Photoreceptor Cells Produce Inflammatory Mediators That Contribute to Endothelial Cell Death in Diabetes

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Purpose. Recent studies suggest that photoreceptor cells regulate local inflammation in the retina in diabetes. The purpose of this study was to determine if photoreceptor cells themselves produce inflammatory proteins in diabetes and if soluble factors released by photoreceptors in elevated glucose induce inflammatory changes in nearby cells.

Methods. Laser capture microdissection was used to isolate the outer retina (photoreceptors) from the inner retina in nondiabetic and diabetic mice. Diabetes-induced changes in the expression of inflammatory targets were assessed by reverse transcription polymerase chain reaction and immunohistochemistry. Cell culture experiments were carried out to determine if photoreceptors in vitro and ex vivo release soluble mediators that can stimulate nearby cells. Photoreceptor contribution to leukocyte-mediated endothelial cell death was tested using coculture models.

Results. Messenger ribonucleic acid and protein expression levels for inflammatory proteins intercellular adhesion molecule 1 (ICAM1), inducible nitric oxide synthase (iNOS), and cyclooxygenase 2 (COX2) were increased in photoreceptors cells in diabetes. In vitro and ex vivo studies show that photoreceptor cells in elevated glucose release mediators that can induce tumor necrosis factor-α in leukocytes and endothelial cells, but not in glia. The soluble mediators released by photoreceptor cells in elevated glucose are regulated by transforming growth factor β-activated kinase 1 and nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) signaling. In contrast to enhanced leukocyte-mediated killing of endothelial cells by leukocytes from wild-type diabetic mice, leukocytes from diabetic mice lacking photoreceptor cells (*opsin*$^{-/-}$) did not kill endothelial cells.

Conclusions. These data indicate that photoreceptor cells are a source of inflammatory proteins in diabetes, and their release of soluble mediators can contribute to the death of retinal capillaries in diabetes.

Keywords: diabetes, retina, photoreceptor cells, inflammation, leukocyte, endothelial cell, diabetic retinopathy

Diabetic retinopathy (DR) is the leading cause of blindness in people aged 24 to 64 years.[1] The pathogenesis of DR remains unclear, and most studies to date have focused on vascular alterations as the primary site of injury in that pathogenic process. There has been speculation that the neural retina might contribute to vascular damage in DR, but without any clear mechanisms by which this might occur.

Recent studies have implicated a specialized neural cell type, photoreceptor cells, in the development of DR. In contrast to the diabetes-induced degeneration of retinal capillaries in wild-type mice, diabetes did not cause capillary degeneration in mice lacking photoreceptors.[2] Consistent with this finding, a survey of diabetic patients who also had retinitis pigmentosa (and thus lacked photoreceptors) suggested that these patients had less retinopathy than the diabetic patients who did not have retinitis pigmentosa.[3]

Previous work by our group and others has provided evidence that inflammation contributes to the pathology of DR. Proinflammatory proteins such as inducible nitric oxide synthase (iNOS), intercellular adhesion molecule 1 (ICAM1), and tumor necrosis factor α (TNF-α) are induced in the retina in diabetes, and whole-body deletion of these proteins inhibits vascular pathologies and the progression of DR in animal models.[4–7] Photoreceptor cells at least initiate the diabetes-induced increases in superoxide and inflammatory proteins (iNOS, ICAM1, TNF-α, and vascular endothelial growth factor [VEGF])[2,8] and are a source of interleukin 1 beta (IL1β) and its receptor components in the retina in diabetes.[9] Nuclear factor kappa light chain enhancer of activated B cells (NF-κB) has been implicated in the induction of many of these proinflammatory proteins in the retina in diabetes.[10] The NF-κB pathway can be activated by many signaling proteins and stimuli such as transforming growth factor β-activated kinase 1 (TAK1)[11–13] and increased oxidative stress.[14,15] NF-κB activation occurs through phosphorylation and activation of IκB kinase (IKK), which phosphorylates nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IKBα) to lead to IKBα ubiquitination and degradation and migration of NF-κB dimers (p65 and p50) into the nucleus to regulate inflammatory gene transcription.[16] Phosphorylation of

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p65 on Serine 536 is a marker for NF-κB activation, p65 nuclear translocation, and transcriptional activity.[11,17–20]

In the present study, we investigated the possibility that retinal photoreceptor cells (1) are a source of inflammatory proteins induced in the retina in diabetes and (2) release soluble factors that can activate neighboring cells and (3) that NF-κB (via TAK1 and nicotinamide adenine dinucleotide phosphate [NADPH]) in photoreceptors regulates the production of these soluble mediators. We also provide evidence that photoreceptor cells can regulate leukocyte-mediated killing of endothelial cells in diabetes.

Experimental Procedures

Experimental Animals

Rhodopsin knockout (*opsin*$^{-/-}$) and wild-type C57BL/6J mice were obtained from J. Lem (Tufts University, Boston, MA, USA) and Jackson Laboratory (Bar Harbor, ME, USA) respectively. Male mice (2 months old) were randomly assigned to become diabetic or remain in a nondiabetic control group. Diabetes was induced by five sequential daily i.p. injections of a freshly prepared solution of streptozotocin in citrate buffer (pH 4.5) at 60 mg/kg of body weight. Hyperglycemia was verified at least three times during the second week after streptozotocin administration, and mice having three consecutive measurements of blood glucose > 275mg/dL were classified as diabetic. Insulin was given as needed to prevent weight loss without preventing hyperglycemia and glucosuria (0–0.2 units of NPH insulin s.c., 0–3 times per week). Food consumption (Harlan Teklad; Indianapolis, IN, USA; 7004 diet) and body weight were measured weekly. The treatment of animals conformed to the Association for Research in Vision and Ophthalmology Resolution on Treatment of Animals in Research as well as to the Case Western Reserve University Institutional Animal Care and Use Committee. At 2 and 8 months of diabetes (4 and 10 months of age, respectively), eyes were collected and their leukocytes were harvested for the measurement of leukocyte-mediated cytotoxicity to retinal endothelial cells.

Laser Capture Microdissection (LCM)

Eyes were enucleated from normal and diabetic mice and frozen at −80°C in the optimal cutting temperature (OCT) compound. Also, 20-μm thick cryosections were cut and placed on frame slides polyethylene naphthalate (PEN)-membrane (Leica, Wetzlar, Germany) for LCM. Slides were placed in 95% ethanol for 1 minute with gentle inversion up and down, followed by 75% and 50% ethanol for 30 seconds each. Sections were then stained with cresyl violet for 40 seconds and dehydrated in 50%, 75%, 95%, and 100% ethanol, respectively, for 30 seconds each. Sections were placed in xylene for 5 minutes and then placed at room temperature to dry. A Leica LMD 7000 LCM machine was used to isolate the outer retina (photoreceptor region; i.e., outer nuclear layer [ONL], inner segment/outer segment [IS/OS]) from the remaining inner retina (i.e., outer plexiform layer [OPL], inner nuclear layer [INL], inner plexiform layer [IPL], and ganglion cell layer [GCL]; Supplementary Fig. S1). Isolated regions were placed in lysis buffer, and RNA was isolated using the Qiagen RNeasy Micro Kit (cat no. 74004; Qiagen, Hilden, Germany).

Cell Culture

The following cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 5 mM glucose and 10% fetal bovine serum: 661W cone photoreceptor cells (our group previously confirmed 661W identity by positive identification of cone opsin mRNA),[21] mouse retinal endothelial cells (mRECs; identity of mRECs was confirmed by positive identification of the platelet endothelial cell adhesion molecule (*PECAM-1*) gene; Supplementary Fig. S2), mouse retinal glial cells (identity of glial cells was confirmed by positive identification of glial fibrillary acidic protein (GFAP); Supplementary Fig. S3), rat Müller cells, and RAW 264.7 (macrophage-like cell line). The 661W cells (200,000 cells) were cultured in 5 mM glucose (normal) and 30 mM glucose (high glucose) in six well plates for 40 to 48 hours. The conditioned medium from the 661W photoreceptor cells was harvested and added to 200,000 mRECs or retinal glial cells in 12 well plates for 24 hours and to 100,000 leukocytes (freshly isolated from wild-type mice) in 96 well plates for 8 hours. Primary photoreceptor cells (generated from vibratome isolation) were incubated in 5 mM and 30 mM glucose for 14 to 15 hours, and the conditioned medium was harvested and transferred undiluted to leukocytes for 8 hours. RNA from mRECs, glial cells, and leukocytes was harvested and reverse transcription polymerase chain reaction (qRT-PCR) was performed.

To inhibit TAK1 or NADPH oxidase, 20 μM of (5Z)-7-oxozeaenol (irreversible TAK1 inhibitor; Sigma, St. Louis, MO, USA) or 100 μM Apocynin (Apo; reversible NADPH oxidase inhibitor; Sigma), respectively, were incubated with 661W cells for 3 hours. The medium was then changed to 5 mM or 30 mM glucose. After 40 to 48 hours of incubation, the conditioned medium was harvested and added to the leukocytes for 8 hours. For detection of phospho-p65 NF-κB in 661W cells, the cells were serum starved overnight, incubated with TAK1 or NADPH oxidase inhibitors for 3 hours, and the medium was changed to 5 mM or 30 mM glucose for 15 to 180 minutes.

To study leukocyte-mediated killing of the endothelial cells, mRECs were grown in DMEM containing 10% fetal bovine serum and placed in either 5 mM or 30 mM glucose. The medium was changed every other day for 4 days. When mRECs reached 90% confluence (300,000 cells), leukocytes (100,000 cells) were added to the mRECs and incubated for 6 hours. Leukocytes isolated from nondiabetic animals were cocultured with mRECs in 5 mM normal glucose, and leukocytes isolated from diabetic animals were cocultured with mRECs in 30 mM high glucose. After 6 hours, mRECs were gently rinsed with phosphate buffered saline to remove leukocytes, incubated with trypsin for 2 minutes, and washed with phosphate buffered saline. mRECs were stained with CD144 (BD Biosciences, San Jose, CA, USA) and 7-Aminoactinomycin D (BD Biosciences) to identify dead cells, and the number of dead cells were counted by flow cytometry. The results were analyzed using Flow Jo 7.6 software (available in the public domain; http://www.flowjo.com) and expressed as percentage nondiabetic.

Vibratome Isolation of Photoreceptor Cells From Mice

We used vibratome to bisect fresh unfixed retina into outer and inner retinas. We prepared a 4% gelatin block and 20% warm gelatin solution as described by Clérin et al.[22] Wild-type mice (3–4 months old) were sacrificed, and the eyes were enucleated. The retinas were isolated, and the 20% warm gelatin was used to seal the retina (with photoreceptor side facing up) to the 4% gelatin block.[22] Cold DMEM was placed in the vibratome chamber containing the retinas. Based on the measured thickness of the photoreceptor layer of mouse retina (Supplementary Table S1), a conservative estimate of 40-μm thick slice was used in isolating the photoreceptor layer from the remaining retina using a Leica VT1000 S vibratome. Elapsed

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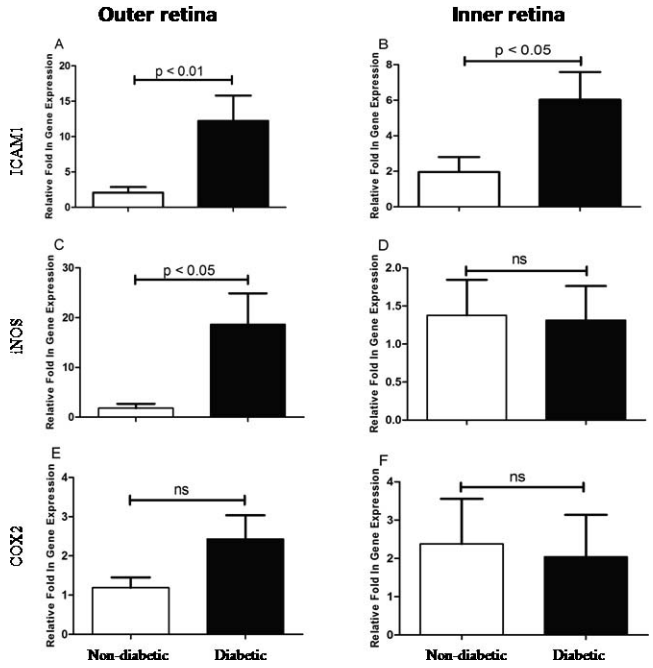

FIGURE 1. Diabetes induces mRNA levels of inflammatory targets in the outer retina (photoreceptors) compared to the inner retina. Retina was bisected into photoreceptors (outer retina) and inner retina using laser capture microdissection, and then, mRNA levels were evaluated using qRT-PCR. (**A**), (**C**), and (**E**) show increases in ICAM1, iNOS, and COX2, respectively, in the outer retina (photoreceptors) in diabetes. (**B**), (**D**), and (**F**) show an increase in ICAM1 in the inner retina, but no change in iNOS or COX2. In the analyses, four to seven animals per group were used. Duration of diabetes was 2 months (4 months of age when killed).

time from eye enucleation to placement of photoreceptor layer in glucose medium was about 15 to 25 minutes.

RNA Isolation and qRT-PCR

Total RNA was isolated from cells (RNeasy kit; Qiagen). cDNA was synthesized (iScript, cDNA synthesis kit; Bio-Rad, Hercules, CA, USA) in a final reaction volume of 20 µL. Real-time PCR was performed using SYBR green (Roche Diagnostics, Indianapolis, IN, USA) with a qPCR system (CFX Connect Real-Time PCR Detection System; Bio-Rad). The following primer sequences were used for 18S forward (5′-ACTCAA CACGGGAAACCTCACC-3′) and reverse (5′-CCAGAC AAATCGCTCCACCAAC-3′), iNOS forward (5′ GAGTCTTGGTG AAAGTGGTGTTC-3′) and reverse (5′-GCAGACAACCTTGGT GTTGA-3′), ICAM1 forward (5′-GCCTTGGTAGAGGTGACT GAG-3′) and reverse (5′-GACCGGAGCTGAAAAGTTGTA-3′), COX2 forward (5′-CACAGCCTACCAAAACAGCCA-3′) and reverse (5′-GCTCAGTTGAACGCCTTTTGA-3′), TNF-α forward (5′-CATCTTCTCAAAATTCGAGTGACAA-3′) and reverse (5′-TGGGAGTAGACAAGGTACAACCC-3′). 18S was used as the reference for normalization. cDNA samples were run in duplicates or triplicates. Quantification cycle (Cq) values were confirmed by agarose gel electrophoresis and melting temperature. $2^{\Delta\Delta Cq}$ = Relative fold in gene expression.

Immunohistochemistry

Eyes were harvested from mice and fixed in 4% paraformaldehyde for 5 hours at room temperature. For cryopreservation, fixed eye samples were placed in sucrose gradients 10%, 15%, and 20% for 1 hour each at room temperature. Eye samples were placed in an OCT compound and frozen at −80°C. The 10-µm thick eye sections were cut using a cryostat. Sections were blocked with 10% normal donkey serum with 0.5% Tween-20 for 1 hour. Sections were stained with primary antibodies (rabbit polyclonal anti-iNOS [Santa Cruz Biotechnology, CA, USA], rabbit polyclonal anti-COX2 [Abcam, Cambridge, MA, USA], and rabbit polyclonal immunoglobulin G (IgG) control [Jackson Immunoresearch, West Grove, PA, USA]) at 1:650 dilution at 4°C overnight. To detect $CD45^+$ cells, rat antimouse CD45 (1:500 dilution; BD Biosciences) was added to the sections at 4°C overnight. Sections were stained with secondary antibodies, fluorescein isothiocyanate goat antirabbit (Jackson Immunoresearch) at 1:100 dilution and DI594 goat antirat IgG (Jackson Immunoresearch) at 1:10000 dilution for 90 minutes. Hoechst deoxyribonucleic acid dye (Thermo, Rockford, IL, USA) at 1:1000 was used to stain nuclei. Retinas were imaged using a fluorescence microscope.

Western Blot

Cells were harvested, sonicated, and centrifuged. Supernatants were used for immunoblotting. Samples (20–30 ug) were fractionated by SDS PAGE and electroblotted onto nitrocellulose membranes, and membranes were blocked in Tris-buffered saline, pH 7.6, containing 0.5% Tween-20 and 5% nonfat milk. Primary antibodies p-NF-κB p65 (S536) (93H1) rabbit monoclonal (1:600; Cell Signaling Technology, Beverly, MA, USA), rabbit polyclonal against p65 NF-κB (1:1000; Abcam), rabbit polyclonal against GFAP (1:1000; Dako, Carpinteria, CA, USA), and mouse monoclonal against β-actin (1:3000; Abcam) were applied overnight at 4°C followed by secondary antibodies goat antirabbit IgG (heavy chain + light chain) − horseradish peroxidase conjugate (1:5000; Bio-Rad) and goat antimouse IgG (heavy chain + light chain) − horseradish peroxidase conjugate (1:5000; Bio-Rad) for 90 minutes at room temperature. After washing, the immunoblots were visualized by chemiluminescence.

Statistical Analysis

Data were described as mean ± SEM and analyzed by Mann-Whitney (nonparametric test). Differences were considered statistically significant at $P < 0.05$ (ns = not significant).

RESULTS

Photoreceptors Increase mRNA Levels of Inflammatory Targets in Diabetes

Using LCM, the outer retinas (photoreceptors) were isolated from the inner retinas (Supplementary Fig. S1) in diabetic and nondiabetic mice. RNA was isolated from the cut samples, and qRT-PCR was used to quantify the change in gene expression of inflammatory targets. Photoreceptors from mice diabetic for 2 months produced increased levels of ICAM1, iNOS, and COX2 mRNA when compared with nondiabetic animals (Figs. 1A, 1C, 1E), but COX2 increase was not statistically significant (Fig. 1E). In contrast, the inner retina produced increased ICAM1 mRNA levels, but did not produce increased mRNA for iNOS or COX2 in diabetes (Figs. 1B, 1D, 1F).

Because it was possible that the photoreceptor layer might contain other cells (such as leukocytes or microglia) that might have infiltrated the photoreceptor region,[23,24] we investigated whether these cells were present in the outer retina of diabetic and nondiabetic mice. We carried out immunohistochemistry with the CD45 antibody to detect hematopoetic cells, such as

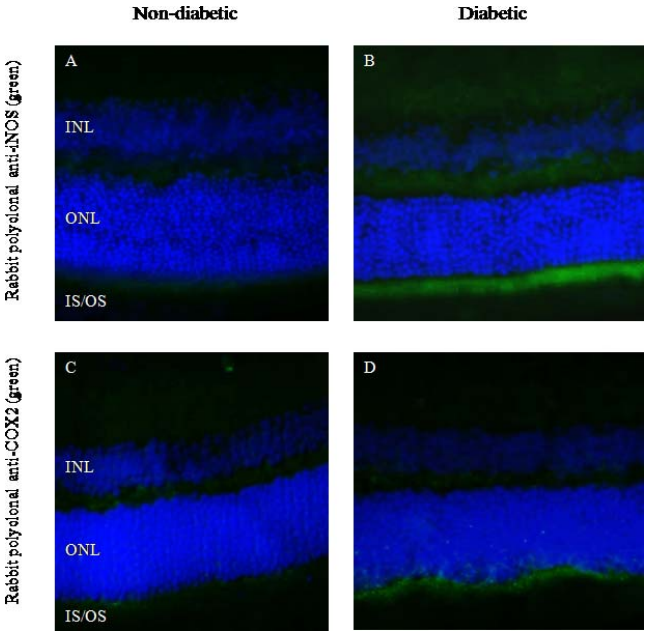

FIGURE 2. Diabetes-induced increase in inflammatory proteins in photoreceptor cells. There was no detection of iNOS in the photoreceptor region in the nondiabetic retina (**A**), but in diabetes, there were increased levels of iNOS in the photoreceptor region (**B**). There was no detection of COX2 in the photoreceptor region of the nondiabetic retina (**C**), but in diabetes, there were increased levels of COX2 in the photoreceptor region in diabetes (**D**). The nuclei were stained with Hoechst DNA dye (*blue*). Micrographs are representative of three or more animals per group.

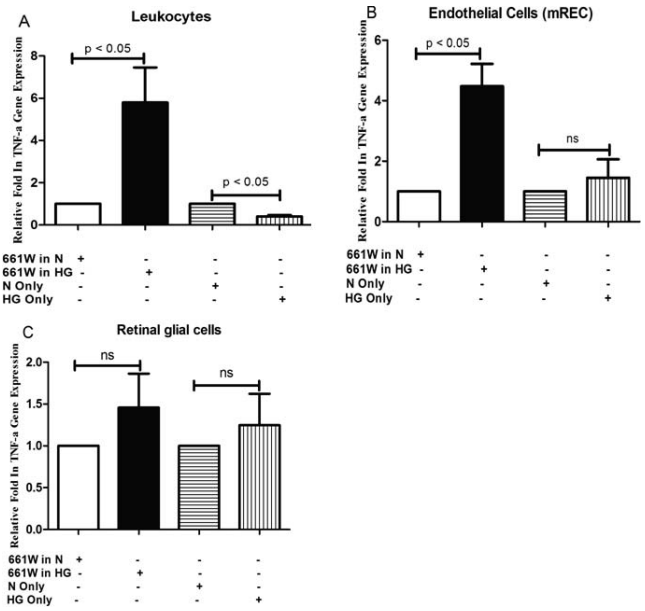

FIGURE 3. 661W photoreceptor cells release mediators in elevated glucose that can stimulate leukocytes and endothelial cells. (**A**) Increase of TNF-α mRNA in leukocytes exposed to conditioned medium from 661W cells incubated in high glucose compared with medium from 661W cells incubated in normal glucose and (**B**) increase of TNF-α mRNA in endothelial cells exposed to conditioned medium from 661W cells incubated in high glucose compared with medium from 661W cells incubated in normal glucose. (**A**, **B**) Photoreceptor cells (661W) in high glucose produce soluble mediators that stimulate TNF-α in leukocytes and endothelial cells. (**C**) No statistically significant change of TNF-α mRNA in retinal glial cells exposed to conditioned media from 661W cells incubated in high glucose. Normal glucose (N) = 5 mM glucose, high glucose (HG) = 30 mM glucose. SEM for leukocytes (**A**) treated with media from 661W in N or N only are 0.778 and 0.426, respectively. SEM for endothelial cells (**B**) treated with media from 661W in N or N only are 0.412 and 0.635, respectively. SEM for glial cells (**C**) treated with media from 661W in N or N only are 0.949 and 0.720, respectively. Results were replicated three to five times.

leukocytes, in the photoreceptor region. There were essentially no $CD45^+$ cells detected in the photoreceptor region (i.e., ONL and IS/OS) in diabetes (Supplementary Fig. S4), leading us to conclude that the mRNA profiles observed in the outer retina samples were likely representative of photoreceptors only.

Photoreceptors Produce Inflammatory Proteins in Diabetes

We supplemented our qRT-PCR data by carrying out immunohistochemistry to detect iNOS and COX2 proteins in the photoreceptor region in mice retinas. We detected increased levels of iNOS and COX2 in the photoreceptors in samples from diabetic compared with nondiabetic animals (Figs. 2A–D). The images demonstrate that most of the increased iNOS and COX2 proteins in the retina in diabetes were localized to the photoreceptor inner segments. As a control, we used an isotype control IgG antibody that showed no staining of proteins in photoreceptors of either nondiabetic or diabetic retinas (data not shown), obviating the possibility that the positive stains were nonspecific. Figures 1 and 2 demonstrate the principle that photoreceptor cells can produce inflammatory proteins in diabetes in vivo.

Soluble Factors Released From Photoreceptors in Elevated Glucose Can Stimulate TNF-α in Leukocytes and Endothelial Cells

Having demonstrated that diabetes can produce inflammatory proteins within photoreceptors, we tested whether soluble factors produced by photoreceptor cells would stimulate inflammation in nearby cells. To do this we used a photoreceptor cell line, 661W, cultured in 5 mM normal glucose (N) or 30 mM high glucose (HG) conditions for 40 to 48 hours. The conditioned media was harvested and added to leukocytes (freshly isolated from the blood of nondiabetic mice), endothelial cells, and glial cells, and qRT-PCR was used to detect changes in the TNF-α mRNA in these cells. As a control, we studied the effect of media (N and HG only), which had not been exposed to 661W cells on leukocytes, endothelial cells, or glial cells. About 6-fold and 4-fold inductions of TNF-α mRNA were observed for leukocytes and endothelial cells, respectively, after exposure to the conditioned media from photoreceptors in 30 mM glucose when compared with cells exposed to the conditioned media from photoreceptors in 5 mM glucose (Figs. 3A, 3B). In contrast, there was no statistically significant induction of TNF-α mRNA observed for glial cells exposed to the conditioned medium from photoreceptors incubated in 30 mM glucose (Fig. 3C). The HG media (30 mM) that had not been conditioned by cells did not induce TNF-α in leukocytes, endothelial cells, or glial cells (Figs. 3A–C), indicating that photoreceptors in elevated glucose release soluble mediators into the medium that can stimulate leukocytes and endothelial cells. Neither IL-1β nor TNF-α were produced by photoreceptors in 30 mM glucose (data not shown), as confirmed by ELISA, ruling out IL-1β or TNF-α as the signaling factors in the medium that stimulate TNF-α in leukocytes and endothelial cells. A potential weakness of

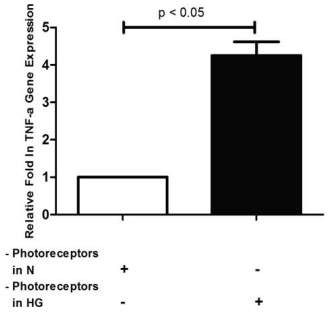

FIGURE 4. Isolated sheet of photoreceptor cells in elevated glucose release mediators that stimulate leukocytes. Increase of TNF-α mRNA in leukocytes by conditioned medium produced by photoreceptor cells in elevated glucose compared with conditioned medium from photoreceptors in normal glucose. Normal glucose (N) = 5 mM glucose, high glucose (G) = 30 mM glucose. SEM for leukocytes treated with media from photoreceptors in N is 0.211. Results were replicated three times.

studies using 661W cells is that they are transformed cells and the extent to which they reflect photoreceptors in vivo is unclear. Therefore, we also bisected fresh retinas using a vibratome to obtain essentially pure photoreceptors.

To ensure that only pure photoreceptor layers were isolated, the thickness of retinal layers were evaluated in preliminary studies (Supplementary Table S1), and based on this information, the retinas were bisected (40 μm thick) with a vibratome to isolate the photoreceptors (outer retinas) from the inner retinas. After each photoreceptor layer was isolated, it was frozen in OCT and sectioned. The inner retina was also frozen in OCT and sectioned. Isolated sections were stained with Hoechst dye to visualize the region of vibratome cut. Compared with the whole retina (Supplementary Fig. S5A), the outer retina was purely photoreceptor cells and devoid of the inner retina (Supplementary Figs. S5B–C). It was estimated that 79% of the outer retina was isolated from whole retinas. The outer retina also expressed more rhodopsin mRNA levels when compared with the inner retina (Supplementary Fig. S5D).

To test if soluble mediators are released by primary photoreceptor cells to stimulate leukocytes, photoreceptors isolated from the retinas of young adult (3–4 months old) C57BL/6 mice using the vibratome were placed in 5 mM or 30 mM glucose for 14 to 15 hours. The conditioned medium was harvested and added to leukocytes (freshly isolated from nondiabetic animals). The conditioned medium from photoreceptors incubated in 30 mM glucose caused an increase in TNF-α gene expression in leukocytes when compared with the conditioned medium from photoreceptors incubated in 5 mM glucose (Fig. 4). This demonstrates that primary photoreceptor cells in elevated glucose can produce soluble mediators that stimulate TNF-α in leukocytes and confirms the previous results with the 661W photoreceptor cell line (Fig. 3).

NF-κB Activation Is Increased in Photoreceptors in Elevated Glucose and Regulates the Release of Soluble Factors

NF-κB is an important regulator of many cellular processes, including inflammation. To determine NF-κB signaling in elevated glucose and if TAK1 or NADPH oxidase regulate NF-κB in those cells, 661W cells were pretreated with 5 mM glucose with or without TAK1 or NADPH oxidase inhibitors (5Z or Apo, respectively) for 3 hours and then incubated in 5 mM or 30 mM glucose in the absence of inhibitors for 15 to 180 minutes. The cells were then harvested and samples were immunoblotted to detect phospho-p65 (Serine 536), which is a

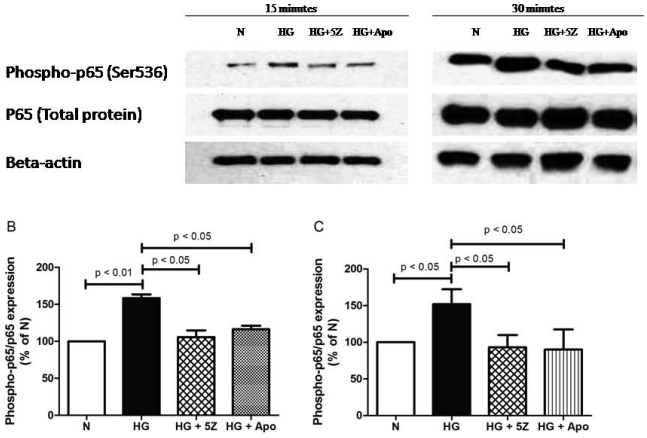

FIGURE 5. NF-κB signaling is increased in photoreceptor cells (661W) incubated in elevated glucose. (**A**) Phosphorylation of p65 in 661W cells exposed to 5 mM or 30 mM glucose with or without inhibitors for TAK1 (5Z-7-Oxozeaenol; 5Z) or NADPH oxidase (Apocynin; Apo) for 15 and 30 minutes. Quantification of phospho-p65 in photoreceptor cells exposed to glucose conditions with or without inhibitors for 15 minutes (**B**) and 30 minutes (**C**). Normal glucose (N) = 5 mM glucose, high glucose (HG) = 30 mM glucose. (**B**) SEM for cells treated with N is 0.042. (**C**) SEM for cells treated with N is 0.082. Results were replicated three to four times.

marker for NF-κB activation, p65 nuclear translocation, and transcriptional activity.[11,17–20] Exposure of 661W cells to 30 mM glucose caused an increase in phosphorylation of p65 when compared with 661W cells in 5 mM glucose (Figs. 5A–C), and this became maximal by 30 minutes, indicating that NF-κB signaling was increased in 661W photoreceptor cells in 30 mM glucose. There was no increase in phosphorylation of p65 when 661W cells were preincubated with 5Z or Apo and then incubated in 30 mM glucose (Figs. 5A–C), suggesting that TAK1 and NADPH oxidase can regulate NF-κB signaling in photoreceptors.

To investigate if the soluble mediators released from photoreceptors in elevated glucose (Fig. 3) were regulated by NF-κB, we incubated 661W cells with 5Z or Apo for 3 hours and then transferred cells to 5 mM or 30 mM glucose media devoid of the inhibitors for 40 to 48 hours. The conditioned medium from 661W cells in 30 mM glucose and exposed to 5Z or Apo caused no increase in TNF-α in leukocytes when

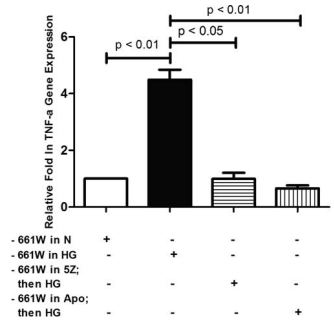

FIGURE 6. Soluble mediators produced by 661W cells in elevated glucose are regulated by TAK1 and NADPH oxidase. There was no TNF-α increase in leukocytes exposed to conditioned medium from photoreceptors incubated with TAK1 or NADPH oxidase inhibitors. Normal glucose (N) = 5 mM glucose, high glucose (HG) = 30 mM glucose. (5Z)-7-Oxozeaenol = TAK1 inhibitor; 20 μm of 5Z was used. Apocynin = NADPH oxidase inhibitor; 100 μm of Apo was used. SEM for leukocytes treated with media from 661W in N is 1.283. Results were replicated four times.

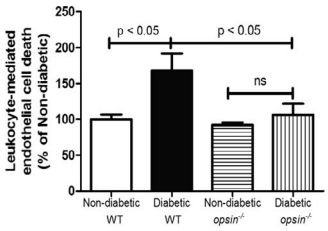

FIGURE 7. Photoreceptor cells contribute to leukocyte-mediated killing of retinal endothelial cells. Leukocytes from wild-type diabetic animals (8 months) cocultured with mouse retinal endothelial cells (mRECs) caused more endothelial cell death than leukocytes from wild-type nondiabetic animals. Leukocytes from animals lacking photoreceptor cells (*opsin*$^{-/-}$) who were likewise diabetic for 8 months caused less endothelial cell death when compared with leukocytes from wild-type diabetic animals. WT = wild-type, *opsin*$^{-/-}$ = rhodopsin knockout. Results were replicated three times.

compared with leukocytes exposed to the conditioned medium from 661W cells in 30 mM glucose alone (Fig. 6). This result is consistent with the interpretation that the release of soluble mediators by photoreceptors in elevated glucose is regulated by NF-κB via TAK1 and NADPH oxidase signaling.

We used trypan blue exclusion to test the possibility that our observations were not an artifact caused by the cytotoxicity of 5Z or Apo. After 3 hours of exposure to 5Z or Apo, 661W cells did not show a significant increase in cell death (Supplementary Fig. S6).

Photoreceptor Cells Activate Leukocytes to Kill Retinal Endothelial Cells

It was previously shown by us that leukocytes mediate the diabetes-induced degeneration of retinal capillaries in vivo, and this leukocyte-mediated cytotoxicity to endothelial cells has also been demonstrated ex vivo.[25–27] To investigate if photoreceptor cells contribute to this leukocyte-mediated killing of endothelial cells, we studied leukocytes from 8-month diabetic rhodopsin knockout mice (*opsin*$^{-/-}$) lacking photoreceptor cells.[8] Leukocytes from wild-type diabetic mice cocultured with endothelial cells led to a 50% to 60% increase in endothelial cell death when compared with the results for leukocytes from wild-type nondiabetic mice (Fig. 7), whereas leukocyte-mediated cytotoxicity to endothelial cells was absent if leukocytes were isolated from diabetic *opsin*$^{-/-}$ mice (Fig. 7). This result supports the conclusion that photoreceptors contribute to the leukocyte-mediated killing of endothelial cells in diabetes.

DISCUSSION

Recent reports provide evidence that photoreceptors contribute to the development of DR.[28] Photoreceptors apparently produce the majority of the superoxide generated in the retina in diabetes,[8] and superoxide production is important because the administration of antioxidants or overexpression of antioxidant enzymes, such as superoxide dismutase, have been shown to decrease retinal vascular lesions in diabetes.[29–31] Increased photoreceptor metabolic activity likely contributes to hypoxia and subsequent neovascularization in the advanced stages of DR.[3,32] Degeneration of photoreceptor cells (such as in opsin deficiency) protects the retinal microvasculature from diabetes-induced degeneration.[2] Consistent with this, a survey of diabetic patients who also had retinitis pigmentosa (i.e., loss of photoreceptors) suggested that these patients had less retinopathy than the diabetic patients who did not have retinitis pigmentosa.[3] Also, electrophysiologic and molecular abnormalities implicated in the pathogenesis of DR were partially inhibited when diabetic animals were illuminated with far-red light for 4 minutes per day.[33] Together, these findings suggest a role for light-sensing photoreceptors in the development of DR.

The retina expresses inflammatory proteins in diabetes. Photoreceptors are important in this response because the diabetes-induced increases in VEGF, TNF-α, ICAM1, and iNOS in the retina were greatly diminished in the absence of photoreceptors.[2,8] Whether these inflammatory proteins are produced by photoreceptor cells themselves, however, was not known. Here, we show that photoreceptor cells per se are a source of the inflammatory proteins (ICAM1, iNOS and COX2) induced in the retina in diabetes and are the major site of induction of some inflammatory proteins (such as iNOS and COX2) in diabetes. When these inflammatory proteins are absent, the development of the vascular lesions is inhibited,[4,5,10] suggesting that inflammatory proteins contribute to the development of DR. There is also evidence that photoreceptor cells produce inflammatory proteins in other retinal diseases, such as in uveitis.[34]

It has been shown that diabetes or elevated glucose conditions give rise to mitochondrial abnormalities (such as increased oxidative stress) in different retinal cells.[30,31,35] Photoreceptor cells, which have a high metabolic demand, produce increased superoxide in the retina in diabetes, and mitochondria and NADPH oxidase contribute to this increase in superoxide production.[8] Oxidative stress can induce the production of inflammatory proteins, including ICAM1 and iNOS.[21,36] It is possible that the diabetes-induced superoxide produced by photoreceptors contributes to the increase in inflammatory proteins in photoreceptors, but this is yet to be determined.

After demonstrating that photoreceptors produce inflammatory proteins in diabetes, we tested whether photoreceptors exposed to elevated glucose produced soluble mediators that stimulate inflammation in nearby cells. Photoreceptors undergoing photic injury previously were shown to produce soluble inflammatory mediators that induced iNOS, IL-10, eotaxin, and Rantes in microglia cells.[37] Here, we showed that photoreceptors in elevated glucose produce soluble mediators that stimulate the transcription of TNF-α in leukocytes and endothelial cells. TNF-α contributes to the development of DR, because when absent, the retinopathy is inhibited.[6,7] We did not see a similar increase of TNF-α in retinal glial cells, indicating that the paracrine effect of photoreceptors on stimulation of TNF-α may be cell-type specific. Also, there was no increase in TNF-α in leukocytes, endothelial cells, or glial cells when exposed to 30 mM glucose media in which photoreceptors were not incubated.

The mediators produced by photoreceptors in the conditioned media and how they may travel physiologically to stimulate leukocytes and endothelial cells in the retina are yet to be determined. Nevertheless, these mediators appear to be regulated by NF-κB in photoreceptors via TAK1 and NADPH oxidase signaling pathways. TAK1 can regulate NF-κB and MAPK pathways,[11–13] so it is possible that MAPK signaling also regulates the production of mediators by photoreceptors in elevated glucose. We previously reported that pharmacologic inhibition of p38 MAPK or NADPH oxidase inhibited the diabetes-induced degeneration of retinal capillaries, superoxide production, and other characteristics of early DR.[8,21,38] It is possible that in elevated glucose, NADPH oxidase mediated-production of superoxide activates TAK1 or vice versa to regulate NF-κB.

Because inhibition or deletion of inflammatory proteins inhibits diabetes-induced degeneration of retinal capillaries,[4,5,10] we tested whether photoreceptors might contribute to vascular damage in diabetes via leukocytes. Leukocytes have

been shown to cause degeneration of retinal capillaries in diabetic mice, and this cytotoxicity against endothelial cells has also been reproduced ex vivo.[25–27] Leukocytes harvested from diabetic mice lacking photoreceptors (*opsin*$^{-/-}$) killed fewer endothelial cells ex vivo than did leukocytes from wild-type diabetic mice, suggesting that photoreceptors had contributed to leukocyte activation in diabetes. Our current studies offer a mechanism by which diabetes causes less capillary degeneration in mice lacking photoreceptor cells.[2]

In summary, we provide evidence that photoreceptor cells (1) produce inflammatory proteins that are known to be important in DR, (2) produce soluble inflammatory mediators (likely via activation of NF-κB) that stimulate TNF-α production in leukocytes and endothelial cells, and (3) contribute to the leukocyte-mediated killing of endothelial cells. Thus, we postulate that photoreceptor cells in diabetes produce inflammatory proteins or mediators (via NF-κB) that stimulate leukocytes and perhaps other cells to mediate the cytotoxicity of endothelial cells, giving rise to vascular damage in DR. Most current studies have focused on vascular abnormalities and how they contribute to DR, but very little attention has been given to the contributions of photoreceptor cells. Pharmaceuticals targeting inflammatory signaling pathways in photoreceptors could offer a novel therapeutic target to inhibit the development of DR.

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