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# Etanercept restores normal insulin signal transduction in $\beta$ 2-adrenergic receptor knockout mice

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## Abstract

**Background:** Inhibition of TNF $\alpha$  protects the retina against diabetic-like changes in rodent models. The mechanism by which TNF $\alpha$  induces deleterious retinal changes is not known. Previously, we have shown that TNF $\alpha$  can inhibit normal insulin signal transduction, leading to increased apoptosis in both retinal endothelial cells (REC) and Müller cells. Additionally,  $\beta$ 2-adrenergic receptor knockout mice ( $\beta$ 2KO) have increased TNF $\alpha$  levels and decreased insulin receptor activity. In this study, we hypothesized that inhibition of TNF $\alpha$  in  $\beta$ 2KO mice would increase normal insulin signaling, leading to improved retinal function.

**Methods:** C57BL6 or  $\beta$ 2KO mice were left untreated or treated with etanercept (0.3 mg/kg subcutaneously, 3 $\times$  a week) for 2 months. Electroretinogram analyses were done before treatment was initiated and after two months of treatment with etanercept on all mice. Western blot or ELISA analyses were done on whole retinal lysates from all four groups of mice for TNF $\alpha$ , suppressor of cytokine signaling 3 (SOCS3), insulin receptor, and apoptotic proteins.

**Results:** Etanercept significantly reduced TNF $\alpha$  levels in  $\beta$ 2KO mice, leading to increased insulin receptor phosphorylation on tyrosine 1150/1151. SOCS3 levels were increased in  $\beta$ 2KO mice, which were reduced after etanercept treatment. Pro-apoptotic proteins were reduced in etanercept-treated  $\beta$ 2KO mice. Etanercept improved ERG amplitudes in  $\beta$ 2KO mice.

**Conclusions:** Inhibition of TNF $\alpha$  by etanercept protects the retina likely through reduced TNF $\alpha$ -mediated insulin resistance, leading to reduced apoptosis.

**Keywords:** TNF $\alpha$ , SOCS3, Apoptosis, Insulin signaling

## Background

The role of sympathetic nerves in diabetes has not been questioned when focusing on peripheral nerve disease [1]. Work has demonstrated that diabetes and aging can both produce significant remodeling of sympathetic ganglia and neurotransmission [2]. However, the role of sympathetic nerve activity in the retina is less clear. Work by our group demonstrated that loss of dopamine beta hydroxylase (a key enzyme required for the production of norepinephrine) produces substantial changes to the retina [3]. Subsequently, we have shown that  $\beta$ -adrenergic

receptors are key to retinal damage, which is similar to retinal changes observed in animal models of diabetic retinopathy [4,5]. Recently, we showed that treatment of  $\beta$ 2KO mice with a novel  $\beta$ 1/ $\beta$ 2-adrenergic receptor agonist, Compound 49b, could prevent impaired insulin receptor signal transduction [6] observed in both diabetic rodent models [7], as well as retinal endothelial [8] and Müller cells [9] grown under hyperglycemia conditions. Retinal endothelial cells express only  $\beta$ 1- and  $\beta$ 3-adrenergic receptors [10], while retinal Müller cells express both  $\beta$ 1- and  $\beta$ 2-adrenergic receptors [11]. Therefore, use of the  $\beta$ 2-adrenergic receptor knockout mice ( $\beta$ 2KO) mice could suggest that the retinal changes observed in these mice are produced by retinal endothelial cells through activation of  $\beta$ 1- and/or  $\beta$ 3-adrenergic receptor signaling. Since we have shown that

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retinal endothelial cells have increased apoptosis in response to high glucose and impaired insulin signaling, it is likely that impaired insulin signaling may be key to apoptosis in the retina [8].

In our previous work in retinal endothelial cells, we have reported that high glucose increases TNF $\alpha$  levels, leading to apoptosis of these cells [8]. Others have also reported that increased cytokine levels can lead to apoptosis of retinal endothelial cells [12]. Increased TNF $\alpha$  can produce insulin resistance in multiple ways. The most direct pathway of TNF $\alpha$ -induced inhibition of insulin signaling is through TNF $\alpha$ -mediated phosphorylation of insulin receptor substrate 1 (IRS-1) on serine 307 [13]. Phosphorylation of serine 307 on IRS-1 inhibits the ability of IRS-1 to transmit the insulin phosphorylation signal to Akt, thus blocking normal insulin signal transduction. In addition to actions on IRS-1, TNF $\alpha$  also leads to increased suppressor of cytokine signaling 3 (SOCS3) levels [14,15]. Increased SOCS3 levels can phosphorylate the insulin receptor on tyrosine 960 which blocks the insulin receptor/IRS-1 interaction [16]. We have previously reported that TNF $\alpha$  and SOCS3 are both increased in response to high glucose in retinal endothelial cells, leading to increased phosphorylation of IRS-1<sup>Ser307</sup> and IR<sup>Tyr960</sup> [8]. The remaining question was whether this occurred *in vivo* and whether inhibition of TNF $\alpha$  could block all downstream responses to restore normal insulin signal transduction.

The suggestion of inhibition of TNF $\alpha$  as a therapeutic for diabetic retinopathy is not novel. Because TNF $\alpha$  is reported to cause insulin resistance in many other tissues, including adipocytes [13] and myeloid progenitor cells [17], it would be expected that inhibition of TNF $\alpha$  would protect cells and normalize insulin signaling. This was directly tested in chronic studies in TNF $\alpha$  receptor 1- or receptor 2-deficient mice fed 30% galactose for up to 20 months. The authors found that inhibition of TNF $\alpha$  with etanercept (Enbrel), a TNF $\alpha$  receptor antagonist, in galactosemic rats led to reduced pericyte loss and degenerate capillary formation [18]. Additionally, in acute studies of retinopathy-like changes, the authors demonstrated that etanercept suppressed caspase activity and apoptosis in Long Evans rats treated with streptozotocin to make them diabetic [18]. Previously, this group had reported that etanercept reduced leukocyte adhesion in diabetic rats [19]. Therefore, it is clear that inhibition of TNF $\alpha$  is protective against diabetic retinopathy changes; however, the mechanism by which this may occur is unknown.

Our hypothesis was that inhibition of TNF $\alpha$  actions in  $\beta$ 2KO mice would restore normal insulin signal transduction, explaining the improvement in markers of diabetic retinopathy reported by others. To test our hypothesis, we treated wildtype mice or  $\beta$ 2KO mice with etanercept for

two months and measured proteins involved in insulin resistance and signaling, including TNF $\alpha$ , SOCS3, insulin receptor (IR), and apoptotic markers. We found that inhibition of TNF $\alpha$  by etanercept treatment to  $\beta$ 2KO mice was able to significantly reduce apoptotic markers through decreased activation of TNF $\alpha$ -mediated insulin resistance.

## Methods

### Mice

All mice experiments, including those for dark-adaptation and tail electrodes for electroretinogram (ERG) analyses, were approved by the Institutional Animal Care and Use Committee at the University of Tennessee Health Science Center (Protocol #1992).  $\beta$ 1/ $\beta$ 2KO mice (*Adrb1<sup>tm1</sup>Bkk Adrb2<sup>tm1</sup>Bkk/J*) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). From the  $\beta$ 1/ $\beta$ 2KO mice, we bred mice to generate only homozygous  $\beta$ 2KO mice. We appreciate that C57BL6 may not be the ideal wildtype control, but the original  $\beta$ 1/ $\beta$ 2KO mice were from a mixed background containing C57BL6, therefore we chose the C57BL6 for wildtype. Since we use these mice at three months of age, other issues from the C57BL6 background should be minimized. We have previously published use of this animal model and genotyping [5] to demonstrate that neuronal markers of diabetic retinopathy are present, as well as increased apoptosis in the retina.

### Etanercept (Enbrel) treatment

A subset of the  $\beta$ 2KO mice were administered Enbrel by subcutaneous injection 0.3 mg/kg, 3 $\times$ /week [19]. After the final treatment, C57BL6 control, C57BL6+ etanercept,  $\beta$ 2KO mice, and  $\beta$ 2KO mice + etanercept (five mice of each gender in each group) were dark-adapted for ERG analyses prior to sacrifice by ketamine and xylazine overdose.

### Electroretinogram

Prior to sacrifice for morphological and biochemical analyses, animals were subjected to ERG analyses to evaluate the changes in the electrical activity of the retina as we have done previously [5,7]. After dark-adaptation 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, Ft. Worth, TX, USA). Methylcellulose (Celluvisc; Allergan, Irvine, CA, USA) drops were applied as well to maintain a good electrical connection and body temperature was maintained at 37 $^{\circ}$ C by a water-based heating pad. ERG waveforms were recorded with a bandwidth of 0.3 to 500Hz and sampled at 2 kHz by a digital acquisition system and were analyzed using a custom-built program (MatLab, Mathworks, Natick,

MA, USA). Statistics were done on the mean  $\pm$  SD amplitudes of the A- and B-wave of each treatment group.

#### Western blot analysis

Equal amounts of protein from the tissue extracts were separated on the pre-cast tris-glycine gel (Invitrogen, Carlsbad, CA, USA), blotted onto a nitrocellulose membrane. After blocking in TBST (10 mM 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 a chemiluminescence reagent kit (Thermo Scientific, Waltham, MA, USA). Primary antibodies used were phosphorylated Akt (Serine 473), Akt, Bax, Bcl-xL, Cytochrome C, SOCS3, phosphorylated insulin receptor (tyrosine 1150/1151), insulin receptor (all purchased from Cell Signaling, Danvers, MA, USA), insulin receptor phosphorylated on Tyr960 (Cell Applications, San Diego, CA, USA), and beta actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

#### ELISA analysis

A cleaved caspase 3 ELISA (Cell Signaling, Danvers, MA, USA) was used to measure levels of the active apoptotic marker in whole retinal lysates. TNF $\alpha$  protein concentrations were measured using a TNF $\alpha$  ELISA (ThermoFisher, Pittsburgh, PA, USA). For cleaved caspase 3 ELISA analyses, equal protein was loaded (50  $\mu$ g) into all wells to allow for comparisons based on optical density (OD). For the TNF $\alpha$  ELISA, 50  $\mu$ l protein was loaded into all wells, with analyses for concentrations based on a standard curve.

#### Statistics

Statistical analyses were done using Prism software (GraphPad, La Jolla, CA, USA). Analyses were done using a Kruskal-Wallis test, followed by Dunn's test. Data are presented as mean  $\pm$  SEM. For Western blots, a representative blot is presented.

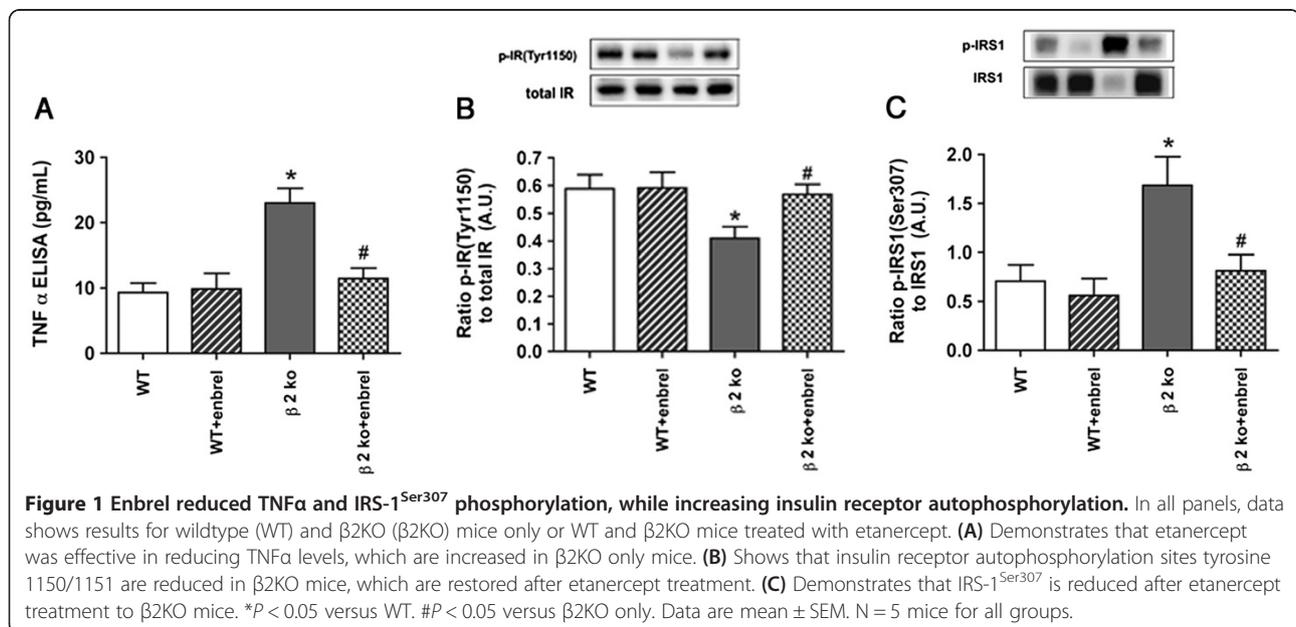
#### Results

##### Etanercept treatment restores normal insulin receptor phosphorylation, while reducing IRS-1<sup>Ser307</sup> levels

To insure that treatment with etanercept was able to significantly reduce TNF $\alpha$  levels in  $\beta$ 2KO mice, we performed an ELISA analysis to show that  $\beta$ 2KO mice have increased levels of TNF $\alpha$ , which were significantly reduced by etanercept treatment (Figure 1A). Since TNF $\alpha$  is a key player in insulin resistance, we wanted to investigate whether blockade of TNF $\alpha$  actions in  $\beta$ 2KO mice could prevent these deleterious effects. Etanercept treatment to  $\beta$ 2KO mice was able to significantly increase insulin receptor autophosphorylation on tyrosine 1150/1151 (Figure 1B). Since TNF $\alpha$  preferentially phosphorylates IRS-1 on serine 307 to inhibit insulin signal transduction [13], we measured IRS-1<sup>Ser307</sup> phosphorylation after etanercept treatment. Data showed that etanercept significantly reduced IRS-1<sup>Ser307</sup> phosphorylation in  $\beta$ 2KO mice (Figure 1C), suggesting that treatment with etanercept prevented TNF $\alpha$ -induced IRS-1 phosphorylation.

##### Etanercept reduced SOCS3 levels, leading to decreased IR<sup>Tyr960</sup> phosphorylation

We have previously reported that  $\beta$ 2KO mice have increased SOCS3 levels and IR<sup>Tyr960</sup> phosphorylation [5], which are both associated with increased TNF $\alpha$  levels



and insulin resistance [15,16]. Figure 2 demonstrated that  $\beta$ 2KO mice have increased SOCS3 (A) and IR<sup>Tyr960</sup> phosphorylation (B), as we reported previously. Additionally, Figure 2 showed that inhibition of TNF $\alpha$  with etanercept was able to significantly reduce both SOCS3 and IR<sup>Tyr960</sup> activity, suggesting that etanercept may prevent insulin resistance in the retina.

#### Pro-apoptotic factors are reduced after etanercept treatment to $\beta$ 2KO mice

Since prevention of apoptosis is a key goal of the insulin signaling pathway, we measured key pro-apoptotic (Figure 3C-E) and anti-apoptotic markers (Figure 3A-B) in wildtype and  $\beta$ 2KO mice. While etanercept had no effects on wildtype mice, etanercept treatment significantly increased levels of key anti-apoptotic markers, Akt (A) and Bcl-xL (B), while decreased pro-apoptotic markers, Bax (C), Cytochrome C (D), and cleaved caspase 3 (E). Taken together, the data suggest that  $\beta$ 2KO mice have impaired insulin signaling, which is restored after inhibition of TNF $\alpha$  through etanercept therapy.

#### Etanercept treatment improves ERG amplitudes

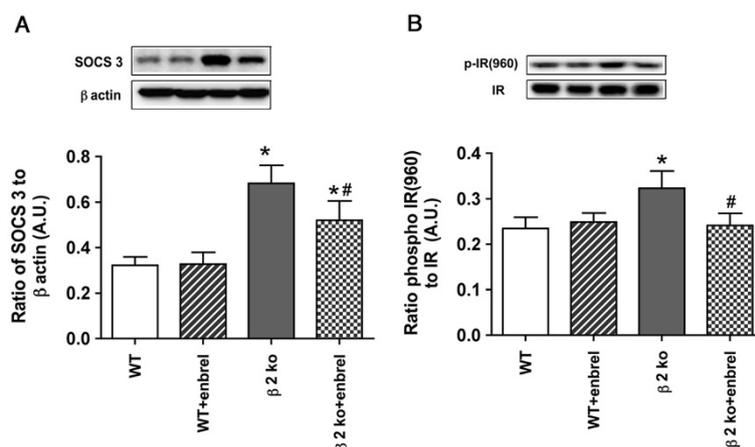
We have previously reported that  $\beta$ 2KO mice have reduced amplitudes of the A-wave, B-wave and oscillatory potentials [5], which was improved with treatment with Compound 49b (Jiang *et al.*, in press). In this study, we found a similar reduction in ERG amplitudes in  $\beta$ 2KO mice compared to wildtype mice (Figure 4, red line), which was resolved with treatment with etanercept for two months (Figure 4, green line). Combining the cell signaling data with functional data, this work demonstrated that TNF $\alpha$  inhibition is effective in preventing

insulin resistance in the retina, leading to normal retinal function.

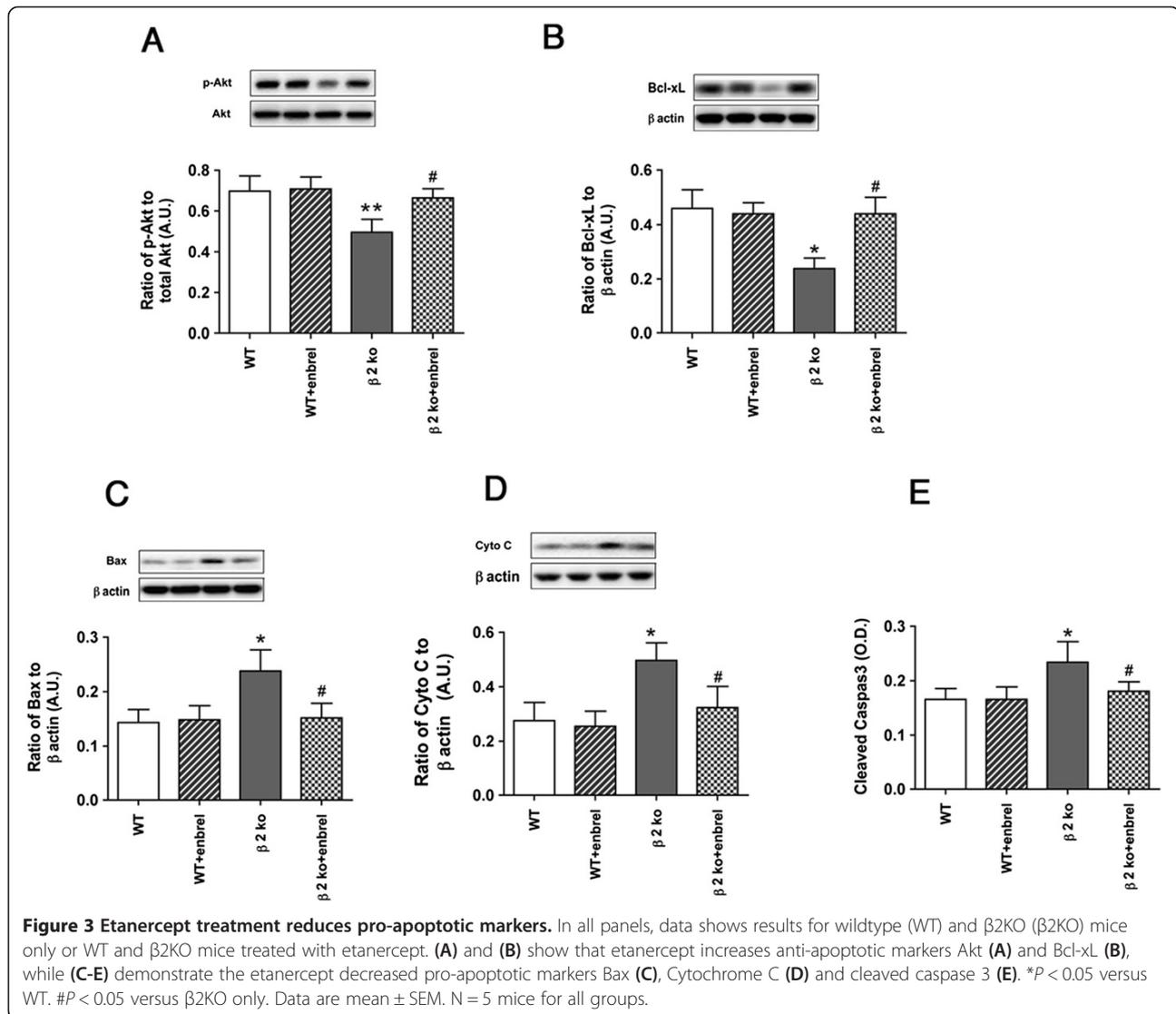
#### Discussion

The goal of this study was to demonstrate that TNF $\alpha$  plays a role in insulin resistance in  $\beta$ 2KO mice. Since others had reported that etanercept was effective in eliminating diabetic-like changes in the rodent eye [18,19], we wanted to determine if inhibition of TNF $\alpha$  with etanercept in  $\beta$ 2KO mice could restore normal insulin signal transduction. We have previously demonstrated that TNF $\alpha$  is key to inhibition of insulin signaling in both retinal endothelial cells (REC) [8] and Müller cells [20] grown in a high glucose concentration, leading to increased apoptosis. In this data set, we demonstrate that etanercept is effective at reducing TNF $\alpha$ -mediated insulin resistance. Two months of etanercept treatment, used at the same treatment regimen as that used in humans with rheumatoid arthritis [19], led to a significant decrease in TNF $\alpha$  in  $\beta$ 2KO mice when compared to untreated  $\beta$ 2KO mice. Since we have reported that one pathway by which TNF $\alpha$  can mediate insulin resistance in REC is through phosphorylation of IRS-1<sup>Ser307</sup> [8], we measured this phosphorylation site *in vivo* and found that etanercept reduced IRS-1<sup>Ser307</sup> in the  $\beta$ 2KO mice. Another potential pathway by which TNF $\alpha$  can mediate impaired insulin signaling is through activation of SOCS3 [15]. Etanercept was able to significantly reduce SOCS3 in  $\beta$ 2KO mice, leading to decreased IR<sup>Tyr960</sup>. Blockade of IR<sup>Tyr960</sup> phosphorylation can promote normal insulin signaling as IR<sup>Tyr960</sup> inhibits the interaction between insulin receptor and IRS-1 [16].

In addition to blockade of TNF $\alpha$ -mediated impairment of insulin signal transduction, etanercept also promoted

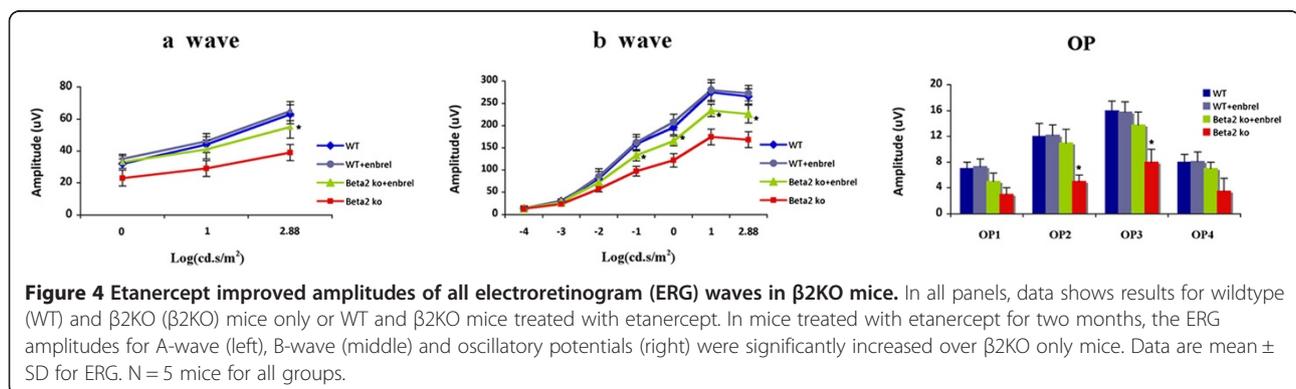


**Figure 2** Inhibition of TNF $\alpha$  with etanercept reduces suppressor of cytokine signaling 3 (SOCS3) and IR<sup>Tyr960</sup> levels. In all panels, data shows results for wildtype (WT) and  $\beta$ 2KO ( $\beta$ 2KO) mice only or WT and  $\beta$ 2KO mice treated with etanercept. (A) Western blot data on SOCS3 levels demonstrating that etanercept reduced SOCS3 levels in  $\beta$ 2KO mice. (B) Western blot data on insulin receptor phosphorylation on tyrosine 960, showing that IR<sup>Tyr960</sup> is reduced after etanercept treatment. \* $P < 0.05$  versus WT. # $P < 0.05$  versus  $\beta$ 2KO only. Data are mean  $\pm$  SEM. N = 5 mice for all groups.



phosphorylation of insulin receptors on tyrosine 1150/1151 in the  $\beta$ 2KO mice, which are autophosphorylation sites, leading to promotion of insulin signaling. We have previously reported that insulin receptor phosphorylation is reduced in  $\beta$ 2KO mice, which appeared to be

localized to the inner retina and ganglion cell layer [5]. Maintenance of normal insulin signal phosphorylation by etanercept likely led to increased Akt activity and decreased cleaved caspase 3 observed in the  $\beta$ 2KO mice. The reduced apoptosis and improvement in insulin



signaling in the inner retina may be correlated to the improvement in the ERG after etanercept treatment.

We focus on our previous work in REC, as  $\beta$ 2KO mice treated with etanercept likely represent effects of TNF $\alpha$  inhibition on REC only, as we have previously reported that  $\beta$ 2-adrenergic receptors are key for TNF $\alpha$  actions in Müller cells [20,21]. Thus, use of the  $\beta$ 2KO mice allows us to dissect TNF $\alpha$ -mediated effects on insulin signaling in REC versus Müller cells' actions *in vivo*. Data from etanercept-treated  $\beta$ 2KO mice suggest that maintenance of normal insulin signaling through TNF $\alpha$  inhibition can reduce inner retinal apoptosis and improve retinal function.

Others have reported that TNF $\alpha$  inhibition using etanercept is effective at reducing diabetic-like changes in the retina [18,19]. This study provided novel information on potential reasons for the improvement in diabetic-like changes; that is, reduced TNF $\alpha$ -mediated impairment of insulin signaling. Data demonstrate that inhibition of TNF $\alpha$  in  $\beta$ 2KO mice led to improved insulin receptor phosphorylation on tyrosine 1150/1151, as well as increased anti-apoptotic proteins. Use of etanercept in  $\beta$ 2KO mice allowed us to dissect TNF $\alpha$ -mediated impairment of insulin signal transduction in REC versus Müller cells. Taken together, the present study provides cellular signaling pathways associated with the beneficial effects of etanercept on  $\beta$ 2KO mice.

#### Abbreviations

$\beta$ 2KO:  $\beta$ 2-adrenergic receptor knockout mice; ELISA: enzyme-linked immunosorbent assay; ERG: electroretinogram; IR: insulin receptor; IRS-1: insulin receptor substrate 1; REC: retinal endothelial cells; SOCS3: suppressor of cytokine signaling 3; TNF $\alpha$ : tumor necrosis factor alpha; WT: wildtype; OD: optical density.

#### Competing interests

No authors have competing interest with this study.

#### Authors' contributions

YJ, QZ, EY completed the studies. JS designed the study and wrote the manuscript. JS, QZ, YJ, EY all edited the manuscript. All authors have read and approved of this manuscript.

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