β-Adrenergic receptor agonist, compound 49b, inhibits TLR4 signaling pathway in diabetic retina

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Diabetic retinopathy has recently become associated with complications similar to chronic inflammatory diseases. Although it is clear that tumor necrosis factor-α is increased in diabetes, the role of innate immunity is only recently being investigated. As such, we hypothesized that diabetes would increase Toll-like receptor 4 (TLR4) signaling, which could be inhibited by a β-adrenergic receptor agonist (Compound 49b) previously shown to have anti-inflammatory actions. In order to investigate β-adrenergic receptor signaling and TLR4 in the diabetic retina, streptozotocin-injected diabetic mice, as well as human primary retinal endothelial cells (RECs) and rat retinal Müller cells (rMC-1) exposed to high glucose (25 mM), were treated with a novel β-adrenergic receptor agonist, Compound 49b (50 μM), or phosphate-buffered saline (control). TLR4 and its downstream signaling partners (MyD88, IL-1 receptor-associated kinase 1, TNF receptor-associated factor 6 and total and phosphorylated nuclear factor-κB) were examined. In addition, we assessed high-mobility group box 1 (HMGB1) protein levels. Our data showed that diabetes or high-glucose culture conditions significantly increased TLR4 and downstream signaling partners. Compound 49b was able to significantly reduce TLR4 and related molecules in the diabetic animal and retinal cells. HMGB1 was significantly increased in RECs and Müller cells grown in high-glucose culture conditions, which was subsequently reduced with Compound 49b treatment. Our findings suggest that high glucose may increase HMGB1 levels that lead to increased TLR4 signaling. Compound 49b significantly inhibited this pathway, providing a potential mechanism for its protective actions. 

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Diabetic retinopathy is associated with significant health care costs (245 billion in 2012), which are expected to substantially increase with the prevailing obesity epidemic (American Diabetic Association, diabetes.org). Unfortunately, current therapies to treat this condition remain limited and currently only delay disease progression until the retinopathy has progressed to a proliferative phase. Although a plethora of potential causal factors are implicated in diabetic retinopathy, none has yet led to the development of successful therapeutic modalities. Most recently, the role of inflammation and the immune system have become of greater interest in understanding diabetic retinopathy.1–5 Work in type 1 diabetic rats has shown that leukocyte activation is induced by diabetes and can be reduced by antibody neutralization of ICAM1 or CD18.6 Others have reported that reductions in tumor necrosis factor (TNF)-α could decrease diabetic-induced retinal damage.7 Similarly, inhibition of interleukin (IL)-1β prevents vascular complications associated with retinopathy.8 Taken together, these results support the notion that inflammation and the immune response may be involved in the diabetic retina.

Toll-like receptor (TLR) pathways are known to have an essential role in the mediation of immune responses. TLRs were originally identified in Drosophila as key receptors for developing embryos.8 Subsequently, it was recognized that these TLRs comprise a group of genetically conserved pattern recognition receptors encoded in germ-line DNA.9 TLRs bind to pathogen-associated molecular patterns, to initiate a proliferative innate immune response,9 leading to activation of a number of cytokines and inflammatory mediators including TNF-α and IL-1. Each TLR has a pathogen group to which it will mount a response. For example, lipopolysaccharide as a potent pathogen signal for TLR4.8,10 In addition to lipopolysaccharide, TLR4 can also respond to endogenous damage-associated molecular patterns, such as hyaluronic acid, high-mobility group box 1 (HMGB1), receptor for advanced glycation end products and heat shock proteins.9 Recent work has suggested that diabetes may represent a sterile inflammation.11 Extending on this work, it has been shown that whole retinal lysates from type 1 diabetic rats have increased TLR4, MyD88 and other downstream proteins associated with TLR4 activation.12 Recent work has demonstrated that reduced MyD88 levels in diabetic mice can prevent leukostasis and decrease ICAM1 levels, as well as reactive oxygen species.13 MyD88 is a common signaling molecule for all TLRs, leading to downstream activation of nuclear factor-κB (NF-κB).8 Retinal endothelial cells (RECs) cultured in high-glucose condition have been shown to express increased levels...
of TLR2 and TLR4, as well as MyD88 and NF-κB. Use of a TLR4 antagonist led to a significant reduction in TNFα, NF-κB, IL-8, ICAM1 and VCAM1, as well as decreased adhesion.

It is clear that a number of cell types may be involved in the retinal damage associated with diabetic retinopathy, as high glucose has been reported to induce expression of inflammatory mediators in RECs and Müller cells as well. In addition, evidence suggests that immune cells may also enter the retina, further regulating the inflammatory response. We have previously reported that β-adrenergic receptor agonists can reduce inflammatory mediator expression by both RECs and Müller cells. TLR4 expression has been detected on mouse Müller cells, whereas photoreceptor studies have shown a role for TLR4 regulation of neuronal apoptosis, further supporting that TLR4 is probably active in the retina. Work in human peripheral blood mononuclear cells has shown that the β-adrenergic receptor agonist, isoprenaline, reduces TNF-α levels following lipopolysaccharide stimulation. Similarly, it has been demonstrated in peritoneal macrophages that ephedrine reduces TNF-α through TLR-mediated pathways. Studies using a THP-1 human monocyte cell line demonstrated that fenoterol, a β2-adrenergic receptor agonist, could inhibit lipopolysaccharide-induced TLR4 levels. Thus, literature suggests that β-adrenergic receptors can regulate TLR4 signaling in multiple targets.

Previously, we reported that the novel β-adrenergic receptor agonist, Compound 49b, reduced TNF-α in both RECs and Müller cells. Following this, we sought to ascertain whether this effect occurs through a reduction in TLR4 signaling. To this end, we hypothesized that hyperglycemia would increase TLR4 signaling, which would then be inhibited by Compound 49b in diabetic mice, RECs and Müller cells.

**RESULTS**

**β-Adrenergic receptor activation reduces diabetes-induced TLR4 signaling in retinal samples** Diabetic mice had significantly lower bodyweight and increased blood glucose levels when compared with control littermates as noted in Table 1. All mice were on a standard chow and glucose measurements were taken on non-fasted mice. In whole retinal lysates from streptozotocin-induced diabetic mice after 2 months of diabetes, TLR4 expression was significantly increased compared with non-diabetic mice (Figure 1a). As TLR signaling leads to pro-inflammatory cytokine production, we also examined protein expression of the TLR4 adaptor molecule, MyD88 together with downstream signaling partners IL-1 receptor-associated kinase 1 (IRAK1), TNF receptor-associated factor 6 (TRAF6) and p-NF-κB (Figures 1b–e, respectively).

Table 1 Body weight and glucose measurements in control and diabetic mice

| Treatments | n | Body weight (g) | Blood glucose (mg dl⁻¹) |
|------------|---|----------------|-------------------------|
| Control (PBS) | 6 | 23 ± 1.6 | 137 ± 6 |
| Control 49b | 6 | 22 ± 1.5 | 134 ± 7 |
| STZ | 6 | 19.5 ± 2.5* | 508 ± 62* |
| STZ+49b | 6 | 19 ± 1.8* | 421 ± 65* |

Abbreviations: PBS, phosphate-buffered saline; STZ, streptozotocin. Data are mean ± s.e.m.

*P<0.05 vs control.

Figure 1 Diabetes increases TLR4 signaling, which is blocked by Compound 49b. Protein levels of TLR4 (a), MyD88 (b), IRAK-1 (c), TRAF6 (d) and the ratio of phosphorylated NF-κB to total NF-κB (e) were measured by western blotting in retinal lysates from control (Ctrl) and diabetic (STZ) mice ± Compound 49b treatment (50 nm) (Ctrl+49b and STZ+49b). *P<0.05 vs Ctrl, #P<0.05 vs STZ. Data are expressed as mean ± s.e.m. (N=6 for all groups) and show one of two similar experiments.
All four molecules were similarly upregulated in retinas of diabetic versus control animals. In contrast, TLR4, MyD88, IRAK1, TRAF6 and p-NF-κB were significantly decreased in diabetic mice treated with topical Compound 49b, potentially explaining a pathway for Compound 49b’s inhibition of TNF-α levels. These data suggest that β-adrenergic receptors can regulate TLR4 signaling in the diabetic retina.

**Compound 49b reduces TLR4 signaling in RECs cultured in high glucose**

In order to dissect potential cell types involved in β-adrenergic receptor regulation of TLR4 signaling in the diabetic retina, human RECs were cultured under normal and high-glucose conditions ± Compound 49b treatment. As demonstrated in Figure 2, high-glucose conditions resulted in significant increases not only in levels of TLR4 (A) but also in the adaptor molecule MyD88 (B) and downstream signaling partners IRAK1 (C), TRAF6 (D) and p-NF-κB (E). Compound 49b treatment significantly decreased expression of TLR4 and aforementioned TLR-related signaling molecules in RECs cultured in high-glucose conditions. This suggests that TLR4 signaling can be regulated by β-adrenergic receptors on RECs.

**Compound 49b reduces TLR4 levels and MyD88-dependent signaling proteins in Müller cells**

Similar to our findings in whole retinal lysates and RECs, high-glucose culture conditions significantly increased TLR4 levels (Figure 3a) in Müller cells. In addition, MyD88, IRAK1, TRAF6 and p-NF-κB (Figures 3b–e, respectively) were increased in response to hyperglycemic conditions. Compound 49b was observed to significantly reduce all of these molecules, indicating that β-adrenergic receptors can regulate the TLR4 signaling pathway in both RECs and Müller cells, two key cell types altered in the diabetic retina.

**Compound 49b significantly reduced HMGB1 levels in both RECs and Müller cells**

To determine a cellular mechanism by which a β-adrenergic receptor agonist can regulate TLR4 signaling, we measured whether Compound 49b could reduce a known damage-associated molecular pattern, HMGB1. High glucose significantly increased HMGB1 levels in both RECs and Müller cells, suggesting that HMGB1 may respond to high glucose and respond as a sterile inflammation. Compound 49b was able to significantly reduce HMGB1 in both cell types (Figures 4a and b), indicating that HMGB1 may be the link between Compound 49b and TLR4 signaling.

**DISCUSSION**

The role of inflammation in diabetes is becoming increasingly evident. It is well established that diabetic retinopathy is associated with elevated leukostasis and upregulated levels of classic pro-inflammatory mediators, TNF-α and IL-1β, by retinal cells. However, only recently studies have started to investigate whether the innate immune response may be involved in diabetic retinal changes despite the fact that observed retinal abnormalities in diabetes are congruous with events of inflammation. As important mediators of inflammation and innate immunity, TLR signaling pathways have become the focus of diabetic retinopathy studies as of late. Results obtained from MyD88 chimeric mice suggest that TLR signaling may regulate leukostasis and leukocyte-induced endothelial cell death. In addition, it has been
demonstrated that TLR4 is increased in the streptozotocin-induced type 1 diabetic rat.12 Similarly, work in human RECs has shown that high glucose activates TLR2/4 downstream signaling pathways. 14 Literature indicates that RECs have increased TLR4 signaling,14 as well as whole retinal lysates from diabetic animals.12 Our data extend those findings to demonstrate that in addition to RECs, Müller cells also demonstrate enhanced TLR4 signaling when cultured under hyperglycemic conditions.

Previous studies have shown that the novel β-adrenergic receptor agonist, Compound 49b, is protective against diabetic retinopathy, ameliorating vascular, neuronal and functional changes in the diabetic retina.24 One mechanism of action for this receptor agonist is by preventing diabetes-induced apoptosis of RECs through increased levels of insulin-like growth factor-1 binding protein 3.24 In addition, Compound 49b has been shown to reduce the potent pro-inflammatory cytokine TNF-α in RECs.25 As TNF-α is known to be upregulated on TLR4 activation, we sought to determine whether this β-adrenergic receptor agonist improves diabetic retinopathy disease pathogenesis, in part, via TLR signaling. As expected, Compound 49b significantly decreased TLR4 levels in both whole retina and in two retinal cell types (RECs and Müller cells).

The remaining key question was the pathway by which Compound 49b could regulate TLR4 activation. Although little work has
investigated β-adrenergic receptor actions on retinal TLR4, studies demonstrated that β-adrenergic receptors can regulate HMGB1 to attenuate hypoxia/reoxygenation, to protect neonatal rat cardiomyocytes.28 These authors demonstrated that dobutamine, a β1-adrenergic receptor agonist used in heart disease, inhibited HMGB1 in a rat model of ischemia/reperfusion.29 Others have reported that HMGB1 may be highly related to autophagy, through its ability to sense stressors and bind TLR2, TLR4 and TLR9.30 Focusing on diabetic retinopathy, HMGB1 can induce cytotoxicity in glial cells and mediate cell death of RECs.31 Based on this literature, we showed that HMGB1 is increased by high-glucose culturing conditions in both RECs and Müller cells. Furthermore, this increase was attenuated following treatment of both cell types with Compound 49b.

Although we feel our findings of β-adrenergic receptor regulation of TLR4 and downstream signaling pathways in the diabetic retina, RECs and Müller cells are novel, we appreciate the limitations of our work. It is highly likely to be that Compound 49b may work on other retinal cell types, including microglia or photoreceptor cells, to protect them against increased TLR4 signaling. This will be a focus in future work. In addition, although beyond the scope of this study, it is clear that β-adrenergic receptors are present in the cornea,32 and that the cornea is significantly affected in diabetes.33 Based on the existing literature, Compound 49b may have detrimental effects to the diabetic cornea in regards to wound healing.34 Future studies may extend our findings into other ocular tissues and investigate whether they may be protected from diabetes-induced damage by Compound 49b.

In conclusion, these data suggest that high glucose causes an increase in HMGB1, leading to enhanced TLR4 signaling. Compound 49b, a novel β-adrenergic receptor agonist, significantly suppressed HMGB1 levels, as well as downstream MyD88-dependent signaling pathways, resulting in reduced NF-κB phosphorylation.

METHODS

Animals

All mouse experiments were approved by the Institutional Animal Care and Use Committee at Wayne State University (Protocol 11-08-14) and adhered to the Animal Policy of the Association for Research in Vision and Ophthalmology. Eight-week-old male C57BL/6J (B6) wild-type mice were purchased from Charles River Laboratories (Chicago, IL, USA). Diabetes was induced by injecting 60 mg kg−1 of streptozotocin dissolved in citrate buffer for 5 consecutive days. Control mice received citrate buffer only. Glucose measurements were done twice weekly, with glucose levels >250 mg dl−1 considered diabetic. Mice were not fasted before blood glucose measurements. Glucose measurements were taken on blood samples obtained from the tail vein using a hand-held measurement device. Table 1 provides body weights and glucose levels of all mice.

After 2 months of diabetes, 6 control and 6 diabetic mice received 1 ml Compound 49b eye drops for 14 days (4 μl onto each eye). Control mice received topical phosphate-buffered saline. Mice were randomly placed into the treatment or no treatment groups without blinding by the investigators. After 14 days of Compound 49b treatment, all mice were killed for further analysis as described below.

RECs culture

Primary human retinal microvascular endothelial cells (RECs) were acquired from Cell System Corporation (Kirkland, WA, USA). Cells were grown in M131 medium containing microvascular growth supplements (Invitrogen, Carlsbad, CA, USA), 10 μg ml−1 gentamycin and 0.25 μg ml−1 amphotericin B but without serum. Three days before the experiment, cells were transferred to high (25 mM) or normal (5 mM) glucose medium (M131 medium with added glucose), supplemented with medium containing microvascular growth supplements and antibiotics. Only primary cells were used within passage 6 only. Cells were quiesced by incubating in high or normal glucose medium without medium containing microvascular growth supplements for 24 h. Compound 49b treatment was carried out at 50 nM, as we have done in the past.24 No signs of mycoplasma contamination were evident.

Müller cell culture

Müller cells (rMC-1; kindly provided by Dr Vijay Sarthy at Northwestern University) were thawed and cultured in DMEM medium under normal glucose (5 mM) conditions. Medium was supplemented with 10% fetal bovine serum and antibiotics. On reaching ~80% confluency, they were passed into dishes containing high glucose (25 mM) or normal glucose medium. Once ready for experimentation, cells were transferred to the appropriate medium without fetal bovine serum, to induce serum starvation for 18–24 h. Compound 49b (50 nM) was then added for 24 h before cell collection. No signs of mycoplasma contamination were evident.

Western blotting

After appropriate treatments and rinsing with cold phosphate-buffered saline, RECs were collected in lysis buffer containing protease/phosphatase inhibitors and scraped into their respective tubes. Retinal extracts were prepared by sonication. Equal amounts of protein from cell or tissue extracts were separated on pre-cast tris-glycine gels (Invitrogen) and 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) bovine serum albumin, membranes were treated with the following primary antibodies: TLR4, IRAK1, (TRAF6), MyD88, HMGB1 (Abcam, San Francisco, CA, USA), NF-xB, phospho-NF-xB (Cell Signaling, Danvers, MA, USA) and β-actin (Santa Cruz, Santa Cruz, CA, USA), and then followed by incubation with secondary antibodies (Fisher Scientific, Pittsburgh, PA, USA) labeled with horseradish peroxidase. Antigen–antibody complexes were detected using a chemiluminescence reagent kit (Thermo Scientific, Pittsburgh, PA, USA). Western blot images were collected on an Azure Biosystems C500 machine (Azure Biosystems, Dublin, CA, USA). Mean densitometry of bands for each protein were measured.

Analyses of samples

Streptozotocin treatment. Treatments for all mice were commenced at 16 weeks of age. Mice were randomly assigned to STZ or control groups to induce diabetes. Two months after STZ treatment, Compound 49b was administered to groups of six control and diabetic mice, which were assigned randomly. Fourteen days after Compound 49b treatment, mice were killed and retinal lysates collected and processed for protein analyses. Enzyme-linked immunosorbent assay was performed on all lysates in parallel and each sample was analyzed in triplicate. Western blot analyses was performed in triplicate and, for each individual blot, a control+phosphate-buffered saline, control+49b, STZ+phosphate-buffered saline and STZ+49b were run as a group of four. Blotting were then probed for α the protein of interest, then stripped and probed for β-actin. Mean densitometry was done for each band and the ratio of the band of protein of interest to β-actin was calculated. The density for each biological replicate was calculated as the mean of the technical replicates for each sample. A representative blot is shown for all western blotting. Data show the mean ± s.e.m. of six independent biological samples for each group.

Cell culture. Samples for RECs and Müller cells were derived from four separate frozen aliquots of Müller or REC cells. These were then expanded to provide sufficient cells for each of the treatment conditions. Following treatment, all cells were collected at the same time and protein analyses were done described for the mouse retinal lysates.

Statistics. Data are presented as the mean ± s.e.m. and were analyzed using the Kruskal–Wallis method, followed by Dunn’s test using Prism 7.0 software (GraphPad, San Diego, CA, USA). P<0.05 was considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.
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