoptotic markers (Figure 6C-E), which were significantly reduced by pioglitazone treatment. Retinal lysates from the BBZDR/Wor obese rats had decreased anti-apoptotic markers, which were increased after pioglitazone therapy (Figure 6A,B). Taken with the other animal findings, these data demonstrate that pioglitazone is highly effective in reducing insulin resistance in whole retina of type 2 diabetic rats, leading to increased survival of retinal cells and improved function.

Pioglitazone increased PPARγ activity in both REC and Müller cells. Pioglitazone was highly effective in protecting whole retinal function and insulin signaling. In order to dissect which cell types might be most responsive to pioglitazone actions, we investigated the effects of pioglitazone on insulin signal transduction in both REC and Müller cells cultured in normal and high glucose to mimic diabetic-like conditions. We chose to focus on these cell types, as we have previously reported that TNFα is key to impaired insulin signaling in both REC (9) and Müller cells (22). Before we could initiate these studies, we first needed to determine the optimal dose of pioglitazone to increase PPARγ activity in each cell type. Because we used 25mg/kg in vivo, we started cell work in both REC and Müller cells at 25μM pioglitazone. This dose was effective in significantly increasing PPARγ activity in REC cultured in high glucose (Figure 7A); however, it did not significantly increase activity in Müller cells. Therefore, we increased the dose of pioglitazone to 50μM pioglitazone and found that this dose was able to significantly increase PPARγ activity (Figure 7B). In the remaining cell culture studies, we treated REC with 25μM pioglitazone and Müller cells with 50μM pioglitazone to maximize PPARγ activation. It is unclear why a higher concentration of pioglitazone is required to significantly increase PPARγ activity in rat Müller cells versus human REC. This will be a focus of further studies.

The pioglitazone-induced increase in PPARγ activity led to a significant reduction in apoptotic markers in both REC and Müller cells. Since the key outcome of restoration of normal insulin signal transduction is prevention of apoptosis, the first question we addressed in both REC and Müller cells was whether pioglitazone reduced apoptotic markers (Bax, cytochrome C, caspase 3), while increasing anti-apoptotic markers (Akt, Bcl-xL). We found that in both REC (Figure 8A-E) and Müller cells (Figure 8F-J), pioglitazone increased levels of key anti-apoptotic factors (Figure 8A-B, F-G). As would be expected with increased anti-apoptotic levels, pioglitazone also decreased pro-apoptotic proteins in both cell types (Figure 8C-E, H-J). Data suggest that pioglitazone likely protects the retina through reduction of apoptosis in both REC and Müller cells.

Pioglitazone treatment reduced TNFα and IRS-1Ser307 phosphorylation in both REC and Müller cells. We have previously reported that high glucose significantly increased TNFα and IRS-1Ser307 in both REC (9) and Müller cells (10), which was associated with increased apoptosis.
In this study, we wanted to ascertain whether pioglitazone could reduce the glucose-dependent increase in TNFα and IRS-1\textsuperscript{Ser307} in REC and Müller cells. Figure 9 showed that high glucose increased TNFα and IRS-1 in both REC and Müller cells, which matches previously published work. Pioglitazone significantly reduced both TNFα (Figure 9A,C) and IRS-1\textsuperscript{Ser307} (Figure 9B-D) in both REC (Figure 9A,B) and Müller cells (Figure 9C, D). This data suggests that pioglitazone works to protect retinal cells by blocking TNFα-induced insulin resistance.

**Pioglitazone treatment also significantly decreased SOCS3 and IR\textsuperscript{Tyr960} in REC and Müller cells.** To insure that pioglitazone works similarly in both REC and Müller cells as it did in whole retinal lysates, we also measured SOCS3 and IR\textsuperscript{Tyr960} in both cell types after high glucose exposure. High glucose significantly increased both SOCS3 and IR\textsuperscript{Tyr960}, as we have reported previously (9,10). Based on the reduced TNFα levels, it was expected that SOCS3 and IR\textsuperscript{Tyr960} would be reduced in both cell types after pioglitazone. Indeed, Figure 10 demonstrates that pioglitazone significantly reduced SOCS3 (Figure 10A, C), as well as IR\textsuperscript{Tyr960} phosphorylation (Figure 10B,D). Combining all the data presented, pioglitazone restored insulin signal transduction in the retina through a reduction in TNFα- and SOCS3-induced insulin resistance in both REC and Müller cells. This restoration of normal insulin signaling inhibited apoptosis in retinal cells, as well as improved retinal function as shown by increased ERG amplitudes in treated diabetic rats.

**PPARγ antagonist T0070907 antagonized pioglitazone effects on TNFα, SOCS3 and IR\textsuperscript{Tyr1150/1151} on REC and Müller cells.** To verify that actions on insulin signaling occur through pioglitazone activity and not through an alternative pathway, we treated both REC and Müller cells with both pioglitazone as we did above, with some cells pre-treated with a highly selective PPARγ antagonist, T0070907, at 50nM for 30 minutes prior to pioglitazone administration. As a control for these studies to verify T0070907 is an effective inhibitor of pioglitazone, the PPARγ activity assay was completed for REC (Figure 11A) and Müller cells (Figure 11E), demonstrating that 50nM T0070907 was effective in reducing pioglitazone actions in the cells. Data demonstrate that pioglitazone decreased TNFα (Figure 11B, F) and SOCS3 (Figure 11C, G) levels, while increasing IR\textsuperscript{Tyr1150/1151} (Figure 11D, H), as we reported above. TNFα levels were much higher in the rMC-1 cells, likely due to the new modification of the TNFα ELISA from Pierce; however, the PPAR inhibitor had the same actions in both REC and Müller cells. Use of the PPARγ inhibitor showed that pioglitazone actions on TNFα, SOCS3 and IR\textsuperscript{Tyr1150/1151} occur through PPARγ activities, as all pioglitazone actions were inhibited by T0070907. Taken together, this data further support that pioglitazone regulates insulin signaling through PPARγ actions.

**Discussion**

The major finding of our study is that pioglitazone treatment in vitro of retinal cells cultured in high glucose, as well as treatment in vivo of type 2 diabetic BBZDR/Wor rats, led to a direct increase in endogenous PPARγ and a concomitant decrease in a variety of cell death markers as well as improvement in retinal function. Pioglitazone is known to increase PPARγ in other tissues; pioglitazone treatment has been reported to improve insulin sensitivity in patients with type 2 diabetes. The intent of our study was to utilize the BBZDR/Wor rat model and retinal cell culture to establish the mechanisms of action of PPARγ in protection of retinal tissue against glucose-induced retinopathy. Our results reported here, show that the BBZDR/Wor model exhibits all the expected features of Type 2 diabetes as reported by previous work from our lab and others, including diabetes-induced changes in TNFα/ SOCS3 pathways. Thus the BBZDR/Wor model provided a valid platform for full evaluation of the mechanisms of action of the target drug, pioglitazone.
Our data demonstrate that pioglitazone is effective in increasing retinal PPARγ levels in vivo, which in turn protects against cell death associated with diabetic retinopathy. This is in agreement with reports that pioglitazone enhancement of PPARγ protects against retinal cell death in a variety of other retinal degeneration models including the streptozotocin-induced diabetic rat model (2), the ischemia/reperfusion damage model in rats (28), and the rat model of optic nerve crush damage (29). The pioglitazone-induced increase in PPARγ activity in whole retina is likely responsible for the improvement in the ERG, as others have reported that pioglitazone improved the ERG in the ischemia/reperfusion model in rats (28).

Our results suggest that pioglitazone likely works to increase PPARγ activity in the rat retina through increased activity in both REC and Müller cells. We found that high glucose culturing conditions significantly reduced PPARγ activity in both human REC and rat Müller cells. Reduced PPARγ is linked to increased apoptosis since pioglitazone treatment enhanced PPARγ and lowered apoptotic rates in these cells. Others have reported a similar link using bovine retinal endothelial cells (2). Likewise the link between PPARγ and protection against apoptosis has been shown in the study by Zhang et al., demonstrating that pioglitazone reduced glial fibrillary acidic protein (GFAP) levels, which is indicative of Müller cell activation and also occurs in Müller cells cultured in high glucose conditions (30). In the rat model of optic nerve crush damage, the authors also report that pioglitazone reduced Müller cell activation (29). Thus, our observations in BBZDR/Wor rats and in REC and Müller cell culture support these earlier findings and strengthen the view that REC and Müller cells mediate the protective actions of PPARγ.

Since pioglitazone improves insulin signaling in type 2 diabetic humans, we used our in vivo and in vitro models to determine which PPARγ pathways are responsible for restoring insulin signaling, thereby reducing apoptosis and improving retinal function. One of the key proteins involved in inducing insulin resistance is TNFα (31,32). Our data clearly demonstrate that pioglitazone reduces TNFα levels, leading to a reduction in IRS-1Ser307 phosphorylation and an increase in insulin receptor resistance through PPARγ actions. These findings were observed in vivo, as well as in cultured REC and Müller cells treated with pioglitazone. Our findings are in agreement with work in cholestatic rats treated with pioglitazone for gastric ulcer (33). Similar results of decreased TNFα after pioglitazone were also noted in the fat tissues of db/db mice (34).

In addition to a direct action of TNFα on insulin resistance through phosphorylation of IRS-1 on serine 307 (24), TNFα can also induce SOCS3, which in turn, can result in insulin resistance through phosphorylation of insulin receptor on tyrosine 960 (26). Kanatani et al., report that pioglitazone reduced SOCS3 in db/db mice, as well as in 3T3 adipocytes (34). Additional work in rats maintained on high cholesterol fructose diet and treated with pioglitazone showed reduced levels of both TNFα and SOCS3 in serum, as well as hepatic tissues (13). Taken together, our findings and the findings of others demonstrate that increases in TNFα and SOCS3 lead to insulin resistance and that pioglitazone enhancement of PPARγ can reverse these effects.

We have previously reported that increased TNFα and SOCS3 levels are associated with apoptosis in both REC and Müller cells (9,10). Our data suggest that TNFα/SOCS3 pathways work through insulin receptor resistance to trigger apoptosis. In both REC (9) and Müller cells (10) we have previously shown that insulin receptor resistance blocks induction of the anti-apoptotic factor, Akt and thus triggers apoptosis.

In conclusion, we have used type 2 diabetic BBZDR/Wor obese rats and cell cultures of REC and Müller cells to establish the pathway by which pioglitazone enhances PPARγ to block TNFα/SOCS3-induced insulin receptor resistance and thereby protect diabetic retinal tissue from apoptosis. Additionally, pioglitazone actions to reduce apoptosis and restore insulin signaling in the retina occur in REC and Müller cells, suggesting that these two cell types are key to insulin actions in the retina.
These data indicate that pioglitazone is a good insulin-sensitizing agent for patients with type 2 diabetes, specifically those with diabetic retinopathy.

Acknowledgements: This work was supported by the National Eye Institute (R01-EY022330 to JJS); Juvenile Diabetes Research Foundation (2-2011-597 to JJS); Oxnard Foundation (JJS); Research to Prevent Blindness (James C. Fleming); National Eye Institute Vision Core Grant (PHS 3P30 EY013080 to Dianna Johnson).
References.


Figure Legends

Figure 1. Pioglitazone increased PPARγ activity in whole retinal lysates of type 2 diabetic rats. Western blot results from whole retina of lean (control), lean+pioglitazone (Ctrl+Pio), BBZDR/Wor obese (Diab) and BBZDR/Wor obese +pioglitazone (Diab+Pio). *P<0.05 vs. control, #P<0.05 vs. diabetic. Data are mean±SEM. N=5 for each group.

Figure 2. B-wave amplitudes are improved in diabetic rats treated with pioglitazone. Top panel shows ERG amplitudes for A-wave (left), B-wave (middle), and oscillatory potentials (right) before treatment was started and the bottom panel shows the effects of 2 months of pioglitazone treatment. *P<0.05 vs. diabetic. Data are mean±SD. N=5 in all groups.

Figure 3. Pioglitazone reduced TNFα and IRS-1Ser307 in type 2 diabetic rats. For both panels, data presented included whole retinal analyses of lean (control), lean+pioglitazone (Ctrl+Pio), BBZDR/Wor obese (Diab) and BBZDR/Wor obese +pioglitazone (Diab+Pio) treated animals. Panel A shows ELISA results for TNFα and Panel B presents Western blot results for IRS-1Ser307. *P<0.05 vs. control, #P<0.05 vs. diabetic. Data are mean±SEM. N=5 in all groups.

Figure 4. SOCS3 and IR Tyr960 are reduced in pioglitazone treated type 2 diabetic rats. For both panels, data presented included whole retinal analyses of lean (control), lean+pioglitazone (Ctrl+Pio), BBZDR/Wor obese (Diab) and BBZDR/Wor obese +pioglitazone (Diab+Pio) treated animals. Panel A shows Western blot results for SOCS3 and Panel B presents Western blot results for IR Tyr960. *P<0.05 vs. control, #P<0.05 vs. diabetic. Data are mean±SEM. N=5 in all groups.

Figure 5. IGFBP-3 and phosphorylation of insulin receptor increased after pioglitazone treatment. For both panels, data presented included whole retinal analyses of lean (control), lean+pioglitazone (Ctrl+Pio), BBZDR/Wor obese (Diab) and BBZDR/Wor obese +pioglitazone (Diab+Pio) treated animals. Panel A shows Western blot results for IGFBP-3 and Panel B presents Western blot results for IR Tyr1150/1151. *P<0.05 vs. control, #P<0.05 vs. diabetic. Data are mean±SEM. N=5 in all groups.

Figure 6. Apoptotic markers are reduced in type 2 diabetic rats treated with pioglitazone. For both panels, data presented included whole retinal analyses of lean (control), lean+pioglitazone (Ctrl+Pio), BBZDR/Wor obese (Diab) and BBZDR/Wor obese +pioglitazone (Diab+Pio) treated animals. Panel A,B shows Western blot results for the anti-apoptotic proteins (Akt and Bel-xL). Panel B presents Western blot results for pro-apoptotic proteins (Bax, cytochrome C, and cleaved caspase 3). *P<0.05 vs. control, #P<0.05 vs. diabetic. Data are mean±SEM. N=5 in all groups.

Figure 7. PPARγ activity is increased in retinal endothelial cells (REC) and Müller cells after pioglitazone. For both REC and Müller cells, cells were grown in normal glucose (NG) or high glucose (HG). For REC, cells were treated with 25uM pioglitazone, while Müller cells were untreated (NT), treated with 25uM or 50uM pioglitazone. *P<0.05 vs. NG NT, #P<0.05 vs. HG NT. Data are mean±SEM. N=4 for all treatments.

Figure 8. Pro-apoptotic proteins are reduced in REC and Müller cells treated with pioglitazone. All work was done in REC and Müller cells left untreated (NT) or treated with pioglitazone. Panels A-E present data in REC, while panels F-J are Müller cell data. Data show that pioglitazone reduced pro-apoptotic proteins (C-E and H-J) in both cell types. *P<0.05 vs. NG NT, #P<0.05 vs. HG NT. Data are mean±SEM. N=4 for all treatments.
Figure 9. TNFα and IRS-1Ser307 are reduced in both REC and Müller cells after pioglitazone treatment. All work was done in REC and Müller cells left untreated (NT) or treated with pioglitazone. Panels A, B present data in REC, while panels C, D are Müller cell data. Data show that pioglitazone reduced TNFα and IRS-1Ser307 in both cell types. *P<0.05 vs. NG NT, #P<0.05 vs. HG NT. Data are mean ± SEM. N=4 for all treatments.

Figure 10. SOCS3 and IR Tyr960 are reduced by pioglitazone. All work was done in REC and Müller cells left untreated (NT) or treated with pioglitazone. Panels A, B present data in REC, while panels C, D are Müller cell data. Data show that pioglitazone reduced SOCS3 and IR Tyr960 in both cell types. *P<0.05 vs. NG NT, #P<0.05 vs. HG NT. Data are mean ± SEM. N=4 for all treatments.

Figure 11. PPARγ antagonist T0070907 (50nm) blocks pioglitazone actions on TNFα, SOCS3, and IR Tyr1150/1151. All work was done in REC and Müller cells left untreated (NT), treated with pioglitazone, treated with T0070907 only, or pioglitazone+T0070907. Panels A-D represent REC, while panels E-H present Müller cell results. Panels A, E show that T0070907 inhibited pioglitazone actions using the PPARγ activity assay. Panels B, F demonstrate that T0070907 antagonized pioglitazone actions on TNFα, while panels C, G show T0070907 actions on SOCS3. Panels D, H show that pioglitazone’s ability to increase IR Tyr1150/1151 requires PPARγ, as it was blocked by T0070907. *P<0.05 vs. NG NT, #P<0.05 vs. HG NT, $P<0.05 vs. HG+Pio. Data are mean ± SEM. N=4 for all treatments.
<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Body Weight (g)</th>
<th>Glucose</th>
<th>IOP(mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>5</td>
<td>475.5±21.8</td>
<td>96.7±20.1</td>
<td>10.1±1.8</td>
</tr>
<tr>
<td>Ctrl+pio</td>
<td>5</td>
<td>470.3±27.5</td>
<td>82.3±15.2</td>
<td>9.8±1.2</td>
</tr>
<tr>
<td>Diab</td>
<td>5</td>
<td>661±16.5*</td>
<td>524.8±34.1*</td>
<td>9.1±1.1</td>
</tr>
<tr>
<td>Diab+pio</td>
<td>5</td>
<td>900±38.1#</td>
<td>89±22.9#</td>
<td>9.5±1.6</td>
</tr>
</tbody>
</table>

Table 1. Summary data for lean (control), lean+pioglitazone (25mg/kg IP, control+Pio) for 2 months, obese (diab), and obese + pioglitazone (Diab+Pio). *P<0.05 vs. lean, #P<0.05 vs. Diab.
Figure 1

Ratio of PPAR γ to β actin (A.U.)

- Ctrl
- Ctrl+pio
- Diab.
- Diab.+pio

Significance indicated by asterisks and hash symbols.
Figure 3

A.

![Graph showing TNF-α ELISA (pg/mL) for different treatments.](image)

- **Ctrl**: Control
- **Ctrl+pio**: Control + Pio
- **Diab.**: Diabetic
- **Diab.+pio**: Diabetic + Pio

B.

![Graph showing ratio of p-IRS1 (Ser307) for different treatments.](image)

- **Ctrl**: Control
- **Ctrl+pio**: Control + Pio
- **Diab.**: Diabetic
- **Diab.+pio**: Diabetic + Pio
Figure 4

A.

SOCS3
β actin

B.

p-IR960
IR

Ratio of SOCS3 to β actin (A.U.)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Ctrl</th>
<th>Ctrl+pio</th>
<th>Diab.</th>
<th>Diab.+pio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio</td>
<td>0.4</td>
<td>0.6</td>
<td>*</td>
<td>#</td>
</tr>
</tbody>
</table>

Ratio of p-IR(960) to total IR (A.U.)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Ctrl</th>
<th>Ctrl+pio</th>
<th>Diab.</th>
<th>Diab.+pio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio</td>
<td>0.6</td>
<td>0.8</td>
<td>*</td>
<td>#</td>
</tr>
</tbody>
</table>
Figure 5

A. Ratio of IGFBP-3 to β-actin (A.U.)

B. Ratio p-IR (Tyr1150) to total IR (A.U.)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Ctrl</th>
<th>Ctrl+pio</th>
<th>Diab.</th>
<th>Diab.+pio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio IGFBP-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio β-actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Ctrl+pio</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diab.</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Diab.+pio</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td>1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Ctrl</th>
<th>Ctrl+pio</th>
<th>Diab.</th>
<th>Diab.+pio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio p-IR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio IR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>Ctrl+pio</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>Diab.</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>Diab.+pio</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
</tr>
</tbody>
</table>
Figure 6

A.  

Ratio of p-Akt to total Akt (A.U.)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Ctrl</th>
<th>Ctrl+pio</th>
<th>Diab.</th>
<th>Diab.+pio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.6</td>
<td>0.4</td>
<td>0.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>

B.  

Ratio of Bcl-xL to β-actin (A.U.)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Ctrl</th>
<th>Ctrl+pio</th>
<th>Diab.</th>
<th>Diab.+pio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.8</td>
<td>0.6</td>
<td>0.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>

C.  

Ratio of Bax to β-actin (A.U.)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Ctrl</th>
<th>Ctrl+pio</th>
<th>Diab.</th>
<th>Diab.+pio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.0</td>
<td>1.5</td>
<td>1.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

D.  

Ratio of Cytochrome C to β-actin (A.U.)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Ctrl</th>
<th>Ctrl+pio</th>
<th>Diab.</th>
<th>Diab.+pio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.75</td>
<td>0.5</td>
<td>0.3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

E.  

Cleaved Caspase-3 (O.D.)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Ctrl</th>
<th>Ctrl+pio</th>
<th>Diab.</th>
<th>Diab.+pio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2</td>
<td>0.1</td>
<td>0.05</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Figure 7

REC

Muller

PPARγ Activity/Control

NG-NT  NG-PIO  HG-NT  HG-PIO

NG-NT  NG-PIO25  NG-PIO50  HG-NT  HG-PIO25  HG-PIO50
Figure 9

A. 

<table>
<thead>
<tr>
<th></th>
<th>NG-NT</th>
<th>NG-PIO</th>
<th>HG-NT</th>
<th>HG-PIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF α ELISA (pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>20</td>
<td>25</td>
<td>15</td>
</tr>
</tbody>
</table>

B. 

<table>
<thead>
<tr>
<th></th>
<th>NG-NT</th>
<th>NG-PIO</th>
<th>HG-NT</th>
<th>HG-PIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio of phospho-IRS-1 (Ser307) to total IRS1 (A.U.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.7</td>
<td>0.6</td>
<td>0.8</td>
</tr>
</tbody>
</table>

C. 

<table>
<thead>
<tr>
<th></th>
<th>NG</th>
<th>NG-PIO</th>
<th>HG</th>
<th>HG-PIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF α ELISA (pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>20</td>
<td>25</td>
<td>15</td>
</tr>
</tbody>
</table>

D. 

<table>
<thead>
<tr>
<th></th>
<th>NG</th>
<th>NG-PIO</th>
<th>HG</th>
<th>HG-PIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio of p-IRS1(Ser307) to IRS1 (A.U.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.7</td>
<td>0.6</td>
<td>0.8</td>
</tr>
</tbody>
</table>
Pioglitazone normalizes insulin signaling in the diabetic rat retina through reduction in tumor necrosis factor α and suppressor of cytokine signaling 3
Youde Jiang, Shalini Thakran, Rajini Bheemreddy, Eun-Ah Ye, Hui He, Robert J Walker and Jena J. Steinle

J. Biol. Chem. published online August 1, 2014

Access the most updated version of this article at doi: 10.1074/jbc.M114.583880

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2014/08/01/jbc.M114.583880.full.html#ref-list-1