Aldose Reductase Inhibition Prevents Endotoxin-Induced Inflammatory Responses in Retinal Microglia

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PURPOSE. Retinal microglia become activated in diabetes and produce pro-inflammatory molecules associated with changes in retinal vasculature and increased apoptosis of retinal neurons and glial cells. We sought to determine if the action of aldose reductase (AR), an enzyme linked to the pathogenesis of diabetic retinopathy, contributes to activation of microglial cells.

METHODS. Involvement of AR in the activation process was studied using primary cultures of retinal microglia (RMG) isolated from wild-type and AR-null mice, or in mouse macrophage cultures treated with either AR inhibitors or small interfering RNA (siRNA) directed to AR. Inflammatory cytokines were measured by ELISA. Cell migration was measured using a transwell assay. Gelatin zymography was used to detect active matrix metalloproteinase (MMP)-9, while RMG-induced apoptosis of adult retinal pigment epithelium (ARPE-19) cells was studied in a cell coculture system.

RESULTS. Aldose reductase inhibition or genetic deficiency substantially reduced lipopolysaccharide (LPS)-induced cytokine secretion from macrophages and RMG. Aldose reductase inhibition or deficiency also reduced the activation of MMP-9 and attenuated LPS-induced cell migration. Additionally, blockade of AR by sorbinil or through genetic means caused a reduction in the ability of activated RMG to induce apoptosis of ARPE-19 cells.

CONCLUSIONS. These results demonstrate that the action of AR contributes to the activation of RMG. Inhibition of AR may be a therapeutic strategy to reduce inflammation associated with activation of RMG in disease.

Keywords: retinal microglia, aldose reductase, LPS, inflammation, migration, MMP-9, aldose reductase inhibitor, β-glucogallin

Chronic inflammation is now recognized as a key factor in the pathogenesis of diabetic retinopathy (DR). Increased levels of pro-inflammatory signaling molecules such as TNF-α and IL-1β have been measured in patients with DR and in diabetic animal models. Tumor necrosis factor-α-induced pathways become activated in the diabetic retina, resulting in increased levels of effector gene products such as intercellular adhesion molecule 1 (ICAM-1) in the retinal vasculature and associated alterations in interactions between circulating leukocytes and the vascular wall. Outside the eye, macrophages are phagocytic cells considered to be the major source of TNF-α and IL-1β, while microglia are thought to be the major cell type responsible for production of similar cytokines in the ocular environment. Microglia are considered the resident macrophages of the eye. Like macrophages, microglia are capable of adopting an “activated” state accompanied by elaboration of a variety of pro-inflammatory cytokines in response to metabolic stress or after encounter with foreign materials. Increased numbers of activated retinal microglia (RMG) have been detected in human eyes with DR, as well as in various experimental diabetic models in rats. Several potential mechanisms have been advanced to link the effects of chronic hyperglycemia on RMG activation. For example, advanced glycation endproducts (AGEs), which accumulate in the diabetic retina, have been shown to potently induce RMG activation. Gardner and colleagues have hypothesized that activated RMG could play a role in the pathogenesis of DR through increased release of VEGF and TNF and associated exacerbation of blood vessel permeability. Many studies have shown that treatment of diabetic rats with aldose reductase inhibitors (ARIs) leads to reduced level of VEGF in the eye. Aldose reductase inhibitors also prevent high glucose-induced TNF-α secretion. Taken together, these observations led us to question whether AR could play a role in regulating the activation of RMG.

Aldose reductase (AKR1B1) is an aldo-keto reductase responsible for polyol synthesis in target tissues of diabetes. In addition, the action of AR has been linked to the expression of pro-inflammatory molecules in a wide variety of tissues and diseases. In animal models, genetic or pharmacologic blockade of AR prevents the onset and/or progression of many defects associated with long-term diabetes, such as retinal pericyte loss and capillary degeneration as well as reduced markers of oxidative stress typically associated with DR. Previous studies demonstrated a potential role for AR in regulation of endotoxin-induced production of cytokines in...
macrophage cultures, as well as in experimental models of endotoxin-induced uveitis.

Given the emergence of AR as a factor in the macrophage response to activation by endotoxin, and in light of the functional similarities between macrophages and RMG, we evaluated whether genetic or pharmacologic downregulation of AR in RMG can influence the activation response. Comparing primary cultures of RMG established from wild-type and AR-deficient mice, we examined whether absence of AR influenced the endotoxin-induced activation response. As a corollary experiment, we measured the effects of two structurally diverse AR inhibitors on various functional parameters associated with activation. Inhibitors included sorbinil, a well-characterized ARI, and β-glucogallin, a novel ARI recently identified from extracts of the Indian gooseberry plant. Our results point to a role for AR in RMG activation and suggest that AR inhibition may offer a strategy to downregulate this process in retinal disorders.

**MATERIALS AND METHODS**

**Materials and Cell Culture**

β-glucogallin (BGG) was isolated and purified as previously reported. Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sorbinil (1-[6]-Fluoro-2,3-dihydro-spiro[4H-1-benzopyran-4,4'-imidazolidine]-2', 5'-dione) was generously provided by Pfizer Central Research (Groton, CT, USA). Control small interfering RNA (siRNA) and AKR1B3 siRNA (siAKR) were purchased from Qiagen (Valencia, CA, USA). Matrix metalloproteinase (MMP)-9 inhibitor was purchase from EMD Millipore (Billerica, MA, USA). RAW264.7 macrophages were cultured in complete Dulbecco’s Modified Eagle Medium (DMEM; Corning Cellgro, Manassas, VA, USA) supplemented with 4 mM L-glutamine, 10% (vol/vol) fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Adult retinal pigment epithelium (ARPE-19) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in low glucose (1 g/L) DMEM supplemented with 4 mM L-glutamine, 10% (vol/vol) fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in a humidified incubator containing 5% carbon dioxide at 37°C.

**Culture of Primary Mouse Retinal Microglia**

This research was conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME, USA) were handled in strict accordance with good animal practice and all animal work was approved by Institutional Animal Care and Use Committee at University of Colorado Anschutz Medical Campus (Aurora, CO, USA). Retinal microglia were isolated from retinas of 8- to 10-week-old C57BL/6 mice using a method modified from a previous study. Briefly, eyes were removed and retinas dissected under aseptic conditions from globes. Tissue was then incubated in Hanks’ balanced salt solution (HBSS; Corning Cellgro) buffer containing papain with 180 units/mL DNase (Worthington Biochemical Corp., Lakewood, NJ, USA) at 37°C for 10 to 20 minutes. Cells were then briefly tritutated, added to an equal volume of ovomucoid protease inhibitor (10 mg/mL, Worthington Biochem. Corp.), and spun at 300g for 10 minutes at 4°C. The dissociated cells were transferred to 75 cm² flasks and cultured for 4 weeks in DMEM formulated as described above for ARPE cells. After this mixed culture had grown to confluency, the flasks were shaken at 100 rpm on an orbital shaker for 1 hour. Detached cells were then grown in 24-well plates for immunofluorescence or in 96-well plates for ELISA.

**Immunofluorescence**

Retinal microglia were incubated on gelatin-coated glass slides in a 24-well plate. Cells were fixed with 4% paraformaldehyde in PBS for 60 minutes at room temperature and then permeabilized with 0.1% Triton X-100 in PBS for 20 minutes. Retinal microglia were identified by incubating with rabbit anti-Iba-1 antibody (1:400; Wako, Richmond, VA, USA) at 4°C overnight, followed by staining with Alexa Fluor 488 Goat Anti-Rabbit immunoglobulin G (IgG; 1:1000; Invitrogen, Carlsbad, CA, USA) and 4',6-diamidino-2-phenylindole (DAPI; 1:5000, Sigma-Aldrich) for 60 minutes. Images were obtained using a Nikon Eclipse 80i light microscope fitted to a Nikon DS Qi1Mc camera (Nikon Instrument, Inc., Tokyo, Japan).

**Western Blotting**

Lysates were prepared by suspending cells in Laemmli sample buffer (Sigma-Aldrich) and heating to 100°C for 10 minutes. After resolution by SDS-PAGE (Bio-Rad, Hercules, CA, USA), materials were transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The following primary antibodies were used for immunodetection: rabbit anti-human AR (1:1000) as reported previously, mouse anti-actin (1:4000; Sigma-Aldrich), or rabbit anti-MMP-9 (1:1000; Abcam, Cambridge, MA, USA). Secondary anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase (1:5000; Millipore, Bedford, MA, USA), as well as the Western blot Substrate kit (Bio-Rad) were used to detect chemiluminescence using a BioRad ChemiDoc XRS+ imaging system.

**ELISA Assay**

Raw264.7 macrophages or RMG were incubated in a 6-well or 24-well plate, and media were collected after the indicated treatment. Secreted TNF-α and IL-1β in media were determined using corresponding Mouse Cytokine and Growth Factor Immunoassays (ElysiaTech, Aurora, CO, USA). The optical density was detected using a BioTek Synergy 4 Hybrid Microplate Reader (BioTek, Winooski, VT, USA) and the levels of each cytokine were deduced from the absorbance value by extrapolation from a standard curve generated in parallel.

**In Vitro Migration Assay**

Using modified 24-well plate Boyden chambers fitted with filter inserts (pore size 8 μm; Greiner bio-one, Monroe, NC, USA), cells (2 × 10⁶) were seeded in the upper chambers. Aldose reductase inhibitors were added to upper and lower chambers, while LPS was added to the lower chamber only. After incubating for 7 hours, cells were fixed with ice-cold methanol for 15 minutes and stained with 2% crystal violet for 30 minutes and the number of migrated cells on the side facing the lower chamber was determined. In the case of RMG, the entire filter area was counted under ×100 magnification to determine the total number of cells that migrated through the membrane. In experiments using the RAW264.7 macrophage cell line, an average number of migrated cells was determined by counting cells in at least three randomly selected fields under ×100 magnification. Results for each condition from three independent experiments was then averaged and reported as percent change relative to vehicle control.
Zymography
Matrix metalloproteinase–9 gelatinase activity was measured in conditioned medium by zymography. This procedure has been shown to estimate both proenzyme and activated MMP-9 enzyme activity. Equal amounts of conditioned medium were subjected to electrophoresis on 10% zymography gels containing 0.1% gelatin (Bio-Rad). Gels were washed with renaturing buffer (Bio-Rad) for 30 minutes, incubated in developing buffer (Bio-Rad) overnight at 37°C, and stained with Coomassie blue (Sigma-Aldrich). Gelatinase activity was detected in sample lanes by the appearance of bands of lighter gel staining due to digestion of gelatin.

Apoptosis of ARPE-19
Retinal microglia were seeded in the upper chamber of a transwell device and treated with LPS for 6 hours. After treatment, RMG were washed twice with PBS. To measure the effects of cytokines released from LPS-activated RMG, the upper chamber was removed and fitted to a lower chamber containing ARPE-19 cells. After coculture for 48 hours, the level of early apoptosis in RPE cells from the lower chamber was determined using the Annexin-V-FITC Apoptosis Detection kit (BD Biosciences, San Jose, CA, USA) as described.33 Adult retinal pigment epithelium–19 cells were released by trypsin treatment and were washed twice with cold PBS and resuspended in 1 mM CaCl2. The cell suspension (100 μL) was transferred to 5-ml tubes, and 5 μL Annexin-V was added. After incubation with Annexin-V for 5 minutes at 4°C, 5 μL of propidium iodide (500 μg/mL) was added. The cells were incubated at 4°C in the dark for 20 minutes, and 400 μL of binding buffer was added before fluorescence-activated cell sorting (FACS) analysis.

Statistical Analysis
Results are shown as the Means ± SEM of at least three experiments. Data were analyzed by ANOVA with P value of less than 0.05 considered significant.

RESULTS
Aldose Reductase Level in Mouse Macrophage and Retinal Microglia
Previous studies have demonstrated high levels of AR in macrophages.27 To determine the level of AR expression in RMG we used primary cultures established from retinas dissected from the eyes of C57BL/6 mice. Using cells cultured for 4 weeks as previously described,34 we estimated purity by immunostaining for the presence of ionized calcium binding adaptor molecule 1 (Iba-1), a well-characterized marker for microglia. By comparison with total cell counting based on DAPI staining, we estimated that 95% (74 of 78) of cells express the Iba-1 marker (Fig. 1A). We then used immunoblotting to confirm the presence of AR in RMG, as well as in the microglial cell line BV2 and murine macrophage cell line RAW264.7 (Fig. 1B).

Aldose Reductase Inhibition or Deficiency Reduces LPS-Induced Inflammatory Cytokine Secretion
Lipopolysacharide is a potent inducer of the inflammatory response, even at very low doses, in macrophages. It has been shown that AR can mediate LPS-induced inflammatory cytokine expression.28 Therefore, we asked whether AR inhibition could attenuate cytokine secretion in RMG. To determine this, LPS-induced secretion of TNF-α and IL-1β was measured in macrophage and RMG cultures treated with the AR inhibitors sorbinil or BGG. Both ARIs attenuated the LPS-induced secretion of TNF-α and IL-1β in macrophages (Fig. 2A), as well as a significant reduction of TNF-α secretion in RMG (Fig. 2B).

Next, we used a genetic approach to further confirm the role of AR in the endotoxin response. By Western blotting, we observed that AR expression was significantly reduced using a siRNA approach in RAW264.7 macrophages and was not detectable in RMG that were cultured from retinas of AR-null mice (Fig. 2C). Both AR knockdown in macrophages (Fig. 2D) and AR gene deficiency in RMG (Fig. 2E) reduced LPS-induced TNF-α secretion. These data demonstrate that endotoxin-induced secretion of TNF by RMG can be suppressed by downregulation of AR by either genetic means or through pharmacologic reduction of its enzymatic activity using ARIs.

Aldose Reductase Inhibition or Deficiency Prevents Cell Migration Under LPS Exposure
Cell motility is a key feature of macrophage and microglial cell biology, and it is known that LPS induces macrophage migration.35 However, a possible role for AR in cell migration.
is still unknown. To explore this possibility, we investigated the effect of AR inhibition on LPS-induced cell migration. In macrophages, LPS increased cell migration to 238% of control. However, pretreatment of cells with Sorbinil or BGG suppressed the LPS-induced increase to 155% and 140% of control, respectively (Fig. 3A). In a similar trend, LPS exposure to RMG increased cell migration to 180% of control, but migration increased only 130% and 127% by pretreatment of cells with Sorbinil or BGG, respectively (Fig. 3B). Likewise, this same effect was also achieved by downregulation of the AR gene expression using siRNA (Fig. 3C). In RMG, the LPS-induced increase in cell migration was largely absent in RMG cultured from AR-null mice (Fig. 3D). These data indicated that AR is a contributing factor important for both macrophage and RMG migration following endotoxin exposure.
Aldose Reductase Inhibition or Knockdown Attenuates LPS-Induced MMP-9 Activation

It has been shown in lens epithelial cells and vascular smooth muscle cells that MMP-9 is activated by LPS exposure in a manner that is sensitive to AR inhibitors.36,37 To examine the effect of ARIs on MMP-9 activation, macrophages were pretreated with Sorbinil or BGG in the absence or presence of LPS. Here, we collected the culture medium and analyzed gelatinase activity by zymography. We also measured total MMP-9 protein expression in cell lysates using Western blot analysis. As shown in Figures 4A and 4B, MMP-9 was activated by LPS exposure and suppressed by ARIs, suggesting that ARIs are potentially effective in preventing cell migration by suppression of MMP-9 activation. Similarly, we found that AR knockdown reduced MMP-9 activation evidenced by zymography (Fig. 4C) and Western blotting (Fig. 4D). We further confirmed this effect using a specific MMP-9 inhibitor, which suppressed LPS-induced macrophage migration (Fig. 4E). These data are consistent with previously studies that AR inhibition suppresses MMP-9 activation.36-37

Aldose Reductase Inhibition or Deficiency Rescues Apoptosis of ARPE-19 Caused by Activated Retinal Microglia

Tumor necrosis factor α is a robust pro-inflammatory cytokine secreted under a variety of conditions. Previous studies showed that TNF-α secreted from activated microglia induces neurodegeneration.38,39 Superoxide and TNF-α have been demonstrated to cause apoptosis in ARPE-19 cells.40 We demonstrate in Figure 2 that AR inhibition or deficiency reduces TNF-α secretion, therefore, we hypothesized that reduction of AR activity in RMG may prevent RMG-induced apoptosis of ARPE-19. To test this hypothesis, we pretreated RMG with Sorbinil in the absence or presence of LPS, which significantly reduced RMG-induced apoptosis (Fig. 5A). We also examined the genetic effect of AR on apoptosis using ARPE-19 cocultured with RMG from AR-null mice, which also decreased apoptosis compared with RMG from wild-type mice (Fig. 5B). Taken together, these results indicate that AR activity is contributing to RMG-induced apoptosis in ARPE-19 cells.
DISCUSSION

Retinal microglia are phagocytic cells that are responsible for cleaning up apoptotic debris throughout the inner and outer plexiform layers of the retina. Retinal microglia can become activated by exposure to a variety of factors, including endotoxin or glycated albumin, where they undergo a morphologic transformation to assume an ameboid shape and migrate to areas of tissue damage. As in macrophages, activated RMG upregulate extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase signaling, which leads to release of TNF-α. Activated RMG have been linked to a variety of ocular disorders such as AMD, light-induced retinal degeneration, DR, glaucoma, and endotoxin-induced uveitis.
As phagocytic cells, macrophages and RMG have many functional similarities. Studies in macrophage cultures have demonstrated that AR inhibition or deficiency prevents endotoxin-induced nuclear factor-κB activation leading to production of pro-inflammatory genes. Similarly, many studies have shown that inhibition of RPE cell migration in response to endotoxin or other factors that cause transition of RMG to an activated state.

In agreement with studies of Ramana and coworkers, our previous studies demonstrated that reduction of AR activity, using either genetic or pharmacologic inhibitors, substantially suppressed the LPS-induced secretion of TNF-α by macrophage cultures. Reactive oxygen species (ROS) is a key factor in the induction of TNF-α through activation of p38 or ERK. Our previous study with the RAW264.7 macrophage cell line showed that AR inhibition attenuates p38 or ERK activation and suppresses ROS production following LPS exposure, indicating that AR inhibition is capable of suppressing TNF-α secretion. In the current study, we evaluated the role of AR activity in response of RMG to activation. Using primary cultures of microglia generated from the mouse eye, we demonstrated that RMG have levels of AR that are similar to murine cell line RAW264.7 (Fig. 1). In addition, we showed that loss of AR activity through BGG or sorbinil inhibition or via AR knockout mice attenuated LPS-induced TNF-α secretion in RMG (Fig. 2). Taken together, these findings demonstrate the functional similarity between macrophages and RMG with regard to the involvement of AR in regulating cytokine production. It has been shown that TNF-α, as one of the major cytokines secreted by activated RMG, may be involved in neurodegeneration and uveitis. Therefore, inhibition of TNF-α through pharmacologic inhibition of AR in microglia may hold promise as a potential new strategy against ocular inflammation.

In the inflamed eye, activated RMG migrate into the subretinal space and cause RPE disorganization. Prevention of RMG migration might be another strategy to minimize collateral damage to cells during inflammation. We demonstrated that AR inhibition or genetic knock down suppresses LPS-induced cell migration in both macrophages and RMG (Fig. 3). This is the first report indicating that AR mediates endotoxin-induced migration of immune cells. This result is also consistent with our previous study that AR inhibition prevents LPS-induced infiltration of inflammatory cells into the eye. The mechanism linking AR activity and cell migration is not yet understood. Thus, to further characterize these mechanisms, we investigated whether AR mediates MMP-9 activation after LPS exposure. Importantly, MMP-9 is an enzyme that induces cellular morphologic changes promoting increased motility. We confirmed that AR inhibition reduces the LPS-stimulated activation of MMP-9 (Fig. 4), which further characterizes the effects of AR inhibition on mechanisms controlling cell migration.

The distribution of RMG throughout the retina changes with age. In younger animals, RMG are found primarily in the inner retina. However, with age or after adopting an “activated” state, RMG can be found in higher numbers in the subretinal space between photoreceptor outer segments and the RPE. Wong and coworkers have shown that activated RMG disrupt the normal organization of RPE and alter the expression of key junctional proteins such as ZO-1. To determine whether AR inhibition or ablation in RMG influences the viability of RPE cells, we carried out coculture experiments with RMG and ARPE-19. Apoptosis was significantly higher when RPE cells were cocultured with activated RMG, which was significantly diminished by pretreating with ARIs. Similarly, RMG derived from AR-null mice were substantially less effective in inducing RPE apoptosis (Fig. 5). Thus, either AR inhibition or ablation reduced the ability of activated RMG to induce apoptosis in ARPE-19 (Fig. 5). Interestingly, it appears that diabetic mice that are null for the AR gene have markedly reduced levels of defects typically associated with DR, including retinal capillary degeneration and iNOS activation. Further study will be required to determine whether the blockade of AR in RMG can translate into reduced markers of inflammation in other cells and tissues in the diabetic eye, including the retinal vasculature and neurons known to be damaged by chronic hyperglycemia such as in diabetes.

Despite discouraging results from clinical trials of ARI against DR and neuropathy, research continues on the development of newer generations of inhibitors for clinical study. We recently identified β-glucogallin as a novel ARI from Indian gooseberry (Emblica officinalis). In addition to having ARI activity in aldo-keto reductase assays, we found that β-glucogallin was effective at reducing inflammatory cells in a murine uveitis model. Results from the current study demonstrate that β-glucogallin suppresses many of the functional responses of macrophages and RMG to LPS exposure, including cytokine production, cell migration, and induction of MMP-9. Further study will be required to determine if β-glucogallin, or structurally-related derivatives, are effective at downregulation of RMG activation in the diabetic retina.

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