Published in final edited form as:

Chem Biol Interact. 2019 April 01; 302: 46–52. doi:10.1016/j.cbi.2019.01.020.

Role of Aldose Reductase in Diabetes-Induced Retinal Microglia Activation

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Abstract

Diabetes-induced hyperglycemia plays a key pathogenic role in degenerative retinal diseases. In diabetic hyperglycemia, aldose reductase (AR) is elevated and linked to the pathogenesis of diabetic retinopathy (DR) and cataract. Retinal microglia (RMG), the resident immune cells in the retina, are thought to contribute to the proinflammatory phenotype in the diabetic eye. However, we have a limited understanding of the potential role of AR expressed in RMG as a mediator of inflammation in the diabetic retina. Glycated proteins accumulate in diabetes, including Amadori-glycated albumin (AGA) which has been shown to induce a proinflammatory phenotype in various tissues. In this study, we investigated the ability of AGA to stimulate inflammatory changes to RMG and macrophages, and whether AR plays a role in this process. In macrophages, treatment with an AR inhibitor (Sorbitol) or genetic knockdown of AR lowered AGA-induced TNF- α secretion (56% and 40%, respectively) as well as cell migration. In a mouse RMG model, AR inhibition attenuated AGA-induced TNF- α secretion and cell migration (67% and 40%, respectively). To further mimic the diabetic milieu in retina, we cultured RMG under conditions of hypoxia and observed the induction of TNF- α and VEGF protein expression. Downregulation of AR in either a pharmacological or genetic manner prevented hypoxia-induced TNF- α and VEGF expression. In our animal study, increased numbers of RMG observed in streptozotocin (STZ)-induced diabetic retina was substantially lower when diabetes was induced in AR knockout mice. Thus, *in vitro* and *in vivo* studies demonstrated that AR is involved in diabetes-induced RMG activation, providing a rationale for targeting AR as a therapeutic strategy for DR.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Keywords

Aldose reductase; Amadori-glycated albumin; retinal microglia; hypoxia; inflammation

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1. Introduction

The global burden of diabetes is enormous. In 2015, there were 415 million adults with diabetes, with the number of affected individuals estimated to increase to 642 million by 2040 [1]. Chronically elevated blood glucose levels lead to diabetic complications affecting the kidneys (nephropathy), peripheral nerves (neuropathy) and cardiovascular system [2–4]. Diabetes is particularly devastating to the eye. Diabetic retinopathy (DR) is one of the leading causes of blindness among adults [5–7].

In efforts to understand the pathogenesis of DR, much research has been focused on aldose reductase (AR), an NADPH-dependent aldo-keto reductase that converts glucose to sorbitol as the first step in the polyol pathway [8]. Hyperglycemia leads to increased AR activity in target tissues of diabetes [9]. Enzymatic or genetic downregulation of AR alleviates diabetes-associated defects in lens [10–13], whereas increased levels of AR such as in AR-overexpressing transgenic mice lead to increased risk for abnormalities in diabetic lens [14, 15] and retina pigmented epithelium [16]. In retina, diabetes-induced vascular endothelial growth factor (VEGF) production is considered a major driver of neovascularization, which is commonly observed in patients with long-standing diabetes and poor metabolic control [17, 18]. Lowering of AR activity by pharmacological inhibition or inactivation of the AR gene protects the retina from retinal barrier leakage and neovascularization by suppressing VEGF production [16, 19, 20]. In addition, hyperglycemia leads to an increased flux of glucose metabolism through the polyol pathway, contributing to oxidative stress [16] and inflammation [21]. Blockade of the polyol pathway by AR inhibitors prevents glucose-induced oxidative stress and retinal inflammation [16, 21]. In light of the importance of AR in variety of diseases, many studies have been focused on developing natural compounds against AR activity [10, 11, 13, 22, 23].

Due to exposure to chronically elevated blood sugar levels, diabetic individuals show increased accumulation of Amadori-glycated proteins that may undergo further irreversible modifications to form advanced glycation end-products (AGEs) [24, 25]. Amadori-glycated proteins are generated by the condensation of one or more sugar aldehydes with a like number of free amino groups on proteins. Albumin is considered one of the major plasma proteins that undergo glycation to form amadori-glycated albumin (AGA; [26]). AGA is found at elevated levels in diabetic patients [24] and animals [27] and has been linked to diabetic complications such as nephropathy [28] and retinopathy [29]. Treatment of diabetic animal models with antibodies to AGA [30] or an inhibitor of albumin glycation [31], showed promising results in prevention of sequelae of diabetes such as changes to the vasculature and proinflammatory phenotype. In the eye, elevated amounts of AGEs are age- and diabetes-related [32]. Accumulation of AGEs is reported to induce oxidative stress [33]. Studies indicate that AGEs induce VEGF production in Muller cells [34] and retinal ganglion cells (RGC) [35], and stimulate cytokine secretion in retinal pigment epithelial

cells (RPE) [36]. Among the mechanisms contributing to inflammatory response, activation of NF- κ B signaling could play a role in AGE-induced inflammation and oxidative stress [37, 38].

Microglia, a type of glial cell that resides in the central nervous system [39], were first discovered in 1933 by Dr. Pio del Rio-Hortega [40]. Retinal microglia (RMG) are eye-specific cell types that reside in the inner and outer plexiform layers. Under stress, RMG become activated and migrate into the neuronal or photoreceptor layers [41] where they may contribute to an inflammatory state. In diabetic animals, increased accumulation of glycation products in the retina is thought to contribute to RMG activation [27, 42]. Gardner and colleagues reported that activated RMG secrete VEGF and TNF, which contribute to the pathogenesis of DR [43]. Studies have demonstrated that AR mediates endotoxin-induced RMG activation [44, 45]. However, the effect of AR and diabetes on RMG activation is still poorly understood. In light of the importance of AGA as an inducer of retinal inflammation, and the potential role of AR as a factor in secretion of proinflammatory cytokines, we studied the role of AGA in induction of AR-dependent inflammatory signaling in RMG in the eyes of diabetic and control animal models.

2. Materials and Methods

2.1. Materials and cell culture

Human amadori-glycated albumin (AGA) and streptozotocin (STZ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sorbinil was generously provided by Pfizer Central Research (Groton, CT, USA). RAW264.7 macrophages were cultured in high glucose (4g/L) Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4 mM L-glutamine, 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin. Primary mouse retinal microglia (RMG) were isolated as previously described [44] and cultured in high glucose DMEM/F12 supplemented with non-essential amino acid, 4 mM L-glutamine, 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin. Cells were maintained in a humidified incubator containing 5% carbon dioxide at 37 °C. For hypoxia condition, RMGs were cultured in a hypoxic incubator chamber containing 1% O₂, 5% CO₂ and 94% N₂ for 24 hour before harvest. [46]

2.2. Animals

This research was conducted in compliance with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by Institutional Animal Care and Use Committee at University of Colorado. C57BL/6 and CX3CR1^{GFP} (JAX stock #005582) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). AR knockout (ARKO) mice were generated from previous studies [47]. CX3CR1 is a chemokine (neurotactin) receptor expressed on a variety of immune cells including microglia. The CX3CR1^{GFP} mouse line was generated by replacement of the *CX3CR1* gene with the cDNA encoding EGFP [48], resulting in a phenotype in which all CX3CR1 expressing cells express autofluorescent GFP. Intercrossing of CX3CR1^{GFP} mice yielded CX3CR1^{GFP/GFP} mice that were homozygous for the mutant allele. Utilizing the CX3CR1^{GFP} mouse line allowed us to visualize RMG activation and migration in the mouse retina. All experimental mice were

also genotyped as homozygous for the wild type allele of the retinal degeneration *rd8* mutation [49]. Experimental diabetes was induced by treatment of mice with streptozotocin (STZ) as described [50]. Briefly, we injected one dose of STZ and checked the blood sugar level 3 days after injection. The mice with blood glucose values exceeding 300 mg/dl were considered diabetic. For AR deficiency study, mice (8-12 week old) were assigned to different groups (WT, ARKO, WT+STZ and ARKO+STZ).

2.3. Small interfering RNA (siRNA) transfection

Control siRNA and AKR1B3 (mouse AR) siRNA were purchased from Qiagen (Valencia, CA, USA). Transient transfection of siRNA was performed using HiPerFect transfection reagent (Qiagen) according to the manufacturer's protocol. Macrophages (5×10^5 cells) were seeded in a 100 mm culture dish. After 16 h cells were ~ 70% confluent and cells were transfected with control or AR siRNA (10 nM) and cultured for an additional 72 h. Efficiency of AR knockdown was confirmed by Western blot.

2.4. Western blotting

Lysates were prepared by suspending cells in Laemmli sample buffer (Sigma-Aldrich) and heated at 100 °C for 10 min, and resolved by SDS-PAGE (Bio-Rad, Hercules, CA, USA). After proteins were transferred to PVDF membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA), primary antibodies were used for immunodetection: rabbit anti-AR (1:1000) [51] or mouse anti-actin (1:4000, Sigma-Aldrich). 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.

2.5. ELISA assay

Macrophages (10^5 cells) or RMG (10^3 cells) were incubated in a 24-well or 96-well plate and media were collected after AGA or hypoxia treatment. Secreted TNF- α and VEGF in media were determined using corresponding Mouse TNF- α DuoSet ELISA Development kit (R&D Systems, Inc., Minneapolis, MN, USA) and Mouse VEGF DuoSet kit (R&D Systems, Inc.). The optical density was detected using a BioTek Synergy™ 4 Hybrid Microplate Reader (Bio Tek, Winooski, VT, USA) and the level of cytokine was deduced from the absorbance value by extrapolation from a standard curve generated in parallel.

2.6. In vitro migration assay

Macrophages (10^4 cells) were cultured in Cultured-Insert (500 μ m cell-free gap, Ibidi, Martinsried, Germany) and incubated with AGA (500 μ g/ml) in the absence or presence of Sorbinil (10 μ M) for 2 days. Migration assay of RMG was carried out as previously described [44]. RMG (10^3 cells) were seeded in Boyden chambers fitted with filter inserts (pore size 8 μ m, Greiner bio-one, Monroe, NC, USA) upper chambers. Sorbinil was added to upper and lower chambers, while AGA was added to the lower chamber only. After incubating for 24 h, cells were fixed with ice-cold methanol for 15 min and stained with 2% crystal violet for 30 min and the number of migrated cells on the side facing the lower

chamber was determined. The entire filter area was counted under 100× magnification to determine the total number of cells that migrated through the membrane.

2.7. Immunofluorescence

Image staining of RMG was completed as previously described [44]. The primary antibodies used for staining were: rabbit anti-Iba1 antibody (1:200; Wako, Richmond, VA, USA) and mouse anti-AR antibody (1:200; Santa Cruz Biotechnology Inc., Dallas, TX, USA). After incubation at 4 °C overnight, the cells were stained with Alexa Fluor® 488 Goat Anti-rabbit, Alexa Fluor® 594 Goat Anti-mouse IgG (1:1000, Invitrogen, Carlsbad, CA, USA), and 4',6-diamidino-2-phenylindole (DAPI) (1:5000, Sigma-Aldrich) for 60 min. Images were obtained using a Nikon Eclipse 80i light microscope fitted to a Nikon DS Qi1Mc camera (Nikon Instruments Inc., Tokyo, Japan).

2.8. Statistical analysis

Results are shown as the Means ± SEM of at least three experiments. Data were analyzed by two-tailed Student's *t* test with *P* value of <0.05 considered significant.

3. Results

3.1. Aldose reductase inhibition or ablation attenuates AGA-induced cytokine secretion and cell migration in macrophages

AGA is known to be involved in diabetic complications including retinopathy [27] and nephropathy [52]. In this study, we first utilized murine macrophages RAW264.7 to study the effect of AR on AGA-induced inflammatory responses. Treatment of AGA in macrophages induces TNF-α secretion in a dose-dependent manner (Fig 1A). Sorbinil, a well-characterized AR inhibitor, suppressed AGA-induced TNF-α secretion (Fig 1B). Genetic ablation experiments showed that AR knockdown not only reduces cytokine secretion (Fig 1C) but also prevents cell migration (Fig 1D). Efficiency of AR knockdown was confirmed using Western blot (Fig 1E).

3.2. Aldose reductase inhibition attenuates AGA-induced cytokine secretion and cell migration in RMG

The receptor for AGE (RAGE) is expressed on RMG [53], indicating that RMG may have the capacity to respond to AGA. We utilized primary RMG cell cultures generated from mouse retinas to investigate the response of immune cells in the diabetic eye. Studies have reported that blockade of AR reduces endotoxin-induced cytokine secretion and cell migration in RMG [44, 45]. In the present study we investigated the effect of AR on AGA-induced inflammatory responses. In this study, we identified RMG in cell cultures using immunostaining with the well-characterized microglia marker Iba1 (Ionized calcium binding adaptor molecule 1). Co-staining with AR antibodies confirmed that cultures were highly enriched for RMG that also express AR (Fig 2A). We observed that AGA strongly induced TNF-α secretion, and that pharmacological inhibition of AR using Sorbinil substantially attenuated the AGA-induced TNF-α secretion (Fig 2B). Similarly, we observed that AGA induced migration of macrophage cells, and that Sorbinil significantly reduced the AGA effect (Fig 2C). These results indicate that AR plays a role in activation of RMG in response

to exposure to AGA. Together, this is the first study to describe the role of AR in AGA-induced inflammatory responses in RMG.

3.3. Pharmacological inhibition or genetic ablation of aldose reductase suppresses hypoxia-induced inflammatory cytokine and VEGF secretion

We next asked whether AR mediates other diabetic phenotype that is induced by RMG. In diabetic retina, high glucose causes hypoxia [54]. Under hypoxic condition, VEGF is the major modulator that causes neovascularization in the eye [55]. While Muller cells and ganglion cells are known to be major sources of VEGF [56], we hypothesized that RMG could be another source of VEGF. To understand the behavior of RMG in diabetic retina, we cultured RMG under hypoxic conditions of 1% oxygen level. Under hypoxia, we observed elevation of TNF- α (Fig 3A and 3C) and VEGF (Fig 3B and 3D) levels. However, the secretion of TNF- α and VEGF were significantly attenuated in RMG treated with Sorbinil (Fig 3A and 3B) as well in RMG isolated from AR null mice (Fig 3C and 3D). Previous animal studies showed that AR knockout prevents DR by suppressing VEGF production [57]. Thus, our findings in RMG provide a possible explanation linking AR to regulation of VEGF secretion in the diabetic retina.

3.4. Aldose reductase deficiency prevents diabetes-induced RMG infiltration

Several studies have observed RMG activation in diabetic retina [27, 58, 59]. We previously showed that AR mediates RMG activation induced by either AGA (Fig 2) or hypoxia (Fig 3) *in vitro*. Therefore, we wanted to further understand the effect of AR on RMG *in vivo*. To observe the RMG activation *in vivo*, we utilized a transgenic mouse (CX3CR1^{GFP}) that expresses green fluorescent protein (GFP) in RMG and followed the distribution of these cells in mouse retinas following STZ-induction of experimental diabetes in animals with or without the functional mouse gene for AR (AKR1B3). In the absence of diabetes, RMG reside in inner plexiform layer; once activated, they migrate to inner nuclear layer (INL) and/or outer nuclear layer (ONL). Without diabetes, there is no significant difference between WT and ARKO in the abundance (Fig 4A, B, and E) or the spatial distribution (Fig 4F) of RMG in the retina. However, after 6 weeks of STZ-induced diabetes, RMG migrate into the INL and ONL of the retina in WT mice (Fig 4C, F). Compared to WT mice, AR null mice demonstrate a reduction in the STZ-induced increase in the abundance of RMG in retina (Fig 4E) as well as loss of diabetes-induced redistribution of RMG to the INL and ONL of the retina (Fig 4F). The lack of STZ-induced RMG movement to outer retinal layers observed in ARKO mice suggests that AR plays an essential role in RMG for its activation in the hyperglycemic diabetic milieu.

4. Discussion

Retinopathy occurs in approximately 80% of patients who have diabetes for 20 years or more [60] and is one of the leading causes of blindness. Glycated proteins, including amadori-glycated albumin (AGA), as well as proteins containing advanced oxidation products of initial glycation intermediates, (advanced glycation endproducts, or AGEs) have been considered as a stressor for pathogenesis of DR [27, 29]. In this study, we found that AGA-induced inflammatory responses are attenuated by either AR inhibition or genetic

ablation in macrophages (Fig 1) and RMG (Fig 2). In diabetic animals, AGEs induce inflammatory responses in RMG through ERK, p38 and ROS activation [27, 61]. Previous studies showed the positive correlation of AR expression and ERK activation [14], and blockade of AR is capable of suppressing activation of either ERK [62] or p38 [23] signaling pathways. Another study also showed that ERK inhibitor is able to reduce AGE-induced inflammatory responses on microglia [63]. Therefore, we speculated that AR inhibition prevents AGE-induced inflammatory responses by suppressing ERK and p38 activation. Suppression of fructose production by inhibiting AR polyol pathway may be another possibility. Glucose is converted into sorbitol by AR and further converted into fructose via NAD⁺-dependent sorbitol dehydrogenase (SDH) [8]. Increased influx of AR in the polyol pathway leads to increased fructose production, which has been shown to form AGEs much faster than using glucose as the precursor [64]. Together, inhibition of AR suppresses AGE-induced inflammatory responses; on the other hand, blockade of AR polyol pathway reduces AGE formation in diabetic retina [65, 66].

VEGF-induced neovascularization is another major cause of DR that involves the abnormal proliferation of retinal capillaries which are unstable. Capillary leakage of plasma contents into the retina leads to retinal edema and visual impairment [55]. Our studies on RMG as a potential mediator of diabetic retinopathy point to AR inhibition as an attractive therapeutic target for reduction of retinal inflammation and VEGF secretion in DR (Fig 3). Previous studies showed that cyclooxygenase-2 (COX-2) plays a regulatory role in the production of VEGF by Müller cells under hypoxia conditions [46]. It is interesting to note that Ramana and colleagues previously reported that AR inhibition suppresses COX-2 expression [67, 68]. In RMG, we found that downregulation of AR protein or enzymatic activity attenuates VEGF production (Fig 3). Further studies will be needed to test whether there is an interplay between AR and COX-2 in the regulation of VEGF secretion in RMG under diabetic or hypoxic conditions.

A previous study showed that blockade of AR *in vivo* prevents RMG migration into the inner and outer nuclear layers in retinas of AR-overexpressing (AR-transgenic) mice [45]. We next investigated whether AR mediates RMG activation in diabetic retina. Here we observed that AR knockout prevents RMG migration in diabetic retina (Fig 4) indicating that RMG is an important therapeutic target mediated by AR in DR. Currently, intravitreal injection of anti-VEGF antibody is commonly deployed to alleviate the severity and progression of vascular changes associated with DR of long duration [69]. However, intravitreal injection therapy may require frequent treatments and associated risk for discomfort and increased risk for side effects including retinal detachment and infection. Our studies suggest that the strategy of AR inhibition in the early stages of DR may suppress retinal inflammation and thus delay or prevent vascular disease that is associated with later stages of DR progression and its associated greater risks for permanent vision loss.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This study is supported by NIH grants EY005856 and EY021498 (JMP), and by a Challenge Grant to the Department of Ophthalmology from Research to Prevent Blindness. We thank Candace Chan for critical review.

Abbreviations

AGA	amadori-glycated albumin
AGE	advanced glycation end-product
AR	aldose reductase
DR	diabetic retinopathy
RMG	retinal microglia
VEGF	vascular endothelial growth factor

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Highlights of “Role of Aldose Reductase in Diabetes-Induced Retinal Microglia Activation”

- In diabetes, aldose reductase (AR) is elevated and linked to diabetic retinopathy.
- Lowering of AR enzymatic activity reduced inflammatory markers.
- Retinal microglia cultured under hypoxia led to TNF- α and VEGF protein expression.
- Diabetes caused an increase in the numbers of activated retinal microglia.
- Targeting AR with inhibitors may prevent inflammation in the diabetic retina.

Fig. 1. Effect of AR inhibition or knockdown on AGA-induced cytokine secretion and migration in RAW264.7.

AGA induces TNF- α secretion in a dose dependent manner (A). Macrophages were treated with vehicle or Sorbinil (10 μM) in the absence or presence of AGA (500 $\mu\text{g/ml}$) for 24 h for TNF- α detection (B). Macrophages were further transfected with control or AKR1B3 siRNA (siAR) for 72 h followed by treatment with AGA (500 $\mu\text{g/ml}$) for 24 h for TNF- α detection (C) or 48 h for migration assay (D). The efficiency of AR knockdown was examined by probing with AR antibody (E). Secreted TNF- α was measured by using mouse TNF- α detection ELISA kit. The width of cell-free gap in (D) is 500 $\mu\text{m} \pm 50 \mu\text{m}$. The migration assay was conducted by using wound-healing method. Data shown are means $\pm$ SEM (N = 3). * $P < 0.05$; ** $P < 0.01$.

Fig. 2. Effect of AR inhibition or knockdown on AGA-induced cytokine secretion and migration in RMG.

RMG cultures were established from C57BL/6 mice and immunostained by microglia marker Iba1 (green) and AR (red) merged with DAPI (blue) to visualize nuclei (A). RMG were treated with vehicle or Sorbinil (10 μ M) in the absence or presence of AGA (500 μ g/ml) for 24 h for TNF- α detection (B) or migration assay (C). Secreted TNF- α was measured by using mouse TNF- α detection ELISA kit. The migration assay was conducted by using transwell method. Data shown are means $\pm$ SEM (N = 3). * P < 0.05.

Fig. 3. AR inhibition or knockdown prevents hypoxia-induced TNF- α and VEGF secretion. RMG were treated with 10 μ M Sorbinil (A and B) or isolated from ARKO mice (C and D) in normoxia or hypoxia (1% O₂) condition for 24 h. Secreted TNF- α (A and C) and VEGF (B and D) were measured ELISA kit. Data shown are means $\pm$ SEM (N = 3). *P, #*P* < 0.05.

Fig. 4. Effect of AR on RMG activation in diabetic mice
 WT-CX3CR1^{GFP} mice (A and B) and ARKO-CX3CR1^{GFP} mice (C and D) were injected with STZ (160 mg/kg body weight) for 6 weeks before sacrificed (B and D). Cryosections were obtained from each group and stained with DAPI (blue). RMG were shown in green color by expressing GFP. White arrows indicate migrated RMG in inner or outer nuclear layers. Statistic data was performed graphically (F). Data shown are means $\pm$ SEM (N = 5). INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar 100 μ m.