Rosiglitazone modifies PEDF gene expression and protein levels in the Koletsky rat choroid-RPE complex

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Abstract

The purpose of this study was to determine whether rosiglitazone, a peroxisome proliferator-activated receptor (PPAR)γ ligand, altered growth factor expression in the choroid-RPE complex of Koletsky rats. Using lean and obese Koletsky rats fed a normal or rosiglitazone-supplemented diet, gene and protein expression of vascular endothelial growth factor (VEGF), pigment epithelial derived growth factor (PEDF), angiopoietin 1, and its receptor, Tie-2, were assessed. Both gene expression and protein expression of PEDF were significantly increased in both obese and lean Koletsky rats treated with rosiglitazone diet. Gene expression of VEGF, angiopoietin-1 and Tie-2 were increased after rosiglitazone treatment, without changes in protein expression. Since PPARγ ligands have been implicated in vascular remodeling, these results suggest that upregulation of PEDF expression may be a potential mechanism in the rat choroid-RPE complex.

Keywords

Choroid • Koletsky • Growth factors • PPAR gamma • RPE

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Introduction

It is clear that neovascularization of the choroid can lead to the vision loss associated with macular degeneration. This is a common component of “wet” age-related macular degeneration [1]. A number of agents are currently in clinical trials to prevent this neovascularization in the choroid, which is the leading cause of blindness in elderly adults. Most are focused on eliminating the stimulus of vascular endothelial growth factor (VEGF), as it is clear that this factor is important in the pathology. In addition to blocking VEGF, other work is focused on pigment epithelial derived factor (PEDF) since it is an endogenous inhibitor of angiogenesis and can protect the immature neuron cells from apoptosis [2]. Since PEDF often changes in the opposite direction of VEGF [3], PEDF may contribute to maintaining the equilibrium of neovascularization in ocular tissues. It is known to be downregulated in human and experimental conditions with ocular neovascularization, such as proliferative retinopathy and exudative AMD [3, 4]. In addition to regulating PEDF and VEGF, other agents are under investigation to regulate the choroidal vasculature, including peroxisome proliferator-activated receptor (PPAR)γ ligands. One such ligand, rosiglitazone has been of particular interest in diabetes, as it has been shown to alter insulin sensitivities, a common problem in type 2 diabetes [5]. Much of the research on the effects of rosiglitazone has been done in animal models of ocular neovascularization, however, little has been done to investigate the effects of PPARγ ligands on the normal (non-diseased) choroid.

In order to best mimic a normal diabetic choroid, a number of animal models are available. One animal that is proposed for such work is the Koletsky rat. This rat strain was obtained by mating a spontaneously hypertensive female rat from the Kyoto Wistar strain with a normotensive Sprague-Dawley male rat in the laboratory of Simon Koletsky in 1969 [6, 7]. This new strain is characterized by genetic obesity, hypertension, hyperlipidemia, and proteinuria with kidney disease, the primary symptoms of type 2 diabetes [6, 7]. The Koletsky rat is rarely used for studies on the primary vascular bed of the eye, the choroid. Furthermore, it is
becoming increasingly clear that the choroid can be damaged in diabetes, in addition to the retina [8].

In order to better understand potential mechanisms by which rosiglitazone could alter the choroidal vasculature, these preliminary experiments investigated a number of choroid growth factors known to be involved in vascular changes. For these studies, we used lean and obese Koletsky rats treated with rosiglitazone (a PPAR γ ligand). Key growth factors investigated include vascular endothelial growth factor (VEGF), angiopoietin1 (Ang1) and its receptor (Tie2) and pigment epithelial derived factor (PEDF).

**Materials and Methods**

**Animals, diets, and experimental design.** Experiments were conducted using lean (F/?) and obese (f/f) male Koletsky rats obtained from Charles River Genetic Models (Indianapolis, IN). Animals were housed individually in stainless steel wire-mesh cages at 21°C in a room with an automatically controlled 12-h light:dark cycle. Rats (3 months old) were acclimated to the environment and were provided unlimited access to non-purified diet (Purina, St. Louis MO) and water prior to receiving drug treatments. Animals were assigned to treatments so that each group was balanced for body weight and provided unlimited access to one of two experimental diets (n = 7 lean and 7 obese for each experimental diet) for 12 wks. Semi-purified, powdered diets based on American Institute of Nutrition recommendations [10] were modified to be high sucrose and high saturated fat. Diets were identical with the following exceptions: the control (C) diet was combined with 0.01% rosiglitazone (CR) to make the treatment diet. All experimental protocols for animal care and use were approved by the Animal Care and Use Committee of Southern Illinois University, Carbondale, IL.

**Body Weight, Organ Weight and Plasma Measurements.** Body weight was measured weekly and weight change was calculated at the completion of the study. At week 12, animals were fasted overnight and decapitated. Trunk blood was collected in heparinized tubes (25µl/ml blood) and placed on ice. In addition,
kidney, heart, and liver were weighed. Extracted plasma was utilized to measure fasting triacylglyceride, cholesterol, glucose and insulin levels.

Quantitative determination of total triacylglyceride and cholesterol was carried out on plasma samples using Triglycerides GPO Reagent (Catalog #445850; Beckman Coulter, Fullerton, CA) and Cholesterol Reagent (Catalog #467825; Beckman Coulter, Fullerton, CA) in conjunction with the Synchron CX Systems CX Multi™ Calibrator (Catalog #442600; Beckman Coulter, Fullerton, CA) and Synchron Multi-Level Controls (Catalog #657365; Beckman Coulter, Fullerton, CA).

Quantitative determination of final glucose concentrations were carried out on plasma samples using the Glucose Reagent (Beckman Coulter, Cat # 442640) in conjunction with the Synchron CX Systems CX Multi™ Calibrator (Beckman Coulter, Cat # 442600) and Synchron Multi-Level Controls (Beckman Coulter, Cat # 657365). The CX-4 requires 30μl of plasma for the timed endpoint assay that monitors the change in absorbance at 340nm. The resultant concentration of glucose is expressed in mg/dl.

Quantitative determination of insulin was carried out on plasma samples using an ALPCO Ultrasensitive Insulin ELISA (Catalog # 008-10-1121-01; Windham, NH). All physiological data were analyzed by one-way analysis of variance (ANOVA) and post-hoc comparisons (Fisher’s Least Significant Difference) were made (significant at P<0.05) when appropriate (SYSTAT® 10.0, SPSS Incorporated, Chicago, IL, USA). Data are presented as mean ± SEM.

RNA Isolation and Reverse Transcription (RT) of choroid from not-treated and treated rats. Twelve weeks after diets were administered to the rats, animals were euthanized, and the eyes were removed. The cornea, lens, retina, and aqueous humor were discarded, and RNA was isolated from the choroid and retinal pigmented epithelial (RPE) complex of 4 rats from each of the control and treated-diet groups using TriReagent® (Molecular Research Center, Inc.). RNA isolation was performed using chloroform and isopropanol. RNA purity was detected by agarose gel electrophoresis, and RNA concentration was measured spectrophotometrically. Reverse transcription of 1 μg RNA for cDNA synthesis was carried
out using an Improm II Kit (Promega, Madison, WI). The reaction mixture (DEPC water, Improm II 5X reaction buffer, 25 mM MgCl₂, 10 mM dNTP, and 20 Units RNAsin, 1μM oligo dT and 1μM reverse strand18srRNA) and 1μg RNA were incubated for 60 minutes at 42°C, followed by heat-inactivation of the reverse transcriptase enzyme at 70°C for 15 minutes. 0.2 μl RNAse inhibitor (10 mg/ml) was added, followed by incubation for 30 minutes at 37°C. Samples were stored at −20°C for real-time PCR.

**Real-time Polymerase Chain Reaction (RT-PCR) Analysis of Gene Expression.** Real-time PCR primers to detect rat VEGF, PEDF, angiopoietin1, and Tie-2 were designed using the GCG Software Prime. Primers were chosen to generate an amplicon smaller than 150 base pairs. The sequences of the PCR primer pairs (5' to 3') that were used for each gene are as follows:

VEGF ACGAAAGCGCAAGAAATCCC (forward) and TAACTCAAGCTGCCTCGCC (reverse);
KDR TAGCACGACAGAGACTGTGAGG (forward) and TGAGGTAGAGATGGGTAGG (reverse);
Angiopoietin 1 CATGCTTGAGATAGGAACCAG (forward) and TTCAAGGAGATGCTTGGATTT (reverse);
Tie-2 CGGCTTAGTTCTCTGTGGAGG (forward) and GGCATCAGAAGACAGGAT (reverse);
PEDF AAGAGTGCTTCCAGAATTGTG (forward) and CCCAGGTCTTAAATCTTCAT (reverse);
18s rRNA TCAAGAACGAAAGTCGGAGGTT (forward) and GGACATCTAAGGGCATCAG (reverse).

Real-time PCR reactions were performed using iQ™SYBR® Green Supermix (100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM of each dNTP, iTaq DNA Polymerase, 50 units/ml, 6 mM MgCl₂, SYBR Green I, 20 nM fluorescein, and stabilizers; Bio-Rad, Hercules, CA). Thermocycling was done in a final volume of 25 μl (8 μl DEPC H₂O, 2 μl cDNA, 1.25 μl=500 nM of each primer, and 12.5 μl of 2 X iQ™SYBR® Green Supermix) using the PCR conditions of initial heating at 95°C for
300 seconds to denature cDNA and activate the Taq DNA Polymerase, followed by 45 cycles consisting of denaturation at 95°C for 20 seconds, annealing at 58°C for 20 seconds, and extension at 72°C for 20 seconds using a Smart Cycler®. One additional step, a melt curve, was added to determine specificity. The melt curve was constructed by increasing the temperature from 60°C to 95°C with a temperature transition rate of 0.2°C/second. To ensure that the correct product was amplified, all samples are separated by 1.2% agarose gel electrophoresis.

To correct for differences in both RNA quality and quantity between samples, data was normalized using the ratio of the target cDNA concentration to that of 18srRNA. To calculate the fold increases in steady-state RNA levels, ΔCT values for lean Koletsy (LK) or obese Koletsy (OK) rat choroid expression of VEGF, PEDF, Ang1, and Tie-2 were calculated by subtracting from their threshold (CT) value the corresponding 18srRNA CT (internal control) value. Then ΔCT values were calculated by subtracting the average of ΔCT values of C diet rat choroid from ΔCT value of rosiglitazone-treated (CR) rat choroidal expression of the gene of interest. Changes in steady-state gene expression are reported as fold increases ($2^{-\Delta\Delta CT}$) relative to not-treated (C) rat choroid. The statistical analysis of steady-state RNA levels is based upon previous work by other groups [10]. Significance in the ($2^{-\Delta\Delta CT}$) between the C and CR fed rats in each of the LK and OK rat colonies were analyzed at P<0.05 using Prism Software (GraphPad, San Diego, CA).

**Western Blot Analysis.** Whole globes were removed from the not-treated and rosiglitazone-treated lean and obese rats. The lens, vitreous, and retina were removed from the eyecup. The remaining choroid-RPE complex was placed into cold lysis buffer (50mM Tris-HCl, pH 7.4; 1% NP-40, 0.25% Na-deoxycholate; 150mM NaCl; 1mM EDTA; 1mM PMSF; 1μg/ml each of aprotinin, leupeptin, pepstatin; 1mM Na3VO4; 1mM NaF; 0.1% SDS) for homogenization. Choroidal-RPE complex samples were assayed for total protein content and then stored at −80°C.

Denaturing sample buffer (1mL 2X GDW, 640μL 1M Tris-HCL pH 6.8, 420μL 30% glycerol, 250μL β-mercaptoethanol, 200μL 0.05% bromophenol blue, and
0.125g recrystallized SDS) was added to 30μg of protein, as determined by Bradford assay. Protein samples were separated on 4–12% pre-cast tris-glycine gels (Invitrogen, Carlsbad, CA). Gels were ran at 130V for one and a half hours and then blotted onto a nitrocellulose membrane at 30V for one and a half hours. For antibody detection, the membrane was blocked overnight at 4°C in block buffer (1mM Tris pH 7.5, 150mM NaCl, and 0.05% Tween) with 5% dry milk. Primary polyclonal antibodies to PEDF (Bioproducts, Middletown, MD, diluted 1:1000), VEGF (isoform 165, Chemicon, Temecula, CA, diluted 1:500), angiopoietin1 (Alpha Diagnostic, San Antonio, TX, diluted 1:1000), and Tie-2 (Chemicon, diluted 1:1000) were applied overnight at 4°C with shaking. The following day, membranes were probed with horseradish peroxidase-conjugated anti-rabbit secondary antibodies applied at a 1:10000 dilution at room temperature for two hours. Immunoreactive bands were detected by enhanced SuperSignal (Pierce, Rockford, IL) and analyzed using the Kodak 2000R image station. Mean densitometry was assessed using Kodak software. A T-test was performed between the LK and LKR and OK and OKR groups with significance accepted at P<0.05. Equal loading was verified by staining of the membrane with Ponceau S after chemilluminescence.

**Results**

*Rosiglitazone prevents a number of the detrimental changes noted in the Koletsky rat.* Lean and obese Koletsky rats that were fed the control diet are compared to their phenotypic littermates fed the rosiglitazone-supplemented diet. It is clear that the obese Koletsky rats have hyperlipidemia and hyperinsulinemia when compared to their lean littermates (Table 1). Insulin levels in the obese Koletsky rats were higher than that noted in other work using this animal model [7].
**Tab. 1.** Fasting plasma cholesterol, glucose, triglycerides, and insulin in lean and obese male Koletsky rats treated for 12 weeks.

| Diet       | Cholesterol (mg/dl) | Glucose (mg/dl) | Triglycerides (mg/dl) | Insulin (ng/ml) |
|------------|---------------------|-----------------|-----------------------|-----------------|
| **LEAN**  |                     |                 |                       |                 |
| Control    | 53.43±1.15          | 120.43±3.43     | 70.43±4.78            | 0.81±0.13       |
| Rosiglitazone | 60.85±3.05¹        | 116.29±3.55     | 63.43±5.96            | 0.50±0.07¹      |
| **OBESE** |                     |                 |                       |                 |
| Control    | 106.85±4.74         | 106.57±16.01    | 142.14±5.73           | 31.35±3.13      |
| Rosiglitazone | 97.28±3.74²        | 90.28±5.01      | 129.71±7.46²          | 19.30±3.44²     |

¹ significantly different vs. lean control
² significantly different vs. obese control

N=7 for all analyses

It was also evident that rosiglitazone treatment significantly (P<0.05) reduced fasting plasma cholesterol, triglyceride and insulin levels as compared to the untreated animals (Table 1). The obese Koletsky rats treated with rosiglitazone (OKR) also exhibited much larger (P<0.05) liver and heart weights versus untreated rats (OK), which is likely due to the significant increase in total body weight of the
obese Koletsky rat on the rosiglitazone diet (Table 2). On the other hand, kidney weight was unchanged in the rosiglitazone treated animals (Table 2).

The lean Koletsky rats treated with rosiglitazone (LKR) demonstrated a reduction ($P < 0.05$) in insulin levels and liver weight when compared to untreated rats (LK). On the other hand, the same animals had a significant ($P<0.05$) elevation in plasma cholesterol versus the untreated animals (Table 1 and 2). Most values for the lean rats are similar to those reported by others for this animal model.

mRNA expression of VEGF is increased in the choroid-RPE complex following rosiglitazone treatment in both lean and obese animals. Both lean and obese animals receiving the diet with rosiglitazone had significantly increased mRNA levels of VEGF ($P<0.05$, Figure 1A). Rosiglitazone did not alter protein expression of VEGF in either lean or obese Koletsky rats, as measured by densitometry (Figure 1B). Baseline protein values for VEGF are increased in the LK rat as compared to the obese rat before rosiglitazone treatment.

Both protein and mRNA expression of PEDF are increased with rosiglitazone treatment. The addition of rosiglitazone to the diet of both lean and obese Koletsky rats resulted in a significant increase in both protein ($P<0.05$, Figure 2B) and mRNA levels ($P<0.05$, Figure 2A) in the choroid-RPE complex. Ang1 mRNA levels in the choroid-RPE complex are increased following rosiglitazone treatment. Ang1 protein expression is not altered by rosiglitazone diet in either lean or obese Koletsky rats (Figure 3B). mRNA expression of Ang1 in the lean Koletsky rat is significantly increased ($P<0.01$, Figure 3A), however not to the level that it is increased in the obese Koletsky rat ($P<0.05$ Figure 3A). mRNA levels for Tie-2, the receptor for Ang1, were significantly increased in both lean and obese Koletsky rats after the rosiglitazone diet (Figure 4A). Protein expression was not altered in either group after rosiglitazone (Figure 4B); however, baseline values for Tie-2 protein were increased in the non-treated lean Koletsky as compared to the non-treated obese rat ($P<0.05$).
Fig. 1. *Panel A*. mRNA levels for VEGF in lean (left) and obese (right) Koletsky rats after feeding on a normal (LK or OK) or a rosiglitazone-supplemented (LKR or OKR) diet. mRNA expression was significantly increased in both lean and obese rats after rosiglitazone. *Panel B*. Bar graphs of protein expression for VEGF in lean (left) and obese (right) Koletsky rats. No significant changes in VEGF protein expression were noted. N=4, *P<0.05.

Fig. 2. *Panel A*. mRNA levels for PEDF in lean (left) and obese (right) Koletsky rats after feeding on a normal (LK or OK) or a rosiglitazone-supplemented (LKR or OKR) diet. mRNA expression was significantly increased in both lean and obese rats after rosiglitazone. *Panel B*. Bar graphs of protein expression for PEDF in lean (left) and obese (right) Koletsky rats. Significant changes in PEDF protein expression were noted in both the lean and obese rat choroid. N=4, *P<0.05,* **P<0.01.
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Fig. 3. **Panel A.** mRNA levels for angiopoietin-1 in lean (left) and obese (right) Koletsky rats after feeding on a normal (LK or OK) or a rosiglitazone-supplemented (LKR or OKR) diet. mRNA expression was significantly increased in both lean and obese rats after rosiglitazone. **Panel B.** Bar graphs of protein expression for angiopoietin-1 in lean (left) and obese (right) Koletsky rats. No significant changes in angiopoietin-1 protein expression were noted in either the lean and obese rat choroid. N=4, *P<0.05, **P<0.01.

Fig. 4. **Panel A.** mRNA levels for Tie-2 in lean (left) and obese (right) Koletsky rats after feeding on a normal (LK or OK) or a rosiglitazone-supplemented (LKR or OKR) diet. mRNA expression was significantly increased in both lean and obese rats after rosiglitazone. **Panel B.** Bar graphs of protein expression for Tie-2 in lean (left) and obese (right) Koletsky rats. No significant changes in Tie-2 protein expression were noted in either the lean and obese rat choroid. N=4, *P<0.05, **P<0.01.
Discussion

On the normal diet, the obese Koletsky rats develop hyperinsulinemia and hypercholesterolemia, as had been previously published for this rat strain [6, 7]. The addition of rosiglitazone to the diet elicited a number of alterations to both the lean and the obese Koletsky rat, including an increase in total body weight in obese animals. Total cholesterol, triglyceride and insulin levels were substantially reduced in the treated obese rat as compared to the obese rat on normal diet. Cumulatively, the addition of rosiglitazone to the diet appeared to lessen the diabetic phenotype in the obese animals.

In addition to characterizing the role of rosiglitazone on physiological and biochemical measurements, experiments were also designed to determine if changes following the rosiglitazone diet were noted in the choroid. Since PPARγ ligands have been suggested to be therapeutic for vascular lesions noted in type 2 diabetes [11], we were interested in whether they could alter gene and protein expression of key growth factors known to alter the vasculature of the choroid. Since VEGF is a key factor in diabetic changes in the retina [12], it was logical to assume that VEGF levels may also be increased in the choroid of a type 2 diabetic rat model. In fact, mRNA levels of VEGF were significantly increased in both the lean and obese Koletsky rat following rosiglitazone diet. While these results are interesting, it is important to note that the Koletsky rat has a mutation of the leptin gene, such that leptin levels in these rats can be up to 100-fold greater in some tissues [13]. Leptin has been reported to initiate an angiogenic response itself and can cause upregulation of VEGF [14]. The effects of rosiglitazone on the retina and choroid have only been investigated in vitro. In some experiments, rosiglitazone or other PPARγ ligands prevented neovascularization of these tissues [15, 16]. The exact mechanisms by which rosiglitazone alter VEGF mRNA levels and not protein expression in the choroid need further investigation in vivo and may be related to plasma leptin levels.
In a manner similar to VEGF, mRNA levels of Ang1 and its receptor Tie2 were significantly increased without changes in protein expression after animals consumed a diet supplemented with rosiglitazone. Since the actions of Ang1 occur later in the angiogenic cascade, it is less clear why protein expression is not increased in a manner similar to steady-state mRNA levels. Until a better understanding of the cellular actions of Ang1 and Tie2 is acquired, the effect of rosiglitazone inducing increased mRNA levels without protein expression will go unanswered.

Since it is proposed that PPARγ ligands prevent retinal and choroidal neovascularization, one potential mechanism, in addition to inhibiting VEGF actions, is to increase PEDF expression, a known angiostatic factor. In fact, both obese and lean rats that received the rosiglitazone-supplemented diet had increased protein and mRNA levels of PEDF. PEDF is reported to be largely anti-angiogenic in function at low doses, while it can promote angiogenesis at higher doses [17]. PEDF is a 50kDa protein that is a potent inhibitor of ischemia-driven retinal neovascularization [18, 19]. PEDF has also been shown to be down regulated in diabetic retinopathy [20]. PEDF was, however, found to be increased in the RPE cells in a case of experimental choroidal neovascularization [21]. Martin et al (2004) suggest that the observed increase in PEDF expression may be the result of completed neovascularization following the experimental damage. In the study by Martin, leptin levels were also increased in the same regions as PEDF. Since PEDF can inhibit the actions of leptin on VEGF in some targets [22], it is expected that they would be expressed in the same locations. The increased PEDF expression following rosiglitazone treatment may decrease leptin levels, leading to unaltered VEGF expression, which may explain the present results. Therefore, it is a strong possibility that rosiglitazone increases PEDF expression in the choroid-RPE complex, which helps to prevent the vascular lesions normally associated with the Koletsky rat. The clinical relevance and the specific mechanisms responsible for the rosiglitazone-induced changes in VEGF, PEDF, Ang1 and Tie2 need further investigation.
Future directions of these studies would include a dissection of whether the changes in VEGF and PEDF are occurring in the vasculature of the choroid or primarily in the RPE cells. Nonetheless, it is clear that RPE cells will signal to the choroid to elicit choroidal vascular changes in a number of disorders. Measurements of PPAR gamma and leptin levels in these experimental conditions would also help to answer some remaining questions and will be addressed in future work.

Overall, it appears that the addition of rosiglitazone, a PPAR$\gamma$ ligand, to the diet of obese and lean Koletsky rats results in substantial changes in the physiological and biochemical indices. These rosiglitazone-induced effects on the physiological and biochemical parameters appeared to lessen the diabetic phenotype in the obese animals. It also increases mRNA levels of VEGF, Ang1 and Tie2 without appreciable changes in protein expression in the choroid. Both gene and protein expression of PEDF are significantly increased, suggesting that this may be the mechanism by which rosiglitazone could prevent vascular remodeling noted in the choroid. These preliminary studies warrant further research investigating the effects of rosiglitazone and other PPAR$\gamma$ ligands on choroidal neovascularization in preclinical models.

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