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14. ABSTRACT Traumatic optic neuropathy (TON) is a type of injury commonly seen in the war. There are currently no proven treatments that reverse the damage in TON. The proposed mechanism of TON involves optic nerve injury-induced activation of retinal microglial cells and their release of pro-inflammatory cytokines, and retinal ganglion cell (RGC) death. As a self-defense system, activated microglial cells also release adenosine, which attenuates inflammation via adenosine receptors (AR)s, including A2AAR. Although AR agonists attenuate inflammation, the way to minimize nonspecific effects associated with systemic administration of these agonists remains unclear. Released adenosine levels in the injured brain are mainly regulated by adenosine kinase (AK). Inhibition of AK potentiates local extracellular adenosine levels at cell and tissue sites which are undergoing accelerated adenosine release. Thus, AK inhibition represents a mechanism to selectively enhance the endogenous protective actions of adenosine during cellular stress. Our studies have shown that microglial activation, retinal inflammation, and RGC death occur in the mouse model of TON, and that A2AAR signaling provides protection from TON. Further, our preliminary data suggest that AK inhibitor (AKI)-enhanced A2AAR signaling provides protection from TON in mice. Therefore, inhibition of AK potentially amplifies the therapeutic effects of site- and event-specific accumulation of extracellular adenosine, which is of highly translational impact.					
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Abbreviations:

TON, traumatic optic neuropathy;

DR, diabetic retinopathy;

AK or ADK, adenosine kinase;

A2AAR, A2A adenosine receptor;

ENT, equilibrative nucleotide transporter;

CNT, concentrative nucleoside transporter;

AKI, adenosine kinase inhibitor;

RGC, retinal ganglion cell;

5'-NT, 5'-nucleotidase;

CD39 and CD73, Ecto-5'-nucleotidases;

I. Introduction

Vision loss related to traumatic optic nerve damage is commonly seen in the theater of war. Unfortunately, there are currently no proven treatments that can prevent the damage associated with an acute retinal inflammation, traumatic optic neuropathy (TON). Retinal ganglion cell (RGC) death is known to be a fundamental pathological process in TON. Several common mechanisms have been hypothesized to underlie apoptotic processes, including interruption of trophic support, oxidative stress, and increased extracellular glutamate levels that result in excitotoxicity. These stimuli associated with the injured RGCs often activate retinal microglia, which release pro-inflammatory cytokines and cytotoxic molecules to further exacerbate the degenerative process, leading to blindness. Although TON is the major cause of blindness in the war, underlying mechanisms of the endogenous anti-inflammation or neuroprotection, which is most critical in reducing inflammation in time, are still not clear. **Our goal is to elucidate the mechanism of anti-inflammation to explore the endogenous neuroprotective mechanism therapeutically.** Adenosine, an extracellular signaling molecule, has widespread effects on the nervous and immune systems with increased concentration at sites of tissue injury. The increased adenosine level at the inflamed sites can protect against cellular damage by activating adenosine receptors (AR)s. We have shown the endogenous neuroprotective effects of A2AAR in animal models of TON [1]. The endogenous inflammation-mitigation activity of A2AAR signaling is remarkable: sub-threshold levels of TON stress that caused minimal retinal inflammation in wild-type mice was sufficient to induce extensive retinal inflammation and more prolonged and higher levels of pro-inflammatory cytokines in A2AAR-knockout mice [1]. However, systemic treatment of retinal inflammation with A2AAR ligands, as was used in this study, is not a therapeutic option because the widespread distribution of A2AAR causes systemic side effects. Extracellular adenosine levels are regulated by the interplay of equilibrative and concentrative nucleoside transporters (ENT and CNT) with enzymes that synthesize it (S-adenosyl homocysteine hydrolase, SAH-H, 5'-nucleotidase, 5'-NT, and ecto-5'-NT), degrade it (adenosine deaminase, ADA) and phosphorylate it (adenosine kinase, AK) (**Illustration 1**).

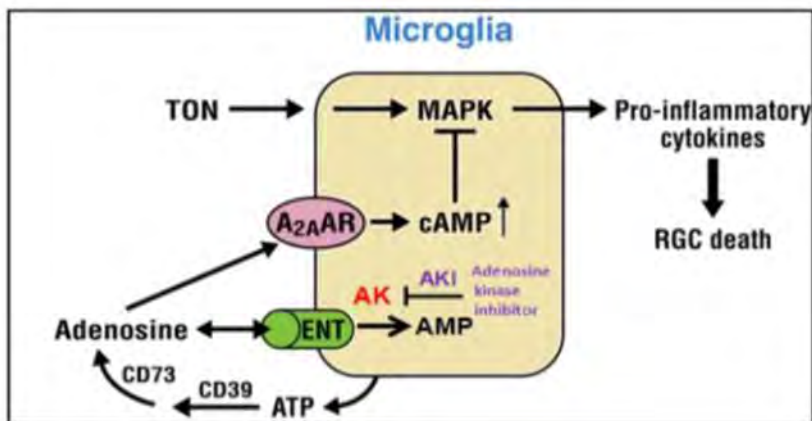


Illustration 1. Concept frame showing how extracellular level of adenosine is regulated.

Extracellular adenosine reuptake by ENT and CNT enhances adenosine degradation, decreases extracellular adenosine, and terminates the neuroprotective effect of AR. Under physiological conditions, the removal of extracellular adenosine is predominantly regulated by AK via conversion of adenosine into AMP. Under diabetic condition, AK is down-regulated, suggesting a potential self-protective effect [2]. However, under TON conditions, AK is surprisingly up-regulated [3]. The up-regulation of AK may further exacerbate neurodegeneration. The notion of AK regulation as a protective response to retinal inflammation is corroborated in our recent studies using an AK inhibitor in the mouse model of diabetes [2]. Based on our recent findings, we hypothesize that adenosine phosphorylation is important in physiology, and that enhanced adenosine degradation by increased AK activity contributes to diabetic retinopathy [2] and TON [3]. We further

hypothesize that AK inhibition by inhibitors or AK gene silencing by siRNA amplifies the endogenous therapeutic effects of site- and event-specific accumulation of extracellular adenosine. We will test these hypotheses in two aims.

II. Statement of Work

We seek to understand the mechanism of inflammation in TON in an effort to control RGC death. This is done under the hypothesis that an imbalance in adenosine formation and metabolism in the retinal microglia may contribute to retinal complications in the setting of TON.

Task 1: To test the hypothesis that a mechanism of anti-inflammation mediated by A_{2A}AR signaling exists in retinal microglial cells. In the setting of TON, however, this process is overwhelmed by the pro-inflammatory state. We further hypothesize that a selective A_{2A}AR agonist effective in reducing inflammation in other disease processes is of utility in TON.

Task 2: To test the hypothesis that an imbalance in adenosine formation and metabolism in the retinal microglia participated by AK may contribute significantly to retinal complications in TON.

III. Progress

Task 1.

Experimental Designs and Methods. Mice were anesthetized according to standard protocol and bilateral limbal conjunctival peritomy was performed posteriorly to the optic nerve in each mouse. Compression by forceps was performed on the right optic nerve in each mouse with the left optic nerve serving as a control. Compression was released at 10 seconds and pupillary dilation was noted. Mice were treated with or without an A_{2A}AR agonist, CGS21680 (25µg/kg; i. p.) every other day for 7 days. All retinas were then harvested for immune-histology, western and molecular analyses.

Results. We examined the role of A_{2A}AR in retinal complications associated with TON. Initial studies in wild-type mice revealed that treatment with the A_{2A}AR agonist resulted in marked decreases in the TON-induced retinal cell death (**Figure 1-a, b, c**) [1], microglial activation (**Figures 3-a, b, c**) [1], and releases of pro-inflammatory cytokines TNF-α and IL-6 (**Figure 4**) [1]. To further assess the role of A_{2A}AR in TON, we studied the effects of A_{2A}AR ablation on the TON-induced retinal abnormalities. A_{2A}AR^{-/-} mice with TON showed a significantly higher mRNA level of TNF-α, Iba1-1 in retinal tissues, and ICAM-1 expression in retinal sections compared with wild-type mice with TON (**Figure 6**) [1]. To explore a potential mechanism by which A_{2A}AR signaling regulates inflammation in TON, we performed additional studies using hypoxia- or LPS-treated microglial cells as an in vitro model for TON. Activation of A_{2A}AR attenuates hypoxia or LPS-induced TNF-α release and significantly repressed the inflammatory signaling ERK in the activated microglia (**Figure 7**) [1].

Task 2.

Experimental Designs. We first confirmed the retinal complication-inhibiting effect of AK inhibitor in type 1 diabetic mice. We then determined this effect in TON.

Results. At 16 weeks, when diabetic mice exhibit significant signs of retinal inflammation including upregulation of oxidative/nitrosative stress, A_{2A}AR, ENT1, Iba1, TNF-α, ICAM1, retinal cell death, and downregulation of AK, the ABT-702 treated group showed lower signs of inflammation compared to control animals receiving the vehicle. The involvement of adenosine signaling in the anti-inflammation effect of ABT-702 was supported by the TNF-α release blocking effect of A_{2A}AR antagonist in AGA-treated microglial cells

(**Figure 1 - 4**) [2]. The use of adenosine receptors agonists are limited by systemic side effects. Therefore, we seek to investigate the potential role of amplifying the endogenous ambient level of adenosine by pharmacological inhibition of AK. We tested our hypothesis by comparing TON-induced retinal injury in mice with and without ABT-702 treatment, a selective AK inhibitor (AKI). The retinal-protective effect of ABT-702 was demonstrated by significant reduction of Iba-1, ENT1, TNF- α , IL-6, and iNOS/nNOS protein or mRNA expression in TON as revealed by western blot and real time PCR (**Figure 1 - 5**) [3]. TON-induced superoxide anion generation and nitrotyrosine expression were reduced in ABT-702 treated mice retinal sections as determined by immunofluorescence (**Figure 6**) [3]. In addition, ABT-702 attenuated p-ERK1/2 and p-P38 activation in LPS induced activated mouse microglia cells (**Figure 7**) [3].

IV. Conclusions. These results suggest a self-protective role of AK in diabetic retina but not in TON. ABT-702, a selective AK inhibitor, has a protective effect on TON-induced retinal inflammation and damage by augmenting the endogenous therapeutic effects of site- and event-specific accumulation of extracellular adenosine.

V. References.

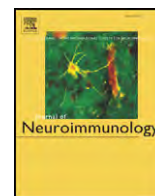
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[3] Ahmad S, Elsherbiny NM, Bhatia K, Elsherbini AM, Fulzele S, Liou GI. Inhibition of adenosine kinase attenuates inflammation and neurotoxicity in traumatic optic neuropathy. *J Neuroimmunol.* 2014 Dec 15;277(1-2):96-104. PMID:25457840

VI. Appendix.

Appendix containing the above three references are attached.



Potential role of A_{2A} adenosine receptor in traumatic optic neuropathy

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ABSTRACT

In traumatic optic neuropathy (TON), apoptosis of retinal ganglion cells is closely related to the local production of reactive oxygen species and inflammatory mediators from activated microglial cells. Adenosine receptor A_{2A} (A_{2A}AR) has been shown to possess anti inflammatory properties that have not been studied in TON. In the present study, we examined the role of A_{2A}AR in retinal complications associated with TON. Initial studies in wild type mice revealed that treatment with the A_{2A}AR agonist resulted in marked decreases in the TON induced microglial activation, retinal cell death and releases of reactive oxygen species and pro inflammatory cytokines TNF α and IL 6. To further assess the role of A_{2A}AR in TON, we studied the effects of A_{2A}AR ablation on the TON induced retinal abnormalities. A_{2A}AR^{−/−} mice with TON showed a significantly higher mRNA level of TNF α , Iba1 1 in retinal tissue, and ICAM 1 expression in retinal sections compared with wild type mice with TON. To explore a potential mechanism by which A_{2A}AR signaling regulates inflammation in TON, we performed additional studies using hypoxia or LPS treated microglial cells as an in vitro model for TON. Activation of A_{2A}AR attenuates hypoxia or LPS induced TNF α release and significantly repressed the inflammatory signaling, ERK in the activated microglia. Collectively, this work provides pharmacological and genetic evidence for A_{2A}AR signaling as a control point of cell death in TON and suggests that the retinal protective effect of A_{2A}AR is mediated by attenuating the inflammatory response that occurs in microglia via interaction with MAPKinase pathway.

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1. Introduction

Traumatic optic nerve injury is commonly seen in motor vehicle accidents, assaults, war and in the natural disaster. Traumatic optic nerve injury is usually the consequence of a severe blunt head trauma, often a frontal blow severe enough to cause loss of consciousness. Unfortunately, there are currently no proven treatments that can prevent the damage associated with an acute traumatic optic neuropathy (TON). Prognosis for the recovery of vision in TON is still poor, nevertheless, animal models

for TON are often used, mostly because they are easy to perform and can be well standardized (Levkovitch Verbin, 2004). Retinal ganglion cell (RGC) death is known to be a fundamental pathological process in traumatic optic injury including TON. Several common mechanisms have been hypothesized to underlie apoptotic processes, including interruption of trophic support, oxidative stress, and increased extracellular glutamate levels that result in excitotoxicity. These stimuli associated with the injured RGCs often activate retinal microglia, which release pro inflammatory cytokines and cytotoxic molecules to further exacerbate the degenerative process (Kreutzberg, 1996). These findings suggest that pharmacological interventions that reduce inflammation may be effective neuroprotectants for TON.

Under stress and ischemic conditions, the local tissue concentration of extracellular adenosine is increased due to its synthesis from the released ATP. This nucleoside has been proposed to modulate a variety of physiological responses by stimulating specific adenosine receptors (AR), which are classified as A1, A2A, A2B, and A3 subtypes (Collis and Hourani, 1993). These receptors can be distinguished based on their affinities for adenosine agonists and antagonists. In addition, these receptors are classified based on their mechanism of signal

Abbreviations: TON, traumatic optic neuropathy; TNF- α , tumor necrosis factor- α ; ELISA, Enzyme-linked immunosorbent assay; ROS, reactive oxygen species; MAP kinase, Mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; AR, adenosine receptor; CGS21680, 2-p-[2-Carboxyethyl]phenethylamino-5'-N-ethylcarboxamidoadenosine; LPS, lipopolysaccharides; NECA, 5'-N-Ethylcarboxamidoadenosine; ZM241385, 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol.

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Table 1

The primer sets used for the detection of mouse genes by quantitative real-time PCR analysis.

Gene	Primer sequence (5'–3')	Accession number
TNF- α	CCCTCAGCTCAGATCATCTTCT GTCACGACGTGGGCTACAG	NM_013693.2
ICAM-1	CGCTGTGCTTTGAGAACTGTG ATACACGGTGATGGTAGCGGA	NM_010493
Iba-1	GTCCTGAAGCGAATGCTGG CATTCTCAAGATGGCAGATC	NM_019467
A _{2A} AR	TCCACTCCGGTACAATGGCTTGGT AGCATGGGGGTGAGCCGAT	NM_009630.2
Mice IL-6	TAGTCCTTCTACCCCAATTTC TTGGTCCTTAGCCACTCTTC	NM_031168.1
GAPDH	CAT GGC CTC CAA GGA GTAAGA GAG GGA GAT GCT CAG TGT TGG	M32599
18S	AGT GCG GGT CAT AAG CTT GC GGG CCT CAC TAA ACC ATC CA	NR_003278

transduction. A1 and A3 receptors interact with pertussis toxin sensitive G proteins of the Gi and Go family to inhibit adenylate cyclase. The A_{2A} receptor stimulates adenylate cyclase through Gs coupling (Fredholm et al., 1994). A_{2B} receptor stimulates phospholipase C activity through Gq (Feoktistov et al., 1999). The increased adenosine at inflamed sites exhibits anti-inflammatory effects to protect against cellular damage through A_{2A}AR (Bong et al., 1996; Ralevic and Burnstock, 1998; Ohta and Sitkovsky, 2001). A_{2A}AR agonist treatment

blocks the inflammation, and functional and histological changes associated with diabetic nephropathy in wild type diabetic mice but not in the A_{2A}AR^{−/−} diabetic mice (Awad et al., 2006). We found that treatment with the A_{2A}AR agonist resulted in marked decreases in diabetes induced retinal cell death and TNF α release (Ibrahim et al., 2011a). We also found that activation of A_{2A}AR in the stressed retinal microglial cells was the most efficient in mediating TNF α inhibition (Liou et al., 2008). Furthermore, our work showed that diabetic A_{2A}AR^{−/−} mice had significantly more TUNEL positive cells, TNF α release, and ICAM 1 expression compared with diabetic wild type mice (Ibrahim et al., 2011a). The proposed mechanism of chronic retinal injury in diabetic retinopathy is RGC death associated with activation of an inflammatory pathway and an anti-inflammatory pathway involving A_{2A}AR signaling. We propose that A_{2A}AR signaling may also play a similar role in the acute treatment of TON.

Recent efforts to understand how neurotoxic inflammatory cytokines are produced have shown that MAPKinase signaling pathway is one of the attractive targets for intervention in human inflammatory associated diseases such as diabetes. However, this pathway does not operate alone, but rather interacts with other signaling systems, such as Gs coupled receptor transducing pathway. Activation of this pathway results in accumulation of cAMP that interacts with the MAPKinase signaling pathway to regulate cell functions (Gerits et al., 2008).

Previously we demonstrated the anti-inflammatory effect of A_{2A}AR in acute (Liou et al., 2008) and chronic (Ibrahim et al., 2011a) retinal

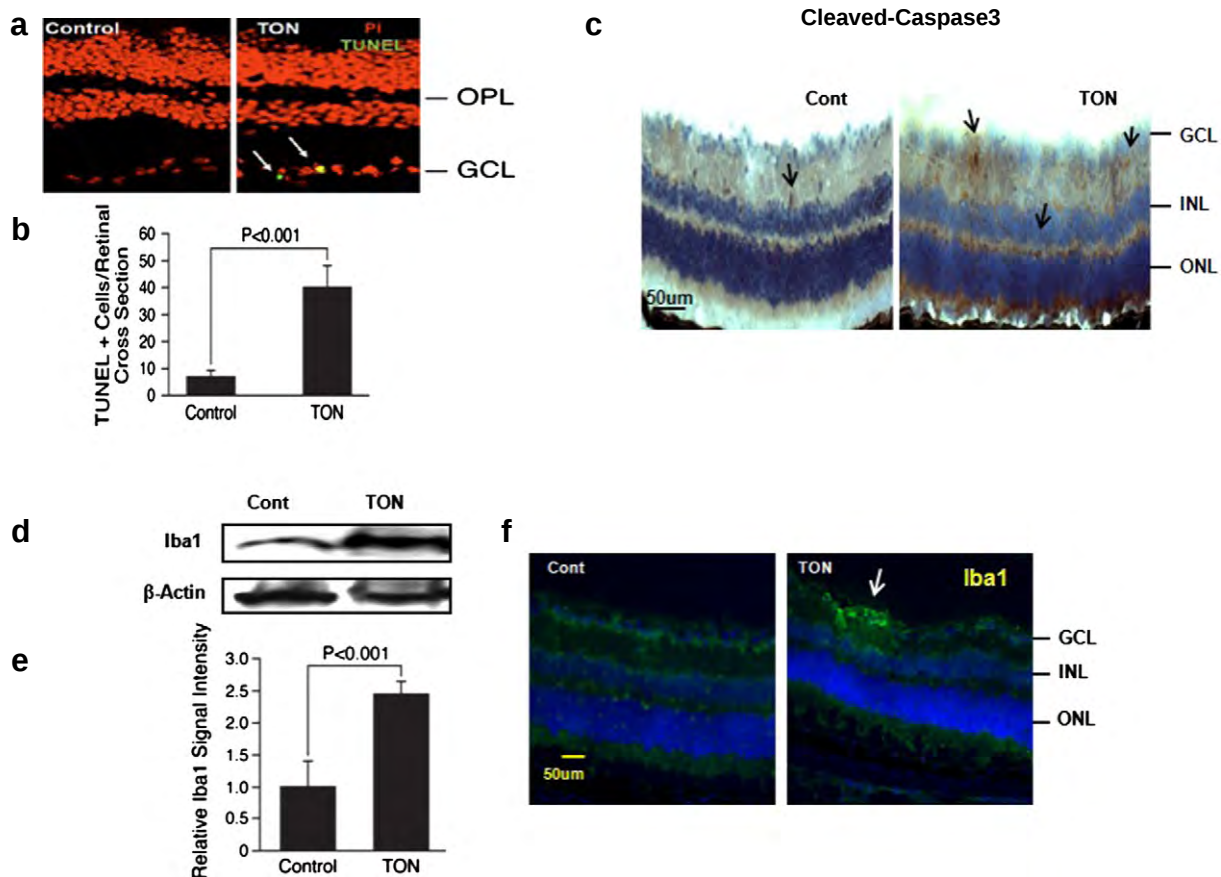


Fig. 1. TUNEL assay, cleaved-caspase3 activation and Iba1 expression on the retina of the mouse model of TON. a–b) Retinal distribution of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells identified in TON and control. Sections were counterstained with propidium iodide (PI). c) Quantitative analysis of TUNEL-positive cells in the retinal cross section of TON and control. TUNEL+ cells were counted in 10 adjacent locations along the vertical meridian within 4 mm of the optic disk (10 fields/retina sections). c) Immunohistochemical analysis of activated caspase3 in retinal section of TON vs. control. DAB (3, 3'-diaminobenzidine) produces a dark brown reaction product of cleaved caspase3. d) Immunoblotting analysis of microglial activation marker Iba1 expression in TON vs. control in the retina. e) Densitometry analysis of Iba1 and actin ratio by Image J software, NIH. f) Immunolabeling of Iba1 (green) with nuclear marker, DAPI (blue) in retinal section of TON vs. control. Data shown are the mean $\pm$ SD (n = 4). *P < 0.01, **P < 0.001, ***P < 0.0001.

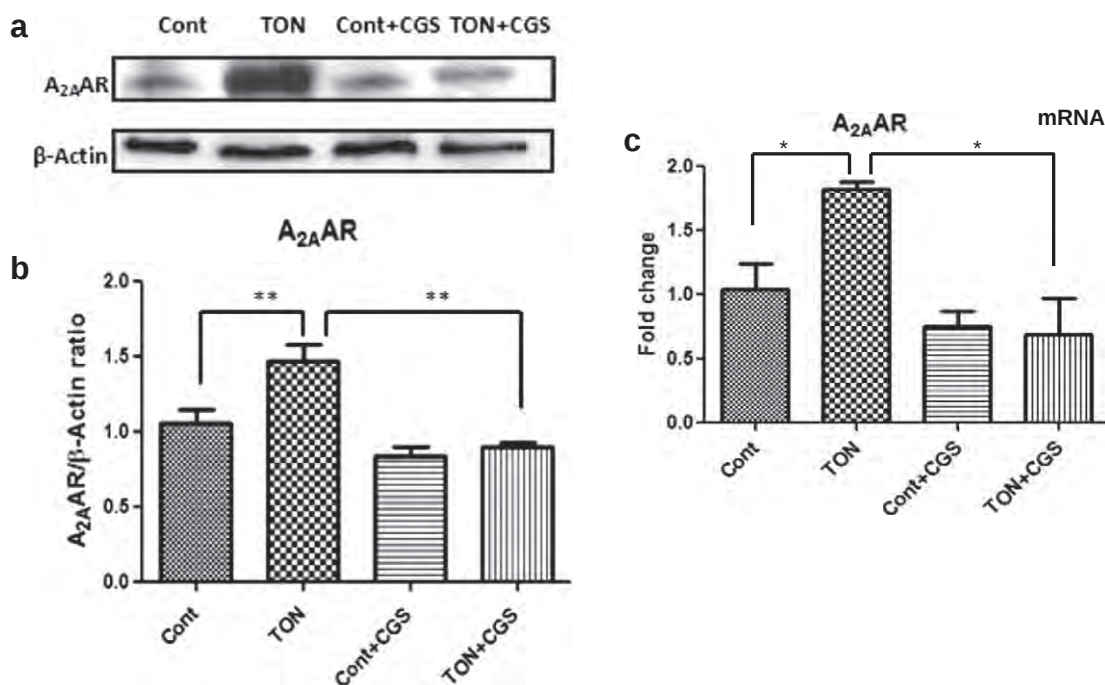


Fig. 2. Effect of A_{2A}AR agonist treatment on A_{2A}AR expression in the mouse model of TON. a–b) Western blot analysis of A_{2A}AR protein expression in the retinal tissue in TON vs. control, with and without CGS21680 treatment. Densitometry analysis was done for A_{2A}AR and actin ratio by Image J software. c) Retinal A_{2A}AR mRNA was determined by real-time PCR in TON vs. control, with and without CGS effect. Data shown are the mean ± SD (n = 4). *P < 0.01, **P < 0.001, ***P < 0.0001.

inflammation. Currently, we seek to determine the contribution of A_{2A}AR in retinal protection against TON induced retinal inflammation and injury. Moreover, we pursue to gain insight into the underlying signaling involved therein. Here, we report evidence that activation of A_{2A}AR plays a

protective role in TON induced retinal cell death by enhancing the anti-inflammatory signaling including the interaction with MAPKinase pathway. These findings suggest that A_{2A}AR agonists might be promising innovative agents in the treatment of TON.

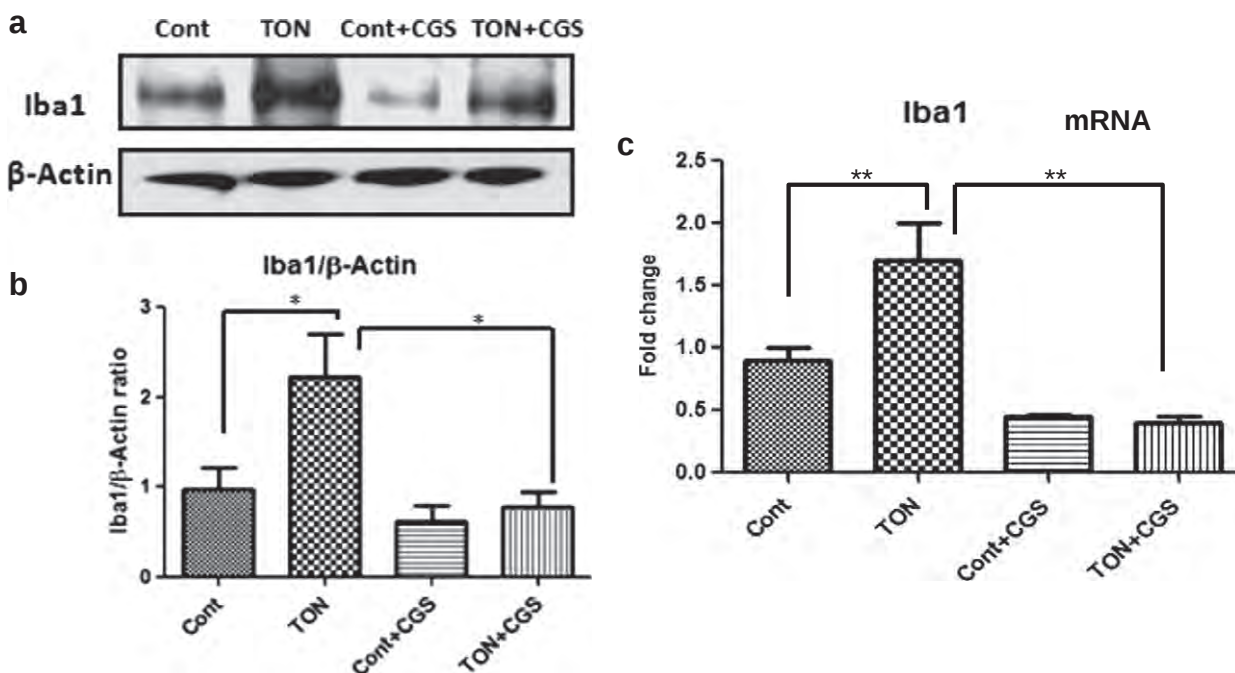


Fig. 3. Effect of A_{2A}AR agonist treatment on the retinal microglial activity in the mouse model of TON. a–b) Analyses of Iba1-expressing retinal cells in TON model with and without agonist treatment by Western blot. Densitometry analysis was done for Iba-1 and actin ratio by Image J software. c) Retinal Iba1 mRNA was determined by real-time PCR in TON vs. control, with and without CGS effect. Data shown are the mean ± SD (n = 4). *P < 0.01, **P < 0.001, ***P < 0.0001.

2. Materials and methods

2.1. Animal preparation and experimental design

All procedures with animals were performed in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare publication, NIH 80 23) and Georgia Health Sciences University guidelines. Eight week old male $A_{2A}AR^{-/-}$ and corresponding littermate controls, wild type (WT) mice in C57BL/6 background, were matched according to sex, age, and weight. $A_{2A}AR^{-/-}$ mice (Chen et al., 1999) were a kind gift from Dr. J F Chen, Harvard Medical School, Boston, MA. Mice were anesthetized according to standard protocol and limbal conjunctival peritomy was performed on one eye of each mouse. Forceps dissection under the conjunctiva posteriorly allowed access to the optic nerve, upon which pressure was placed 1 mm posterior to the globe until pupillary dilation was noted (approximately 10 s). Blood vessel close to optic nerve was carefully avoided in TON surgery. Mock operated contralateral eye served as the control. After one week, all mice were sacrificed. Eyes were enucleated and sectioned for immunohistochemistry. Retinas were harvested for Western or Real Time PCR analysis. In pharmacologic studies, age, weight and sex matched C57BL/6 mice were rendered optic nerve crush and then injected i.p. with vehicle (DMSO), or CGS21680 (25 μ g/kg) every other day for the duration of the study ($n = 4$ 6/group) (Genovese et al., 2009).

2.2. Terminal dUTP nick end labeling (TUNEL)

TUNEL was performed in frozen sections using the TACS 2 TdT Fluorescein In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD)

counter stained with propidium iodide, according to the manufacturer's suggestions. Briefly, sections were hydrated with alcohol 100%, 95%, and 70%, then fixed in 3.7% paraformaldehyde. After washing, slides were incubated in mixture of TdT, Mn^{+2} , and TdT dNTP for 1 h at 37 °C. The reaction was stopped with TdT Stop Buffer for 5 min. After washing with deionized water, the slides were incubated with Streptavidin HRP (diluted 1:200) solution for 20 min at room temperature. Slides were counter stained, mounted, covered with coverslips and visualized by a confocal microscopy (LSM 510, Carl Zeiss, Inc.). Apoptotic cells were identified as doubly labeled with TdT Fluorescein and propidium iodide and only nuclei that were clearly labeled yellow were scored.

2.3. Immunohistochemical analysis

Immunofluorescence analysis was performed using frozen retinal sections. Briefly, cryostat sections (15 μ m) were fixed in 4% paraformaldehyde, blocked with 10% normal goat serum (NGS) and then incubated overnight at 4 °C with primary antibodies: rabbit anti Iba 1 (Wako Pure Chemical, Wako, TX), or mouse anti ICAM 1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) together with Texas Red labeled Isolectin B4 (Invitrogen, Carlsbad, CA), anti gp91phox (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti GFAP (Dako, USA) with FITC green, rabbit anti $A_{2A}AR$ (Abcam, USA), and rabbit anti cleaved Caspase3 (Cell Signaling, USA). Thereafter, sections were briefly washed with PBS and incubated with appropriate secondary antibodies. Slides were examined by confocal microscopy (LSM 510, Carl Zeiss). Specificity of the reaction was confirmed by omitting the primary antibody. Data (10 fields/retina, $n = 4$ 6 in each group) were analyzed using fluorescence microscopy and Ultra View morphometric software or Image J software (NIH) to quantify the intensity of immunostaining.

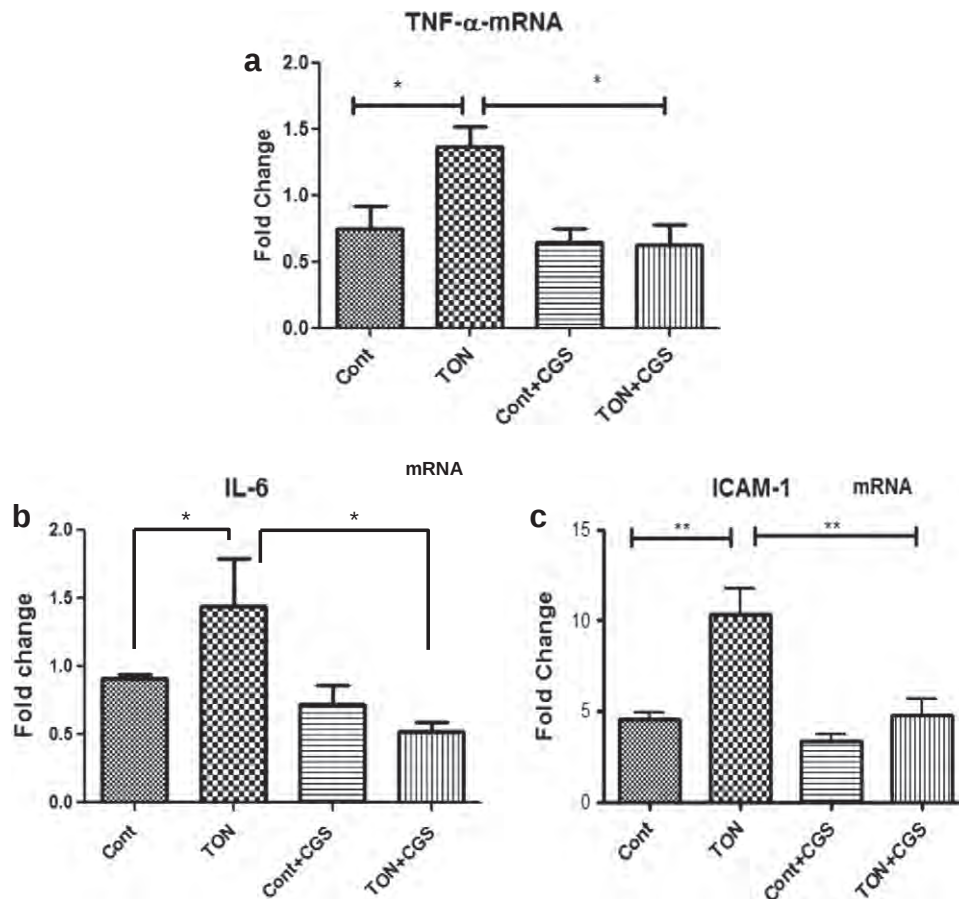
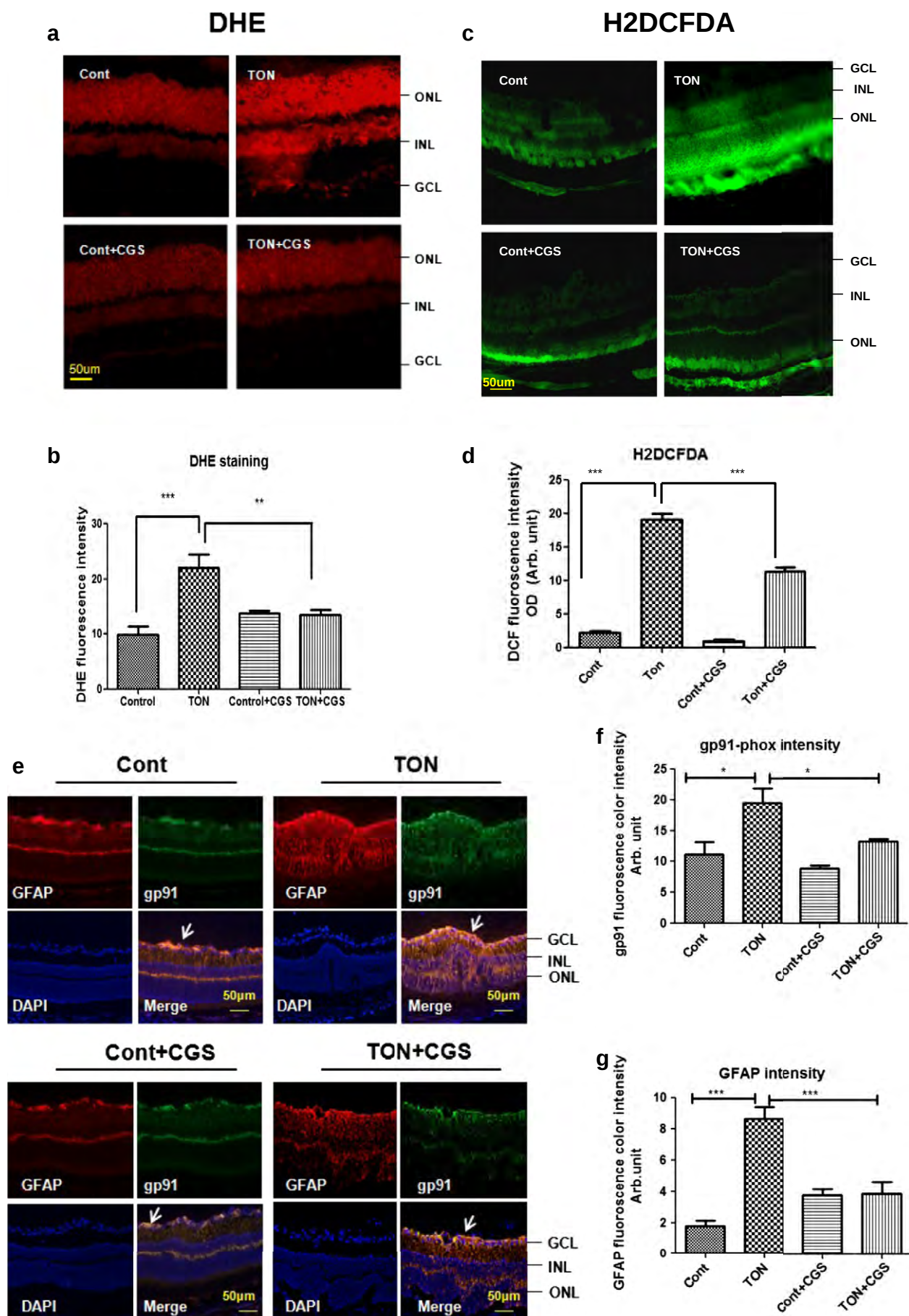


Fig. 4. Effect of $A_{2A}AR$ agonist treatment on the retinal expression of pro-inflammatory cytokines in the mouse model of TON. a–c) Real-time PCR analysis of TNF- α , IL-6 and ICAM-1 mRNA expression in the retina of TON, with and without agonist. Data shown are the mean $\pm$ SD ($n = 4$). * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$.



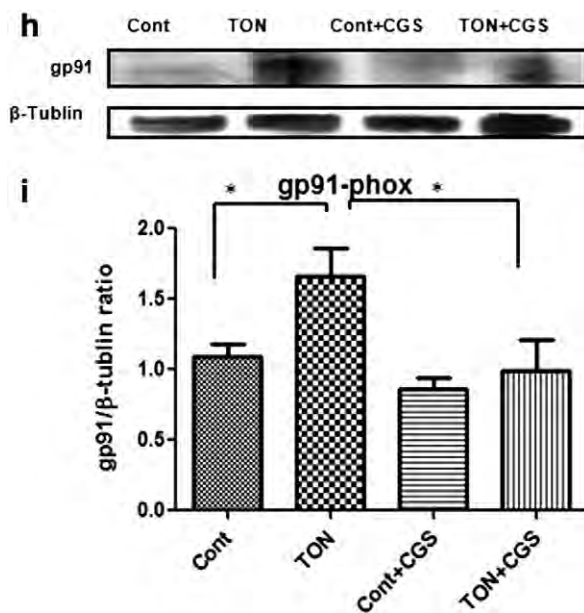


Fig. 5. Effect of $A_{2A}AR$ agonist treatment on the retinal levels of oxidative stress in the mouse model of TON. a–b) Superoxide levels determined by dihydroethidium (DHE) fluorescence. Fluorescence intensities were quantified by Image J software, NIH. c–d) ROS activities determined by 2,7-dichlorodihydro-fluorescein diacetate (H2DCFDA). Fluorescence intensities were quantified by Image J software, NIH. Data shown are the mean $\pm$ SD ($n = 4$ –5). e–g) Immunofluorescence analysis of NADPH oxidase subunit gp91-phox expression in retina. Gp91-phox (green), GFAP (red) with DAPI (blue). Fluorescence intensities of gp91 and GFAP were quantified by Image J software, NIH. h–i) Retinal gp91-phox protein expression was determined by Western blotting in TON vs. control with and without agonist. Densitometry analysis of gp91 and tubulin band ratio was done by Image J software, NIH. Data shown are the mean $\pm$ SD ($n = 4$). GCL = ganglion cell layer; INL = inner nuclear layer; ONL = outer nuclear layer. * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$.

Immunohistochemistry analysis was done by MOM kit (Vector Laboratories, USA). Protocol was used according to Kit.

2.4. Analysis of dihydroethidium (DHE) fluorescence for the detection of superoxide

The detection of superoxide anion in the mouse eye sections was performed as described previously (Inaba et al., 2009). In brief, mouse eyes were frozen in OCT and stored at -80°C until use. Enzymatically intact eye sections were thawed in room temperature, rehydrated with PBS, incubated with dihydroethidium (DHE; $10\text{ }\mu\text{M}$ /L in PBS) for 30 min at 37°C in a humidified chamber protected from light. After incubation, sections were washed with PBS. DHE is oxidized on reaction with superoxide to ethidium, which binds to DNA in the nucleus and emits red fluorescence. For the detection of ethidium, samples were examined with a fluorescence microscope (Axioskop 2 plus with AxioCam; Carl Zeiss, Germany; Excitation/Emission wavelengths: 518/605 nm). DHE fluorescence was quantified using Image J software (NIH).

2.5. Measurement of ROS activity by H2DCFDA for oxidative stress

Dichlorofluorescein (DCF), the oxidation product of 2,7-dichlorodihydro fluorescein diacetate (H2DCFDA; Invitrogen) emits a green fluorescent signal and is a marker of cellular oxidation by reactive oxygen species (ROS) including hydrogen peroxide, peroxynitrite, and hydroxy radicals (Al Shabraway et al., 2008). Retinal sections were incubated with H2DCFDA ($10\text{ }\mu\text{M}$) in HBSS buffer for 20 min at 37°C .

DCF formation was measured with fluorescence microscopy to collect the images, and with computer assisted morphometry to determine the fluorescence intensity.

2.6. Protein extraction and Western blot analysis

Washed cultured cells and retinal tissue were lysed in modified RIPA buffer (Upstate, Lake Placid, NY), containing 50 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 1% Nonidet P 40, 0.25% deoxycholate, supplemented with 40 mmol/L NaF, 2 mmol/L Na_3VO_4 , 0.5 mmol/L phenylmethylsulfonyl fluoride and 1:100 (v/v) of proteinase inhibitor cocktail (Sigma). Insoluble material was removed by centrifugation at $12,000\times g$ at 4°C for 30 min. Protein was determined by DC Protein Assay (Bio Rad, Hercules, CA) and 50–100 μg was boiled in Laemmli sample buffer, separated by SDS PAGE on a gradient gel (4 to 20%) (Pierce, Rockford, IL), transferred to nitrocellulose membrane and incubated with specific antibodies. Antibodies for β actin (Sigma), Iba1, $A_{2A}AR$, phospho ERK and ERK (Cell Signaling Technology, Beverly, MA) were detected with a horseradish peroxidase conjugated antibody and ECL chemiluminescence (Amersham BioSciences, Buckinghamshire, UK). Intensity of immunoreactivity was measured by densitometry.

2.7. Isolation of RNA, synthesis of cDNA, and real time PCR

Total RNA was isolated from mouse retina using SV Total RNA Isolation kit (Promega, Madison, WI) following manufacturer's instructions, and the quality of the RNA preparations was monitored by absorbance at 260 and 280 nm (Helios Gamma, Thermo Spectronic, Rochester, NY). The RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA) using iScript reagents from Bio Rad in a programmable thermal cycler (PCR Sprint, Thermo Electron, Milford, MA). Fifty ng of cDNA was amplified in each real time PCR using Bio Rad iCycler, ABgene reagents (Fisher scientific) and appropriate primers. Average of glyceraldehyde 3 phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA was used as the internal control for normalization (Table 1).

2.8. Primary retinal microglial culture

Microglial cells were isolated from retinas of newborn SD rats according to a previous procedure (Wang et al., 2005) with minor modifications. Briefly, retinas were dissected from newborn SD rat pups. Tissues were collected into 0.01 M PBS and washed with ice cold 0.01 M PBS, digested with 0.125% trypsin for 3–5 min and mixed with DMEM/F12 (1:1) (Invitrogen, CA) containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, GA) and 1% penicillin/streptomycin (Mediatech, Herndon, VA). Retina pieces were triturated by passing them through a disposable pipette several times until cells were dispersed. Cells were then filtered through a mesh ($100\text{ }\mu\text{m}$), collected by centrifugation, resuspended in the culture medium and plated onto T150 cell culture flasks (Corning, NY) at a density of 2×10^5 cells/ cm^2 . All cultures were maintained in a humidified CO_2 incubator at 37°C and 5% CO_2 and fed on the third day, then once every 4 days. After 2 weeks, microglial cells were harvested by shaking the flasks at 100 rpm for 1 h. The cell suspension was centrifuged and detached cells were replated in DMEM/F12 (1:1) + 10% FBS overnight and then in serum free/low protein media (Cellgro Complete containing 0.1% bovine serum albumin; Mediatech, Manassas, VA) at designated densities for various experiments. The purity of microglial cultures was 98%, as determined by immunocytochemical staining analysis and by cytometry (Ibrahim et al., 2011a) for Iba1, a microglial marker. The morphology of microglia in culture was carefully examined by phase contrast and fluorescence microscopy.

2.9. Drug treatment effects on cultured microglial cells

Microglial cells were seeded at a density of 5×10^5 cells/well in 24 well tissue culture plate, or 1×10^5 cells/well in 96 well plate. One day after seeding, the wells were washed with Cellgro Complete (Mediatech, Manassas, VA) and incubated in the same media with various treatments. Cells were pretreated with $A_{2A}AR$ selective agonist (CGS21680, Tocris, Ellisville, MO, 20 μM) or $A_{2A}AR$ selective antagonist (ZM 241385, Tocris, Ellisville, MO, 2 μM) at the indicated concentrations reported previously or vehicle dimethylsulfoxide (DMSO) for 30 min at 37 °C before hypoxia or LPS treatment (30 ng/ml). NECA (2 μM) was used as nonselective AR agonist. Microglial cells were placed in 1% oxygen (hypoxic) and room air (normoxic) conditions at 37 °C for the indicated time. Both groups were then placed under normoxic conditions for 24 h. At indicated time points, cells were homogenized for Western blot analysis and culture media were taken and analyzed for TNF α by ELISA.

2.10. Enzyme linked immunosorbent assay (ELISA) for TNF α

TNF α levels in the supernatants of culture media were estimated with ELISA kits (R&D, Minneapolis, MN) per the manufacturer's instructions. Standards and samples were added and bound by the immobilized antibody. After washing, an enzyme linked polyclonal antibody specific for the cytokine was added to the wells followed by a substrate solution yielding a colored product. The intensity of the color was measured at 450 nm. The sample levels were calculated from the standard curve and corrected for protein concentration.

2.11. Statistics

The results are expressed as mean $\pm$ SD. Differences among experimental groups were evaluated by analysis of variance (one way ANOVA), and the significance of differences between groups was assessed by the posthoc test (Newman Keuls multiple comparison). Significance was defined as $P < 0.05$. Data were analyzed by GraphPad PRISM software.

3. Results

3.1. TON induced microglial activation and retinal cell death

Because activation of the $A_{2A}AR$ has been implicated in the anti inflammatory actions of adenosine in experimental diabetes (Ibrahim et al., 2011a), we hypothesized that this receptor may also be effective in protecting retinal neurons from TON induced inflammation and neurotoxicity. Quantitative analysis of TUNEL FITC and propidium iodide double labeled cells in retinal tissue showed a significant increase in the frequency of retinal cell death mostly in the ganglion cell layer 7 days after optic nerve crush (Fig. 1a & b). That the retinal cell death was through apoptosis was confirmed with activated caspase 3 by immunohistochemistry (Fig. 1c). Following this, we addressed an interesting feature, acquisition of reactive microglial phenotype that could be an important determinant for understanding the mechanisms by which optic nerve crush induces RGC death. In this regard, we noted that when microglia encountered TON milieu, they became activated as indicated by increased Iba 1 expression (Fig. 1d, e & f).

3.2. Role of $A_{2A}AR$ agonist in microglial activation and $A_{2A}AR$ expression

To test this hypothesis, we treated mice with the procedures that crushed optic nerve in one eye. Treated mice were further treated with the $A_{2A}AR$ selective agonist, CGS21680, or with vehicle via i.p. injection every two days. At day 7, retinal microglial activation, retinal cell death, pro inflammatory cytokine release, and ROS production were compared. We determined the effect of optic nerve crush on the expression of $A_{2A}AR$

in the retina. Compared with the contralateral eyes, the eyes with crushed optic nerves demonstrated a significant increase in the levels of $A_{2A}AR$ protein expression (Fig. 2a & b). The treatment with CGS21680 resulted in a marked reduction of TON associated $A_{2A}AR$ protein and mRNA up regulation (Fig. 2c). These results demonstrated that under TON associated stress, a self defense system including $A_{2A}AR$ assumes a compensatory effect. However, CGS21680 treatment in the eyes with crushed optic nerves significantly reduced Iba 1 protein and mRNA expression (Fig. 3a c), suggesting reduced microglial cell activity.

3.3. Role of $A_{2A}AR$ in retinal inflammation

We next determined the effect of optic nerve crush on the levels of pro inflammatory cytokines in the retina. Compared with the contralateral eyes, the eyes with crushed optic nerves demonstrated a significant increase in the levels of cytokines (TNF α and IL 6) along with intercellular adhesion molecule, ICAM 1. CGS21680 treatment in the eyes with crushed optic nerves significantly reduced TON associated cytokines and ICAM 1 mRNA expression (Fig. 4a c).

3.4. Role of $A_{2A}AR$ in retinal oxidative stress

We then determined the effect of optic nerve crush on the levels of oxidative stress in the retina by two methods (DHE and DCF) that measure superoxide generation and different ROS. Compared with the contralateral eyes, the eyes with crushed optic nerves demonstrated a significant increase in the levels of all the ROS determined (Fig. 5). The treatment with CGS21680 resulted in a marked reduction of TON associated ROS release (Fig. 5a d). As the report suggests that NADPH oxidase is the main source of free radical generation (Al Shabrawey et al., 2008; Brennan et al., 2009), we checked the expression level of gp91phox (NADPH oxidase subunit) and GFAP by immunofluorescence and Western blotting. The expression level of gp91phox and GFAP was significantly high in TON compared with the control eye and CGS21680 treatment significantly modulated the NADPH oxidase and GFAP activation.

3.5. Role of inflammation in $A_{2A}AR$ $-/-$ mice

To further assess the role of $A_{2A}AR$ in TON, we studied the effects of $A_{2A}AR$ ablation on TON induced retinal inflammation. As shown in Fig. 6a, mRNA expression of TNF α in the retinas of TON associated $A_{2A}AR$ $-/-$ mice were notably increased with their age matched TON associated WT control mice. Furthermore, Iba1 mRNA in the $A_{2A}AR$ $-/-$ mice retina was significantly increased (~1.5 fold) compared with WT (Fig. 6b). Immunofluorescence analysis of ICAM 1 expression in retinal section showed marked increase in $A_{2A}AR$ $-/-$ retina compared with WT littermates with TON (Fig. 6c). Taken together, these results suggest that $A_{2A}AR$ plays a crucial role in limiting retinal inflammation and neuronal cell injury associated with TON.

3.6. Activation of $A_{2A}AR$ attenuates hypoxia or LPS induced TNF α release in retinal microglial cells

After having shown that $A_{2A}AR$ $-/-$ mice with TON exhibit profound retinal cell death and inflammation, we next sought to explore a potential mechanism by which $A_{2A}AR$ signaling regulates inflammation in TON. To do this, additional studies using microglial cells treated with hypoxia or LPS were performed. Treatment of microglia with hypoxia has been shown to simulate inflammation (Wang et al., 2005; Quan et al., 2007; Wang et al., 2007). As shown in Fig. 7b, treatment of retinal microglial cells with hypoxia, but not with normoxia, triggered a prominent increase in TNF α release. To determine the role of the $A_{2A}AR$ in regulating TNF α release, we first examined the effect of the selective $A_{2A}AR$ agonist, CGS21680, in hypoxia or LPS induced TNF α release. As shown in Fig. 7f & g, activation of $A_{2A}AR$ with CGS21680 inhibited TNF α release. To confirm this point, we used

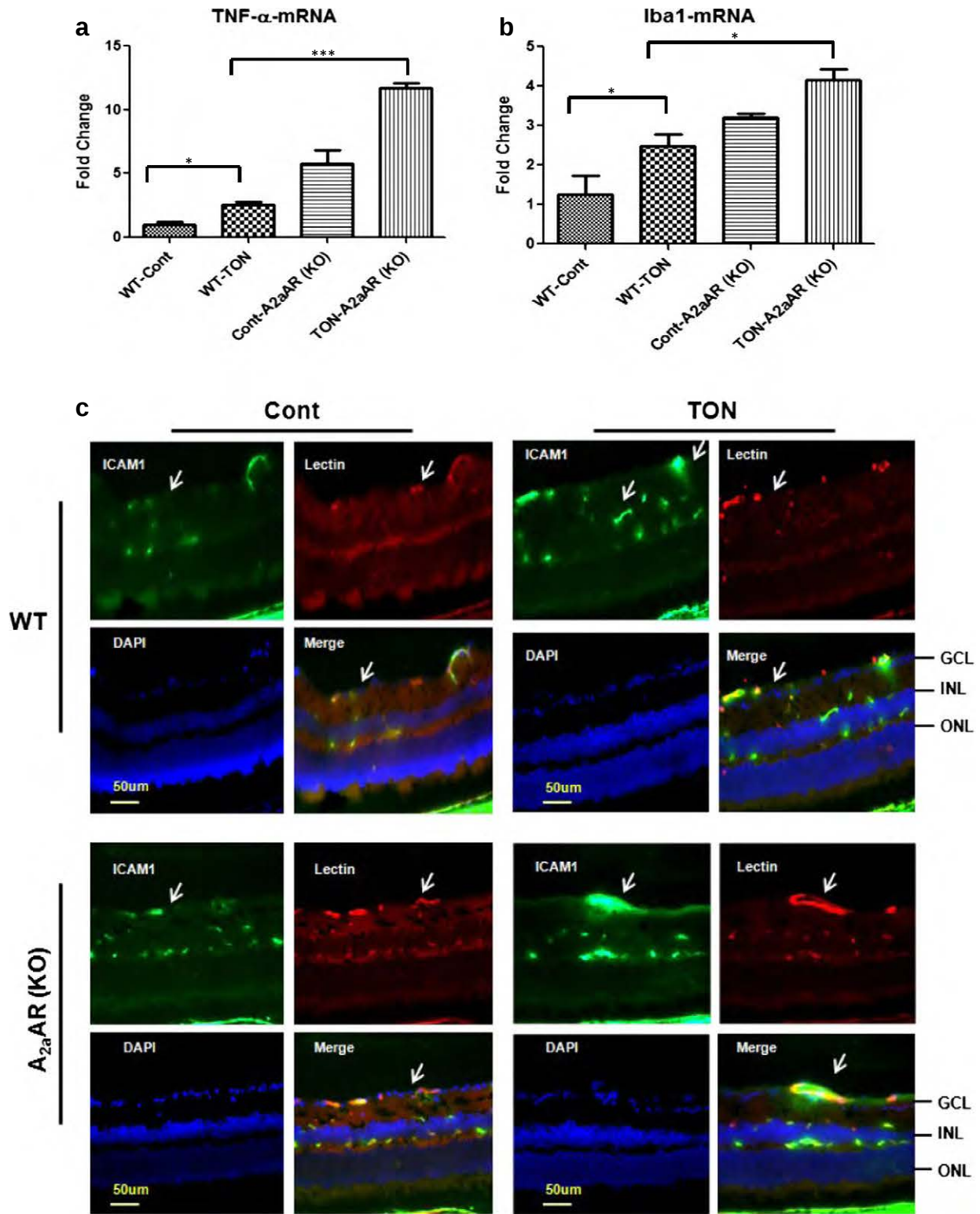


Fig. 6. Effect of A_{2A}AR depletion on the retinal mRNA expression of TNF- α , Iba1 and ICAM-1 in the mouse model of TON. a–b) Real-time PCR analysis of Iba1 and TNF- α expression, WT vs. A_{2A}AR-KO mice. c) Immunofluorescence analysis of intercellular adhesion molecule 1 (ICAM-1) distribution in the retinal sections, WT vs. A_{2A}AR-KO. Sections were stained with ICAM1 (green), endothelial cells marker Isolectin B4 (red) and DAPI (blue). Data shown are the mean $\pm$ SD (n = 4). *P < 0.01, **P < 0.001, ***P < 0.0001.

A_{2A}AR non selective agonist (NECA) and A_{2A}AR selective antagonist (ZM241385) to treat the microglial cells followed with LPS treatment. We showed that LPS significantly elevated TNF α release as compared with control. ZM241385 treatment alone did not alter the TNF α

release. NECA treatment significantly decreased TNF α level. LPS + NECA + ZM treatment reversed the effect of TNF α release by LPS + NECA, suggesting that A_{2A}AR is responsible for the reduction of TNF α release. Together, these findings identify a signaling through

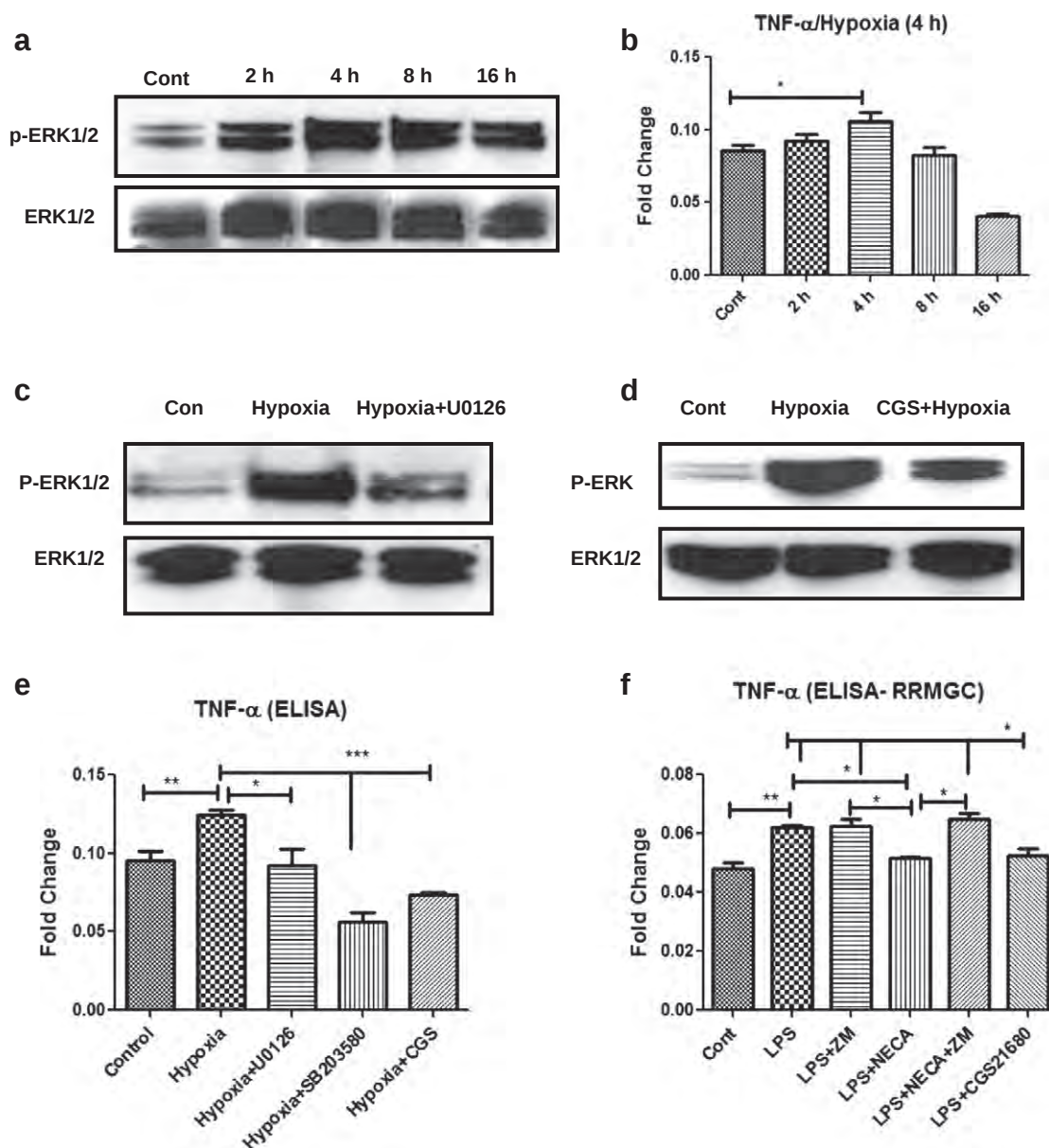


Fig. 7. Role of MAPKinase in the $A_{2A}AR$ -mediated anti-inflammation: analysis of hypoxia- & LPS-induced TNF- α release in the retinal microglial cells. a) Time-dependent, hypoxia induced activation of ERK in the retinal microglial cells. Cells were treated with hypoxia for 2–16 h. Phosphorylated (p) ERK and its total protein in cell lysate were determined by Western analysis. b) TNF- α release was measured by ELISA in time-dependent hypoxia. c–d) Selected time point (4 h) study in retinal microglial cells for hypoxia-induced pERK activation and TNF- α release, and its inhibition with U0126 (MEK inhibitor). e–f) Retinal microglial cells were treated with CGS21680 (20 μ M) for 4 h. p-ERK and TNF- α releases were determined by Western and ELISA analyses, respectively. Retinal microglial cells were treated with LPS (30 ng/ml), NECA (2 μ M), ZM241385 (2 μ M) and CGS21680 (20 μ M). TNF- α release was determined by ELISA. Data shown are the mean $\pm$ SD (n = 4). *P < 0.01, **P < 0.001, ***P < 0.0001.

$A_{2A}AR$ as a critical control point for TNF α release in hypoxia or LPS treated retinal microglial cells.

3.7. $A_{2A}AR$ signaling mediates the anti inflammatory effect via interaction with hypoxia activated MAPK pathway in retinal microglial cells

The role of MAPKinases in the hypoxia induced TNF α release was studied. Whether or not MAPKinases are modulated by the $A_{2A}AR$ signaling was also examined here. As shown in Fig. 7a & c, ERK was hypoxia activated, although following different time courses. As shown in Fig. 7e & f, CGS21680 inhibited hypoxia induced ERK activation as well as TNF α release. Collectively, these results suggest that the $A_{2A}AR$ signaling cross talks with MAPK pathway to modulate hypoxia induced TNF α expression in retinal microglial cells.

4. Discussion

In traumatic optic neuropathy (TON), the injured optic nerve may lead to visual loss. Visual loss results from loss of retinal ganglion cells (RGC), a layer of cells that is continuous with the optic nerve. In TON, stress causes vascular and neuronal damage of the retina and activates microglial cells. It has been reported that the activated microglia increased the damage by secreting pro inflammatory cytokines and cytotoxic molecules in response to oxidative stress (Kreutzberg, 1996). In the retinal microglial cells near RGC, extracellular adenosine is responsible for a mechanism of anti inflammation mediated by adenosine receptor A_{2A} ($A_{2A}AR$) signaling (Ibrahim et al., 2011b). Adenosine, which is a ubiquitous purine nucleoside, has been reported to have many diverse functions in metabolic stresses such as hypoxia and inflammation. Adenosine works through its interaction with specific 7

transmembrane receptor subtype. Evidence suggests that $A_{2A}AR$ has a potent anti inflammatory function and is widely expressed on cells of immune system like microglia and macrophages (Milne and Palmer, 2011). The previous report of our group demonstrated that the activation of $A_{2A}AR$ blocks endotoxin induced inflammation in these cells (Liou et al., 2008). In this study, pharmacological experiments with $A_{2A}AR$ agonist CGS21680 is showing that $A_{2A}AR$ is the main candidate for mediating the adenosine effect on suppression of pro inflammatory cytokines in activated microglial cells. The ability to offer protection of $A_{2A}AR$ and its agonists against inflammation and tissue injury in the kidney, liver, heart, lung, vasculature and brain have been demonstrated in a number of studies (Sheardown and Knutsen, 1996; Okusa et al., 1999; McPherson et al., 2001; Ohta and Sitkovsky, 2001; Fozard et al., 2002; Yang et al., 2005).

The present study showed that mouse retina with TON exhibits significantly increased oxidative stress, activated microglial cells, TNF α release, and retinal cell death as compared with control retina and which may be effectively treated with $A_{2A}AR$ agonist. We have shown the data that treatment of hypoxia or LPS induced MAPK activation increased the accumulation of TNF α in primary culture of rat retinal microglial cells, which further may lead to neurodegeneration. Treatment with $A_{2A}AR$ agonist in both the cases (in vitro and in vivo study) significantly blocked the MAPK activation and TNF α release. To the best of our knowledge, this is the first report demonstrating that $A_{2A}AR$ agonist could be an effective therapeutics for TON. In in vivo study, we have shown increased cell death, cleaved caspase 3 activation and microglial marker Iba 1 expression in TON retina compared with normal retina. Pro inflammatory cytokine (TNF α and IL 6) production was significantly higher in the retina with TON. The $A_{2A}AR$ agonist administration significantly attenuated the expression of these inflammatory and cell death markers, and results show that $A_{2A}AR$ selective agonist evokes the anti inflammatory activity in TON activated microglia cells via MAPKinase signaling. Using primary culture of microglial cells, we found that treatment with hypoxia or LPS significantly increased TNF α level and activated ERK1/2 signaling. $A_{2A}AR$ agonist reduced ERK1/2 activation and TNF α release in microglial cells (Fig. 7a–f). Recent study of our group has shown that $A_{2A}AR$ selective agonist inhibits Ras/C Raf/MEK/ERK signaling in AGA activated microglial cells (Ibrahim et al., 2011b). Stork and Schmitt (2002) has reported that on activation of Gs coupled receptor to regulate cell function, cAMP increases to interact with the Ras/C Raf/MEK/ERK signaling (Stork and Schmitt, 2002). The interaction between cAMP and Ras/Raf/MEK/ERK signaling may lead to activation or inhibition of ERK activity, which further regulates cytokine production in immune cells. Report suggests that $A_{2A}AR$ inhibits two major pro inflammatory signaling pathways, the NF κ B and the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways (McPherson et al., 2001). These pathways regulate inflammatory cytokines such as TNF α and IL 6 production (Karin and Ben Neriah, 2000; Ding et al., 2009). These studies further support our findings that $A_{2A}AR$ agonist suppresses pro inflammatory cytokine release via different signaling pathways and in the case of TON, inhibition of MAPKinase pathway through $A_{2A}AR$ may regulate the increased TNF α release in activated microglial cells.

We further investigated the role of $A_{2A}AR$ agonist on superoxide generation/ROS production in TON. Previous studies have shown that $A_{2A}AR$ inhibits superoxide/ROS production in leukocytes (Sullivan et al., 2001; Nadeem et al., 2009). However, our DHE and DCF data showed that $A_{2A}AR$ agonist significantly inhibits superoxide/ROS generation via inhibition of NADPH oxidase subunit gp91phox activation. NADPH oxidase and GFAP activation have been implicated in pathogenesis of several diseases including hypertension, stroke and diabetes, where increased generation of superoxide/ROS contributes in cell death (Roe et al., 2011; Bhatia et al., 2012; Tang et al., 2012). In agreement, another study has shown that $A_{2A}AR$ agonist CGS21680 inhibits NADPH oxidase activity via cAMP PKA signaling pathway (Nadeem

et al., 2009). Together, these results suggest that microglial NADPH oxidase could be the major source of ROS formation in TON and CGS21680 may attenuate its activation.

We also studied the role of $A_{2A}AR$ in TON by using $A_{2A}AR$ KO mice. Retinas from KO mice exhibit significantly more TNF α release, ICAM 1 expression, activated microglial cells, and cell death compared to retinas from wild type mice. Increased ICAM 1 expression has been implicated in increased retinal inflammation and permeability in diabetes (Joussen et al., 2004). Under stress conditions, the local levels of extracellular adenosine are elevated due to the increased need for energy supplied by ATP (Johnson et al., 1999) and the degradation of released ATP (Wurm et al., 2008). This increased adenosine can protect against excessive cellular damage in a negative feedback manner (Ralevic and Burnstock, 1998). However, the apparently impaired protection in mice with TON reflects that protection by endogenous adenosine is insufficient and CGS21680 treatment modulates this effect.

To prove the anti inflammatory effect of $A_{2A}AR$, we used LPS to activate primary retinal microglial cells. In our earlier work, we have shown that LPS treatment activates microglial cells to stimulate inflammation and p38 mitogen activated protein kinase activation, which causes neuronal degeneration (El Remessy et al., 2008). We pre treated the microglial cells with an $A_{2A}AR$ selective agonist CGS21680, and the role of $A_{2A}AR$ in reducing TNF α release was confirmed by non selective agonist NECA and $A_{2A}AR$ selective antagonist ZM241385 followed by LPS treatment as shown in Fig. 7g. Our findings are supported by earlier work where CGS21680 reduces ischemic or excitotoxic hippocampal damage (Jones et al., 1998). In this study, we showed that in vivo efficacy of CGS21680 reduces retinal inflammation and cell death associated with TON. This evaluation of the in vivo effect of CGS21680 is important for the development of a receptor based therapy for TON. Future challenges include the development of compounds with high and selective binding affinity to $A_{2A}AR$ approaches to deliver $A_{2A}AR$ agonists to retina, and the definition of pharmacologic strategies to safely use $A_{2A}AR$ agonists in patients with TON.

In summary, we may conclude that this present study yields preclinical evidence which demonstrates that $A_{2A}AR$ mediated signaling is a critical pathway for debilitating retinal cell death associated with TON (Fig. 8). Furthermore, the current study substantiates that the function

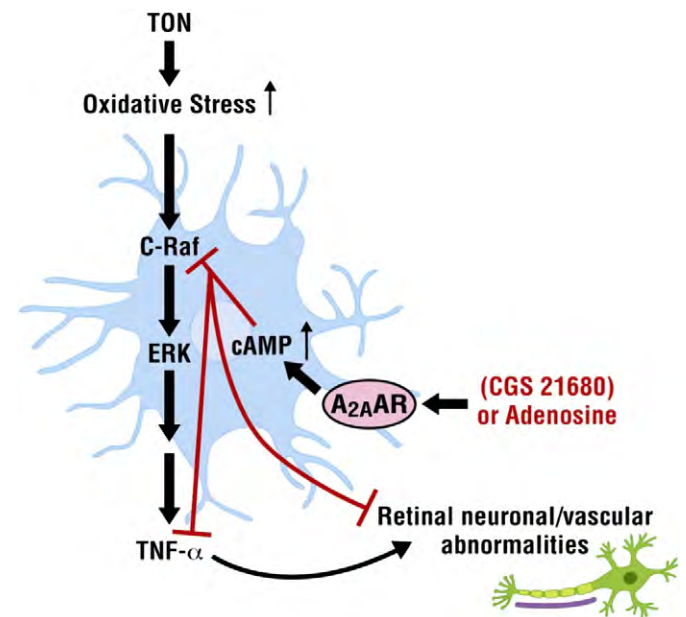


Fig. 8. Proposed pathway of oxidative stress and MAPKinase mediated TNF- α release, and A_{2A} adenosine receptor ($A_{2A}AR$) agonist mediated anti-inflammation in the retinal microglial cells during traumatic optic neuropathy.

of A_{2A}AR agonist to inhibit oxidative stress and MAPK mediated inflammatory cytokine release in activated microglia represents a novel therapeutic approach to treat retinal degeneration associated with TON. Thus, A_{2A}AR has significant potential as a therapeutic target in TON.

Disclosure

This report is according to journal guidelines and ethical issues. All authors have no conflicts of interest.

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ABT-702, an adenosine kinase inhibitor, attenuates inflammation in diabetic retinopathy

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ABSTRACT

Aims: This study was undertaken to determine the effect of an adenosine kinase inhibitor (AKI) in diabetic retinopathy (DR). We have shown previously that adenosine signaling via A_{2A} receptors ($A_{2A}AR$) is involved in retinal protection from diabetes induced inflammation. Here we demonstrate that AKI enhanced adenosine signaling provides protection from DR in mice.

Main methods: We targeted AK, the key enzyme in adenosine metabolism, using a treatment regime with the selective AKI, ABT 702 (1.5 mg/kg intraperitoneally twice a week) commencing at the beginning of streptozotocin induced diabetes at the age of eight weeks. This treatment, previously demonstrated to increase free adenosine levels in vivo, was maintained until the age of 16 weeks. Retinal inflammation was evaluated using Western blot, Real Time PCR and immuno staining analyses. Role of $A_{2A}AR$ signaling in the anti inflammation effect of ABT 702 was analyzed in Amadori glycated albumin (AGA) treated microglial cells.

Key findings: At 16 weeks, when diabetic mice exhibit significant signs of retinal inflammation including up regulation of oxidative/nitrosative stress, $A_{2A}AR$, ENT1, Iba1, TNF α , ICAM1, retinal cell death, and down regulation of AK, the ABT 702 treated group showed lower signs of inflammation compared to control animals receiving the vehicle. The involvement of adenosine signaling in the anti inflammation effect of ABT 702 was supported by the TNF α release blocking effect of $A_{2A}AR$ antagonist in AGA treated microglial cells.

Significance: These results suggest a role for AK in regulating adenosine receptor signaling in the retina. Inhibition of AK potentially amplifies the therapeutic effects of site and event specific accumulation of extracellular adenosine, which is of highly translational impact.

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Introduction

Diabetic retinopathy (DR) is the leading cause of acquired vision loss among adults of working age in developed countries worldwide and has been perceived as the most common microvascular complication of diabetes (Zhu and Zou, 2012). Despite many years of research, treatment options for DR, including photocoagulation, vitrectomy and repeated intraocular injections of steroids and anti vascular endothelial growth factor (VEGF), remain invasive, limited and with adverse effects. This is because VEGF, although induces angiogenesis, is also required for the maintenance of retinal neurons. By neutralizing VEGF with anti VEGF, angiogenesis could be solved at the expense of neuronal degeneration. Therefore, there is a great need for the development of new non invasive therapies.

The early signs of DR in experimental diabetic models include vascular inflammatory reactions due to oxidative stress, pro inflammatory cytokines, and the consequent upregulation of leukocyte adhesion molecules (Tang and Kern, 2011). These reactions lead to breakdown of the blood retinal barrier, vascular occlusion and tissue ischemia, which in turn leads to neuronal cell death (El Remessy et al., 2006). Under these conditions, normally quiescent microglial cells become activated. Activated microglia release reactive oxygen species and proinflammatory mediators, such as tumor necrosis factor TNF α (Xie et al., 2002). Thus, research on retinal microglia activation may provide insights into the pathogenesis of DR (Ibrahim et al., 2011a).

Adenosine is centrally involved in the signaling cascade of related events, including anti inflammatory actions, angiogenesis, oxygen supply/demand ratio, and ischemic pre and postconditioning (Johnston Cox and Ravid, 2011). Under these circumstances, the local levels of extracellular adenosine are increased due to the increased need for energy supplied by ATP (Vallon et al., 2006). The increased extracellular adenosine at inflamed sites can protect against cellular damage by activating the A_{2A} adenosine receptor ($A_{2A}AR$), a Gs coupled

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receptor (Ibrahim et al., 2011b). Extracellular adenosine re uptake by the equilibrative and concentrative nucleoside transporters (ENT and CNT) allows for adenosine conversion to AMP by adenosine kinase (AK) (Löffler et al., 2007), decreases extracellular adenosine levels, and terminates the protective effect of A_{2A} AR. The removal of extracellular adenosine is predominantly regulated by AK via conversion of adenosine into AMP. The extracellular levels of adenosine are largely dependent on the intracellular activity of AK whereas the degradation of adenosine into inosine by adenosine deaminase (ADA) plays only a minor role in regulating adenosinergic function (Pak et al., 1994).

We aim to evaluate the AK in regulating adenosine signaling in the retina. It was reported that the degree of brain injury directly depends on expression levels of AK and the resulting extracellular levels of adenosine (Boison, 2006). Indeed, transgenic mice overexpressing AK are highly susceptible to stroke induced brain injury (Shen et al., 2011). We therefore hypothesized that adenosine kinase inhibitors (AKI) could play the same protective role in the diabetic retina.

Methods

Preparation of AKI

A selective AKI, 4 amino 5 (3 bromophenyl) 7 (6 morpholino pyridin 3 yl)pyrido[2,3 dipyrimidine (ABT 702, 5 mg) from Santa Cruz was dissolved in 0.25 mL of DMSO (20 mg/mL) and then in 9.75 mL of distilled water to prepare a 0.5 mg/mL stock solution. The solutions were aliquoted and stored at -20°C for later use. An equivalent volume of vehicle solution was administered to the control animals. ABT 702 was used previously to study the effect of AK inhibition on neuronal inflammation (Suzuki et al., 2001) and age related hearing loss (Vlajkovic et al., 2011). ABT 702 was 1300 to 7700 fold selective for AK compared with a number of other neurotransmitter and peptide receptors, ion channel proteins, neurotransmitter/nucleoside reuptake sites, and enzymes, including cyclooxygenases 1 and 2 (Jarvis et al., 2000).

Animal preparation and experimental design

All procedures with animals were performed in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare, National Institutes of Health Publication No. 80-23) and the Georgia Health Sciences University guidelines. Male, eight week old mice in C57BL/6J (Jackson Laboratory, Bar Harbor, ME) background were used. Animals were given i.p. injections of vehicle or freshly prepared streptozotocin in 0.01 mol/L sodium citrate buffer, pH 4.5 (45 mg/kg) after a 4 hour fast each day for 5 consecutive days. Diabetes was confirmed by fasting blood glucose levels >250 mg/dL. The diabetic and normal, non diabetic mice were randomly divided into four subgroups: ABT 702 treated diabetic, ABT 702 treated normal, vehicle treated diabetic and vehicle treated normal (1.5 mg/kg intraperitoneally, twice a week).

Eight weeks after the establishment of diabetes, the retinas were removed, snap frozen in liquid nitrogen, stored at -80°C , and analyzed by Quantitative Real Time PCR (qRT-PCR) or Western blot. Frozen eye sections were prepared for immunofluorescence or immunohistochemistry.

Measurement of blood glucose

Blood glucose was measured by blood glucose meter (OneTouch UltraEasy, USA).

Primary retinal microglia culture

Microglial cells were isolated from retinas of newborn Sprague Dawley (SD) rats according to a previous procedure (El Remessy

et al., 2008) with minor modifications. Briefly, retinas were collected into phosphate buffered saline and digested with 0.125% trypsin for 3–5 min before mixing with Dulbecco's Modified Eagle Medium (DMEM)/F12 containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Retina pieces were then filtered through a mesh (100 μm), collected by centrifugation, resuspended in culture medium and plated onto T75 cell culture flasks (Corning, NY) at a density of 2×10^5 cells/cm². After 2 weeks, microglial cells were harvested by shaking the flasks at 100 rpm for 1 h. Immunocytochemical studies showed that more than 95% cultured cells stained positively for Iba1. Almost none of these cells showed positive staining for GFAP, indicating that majority of the isolated cells were microglia and were not contaminated with astrocytes or Müller cells (data not shown).

Drug treatment effects on cultured microglial cells

Microglial cells were seeded at a density of 5×10^5 cells/well in a collagen 1 pretreated 24 well tissue culture plate. One day after seeding, the cultured wells were washed with Cellgro Complete (Mediatech, Manassas, VA) and incubated in the same media with various treatments. Cells were pretreated with AR antagonists (all are from Sigma Aldrich except ZM 241385, which is from Tocris) at the indicated concentrations for 30 min at 37°C , followed with ABT 702 or vehicle for 30 min at 37°C . Microglial activation was then achieved by addition of Amadori glycated albumin (AGA; Sigma) with undetectable endotoxin (<0.125 units/mL, 10 EU = 1 ng lipopolysaccharide; Lonza, Basel, Switzerland) (Ibrahim et al., 2011a) to each well at a final concentration of 250 or 500 $\mu\text{g/mL}$ at indicated time points (Ibrahim et al., 2011a,b). After the indicated time course, culture media were collected and assayed for TNF α by ELISA.

ELISA for TNF α

TNF α levels in the supernatants of culture media were estimated with ELISA kits (R & D, Minneapolis, MN) per the manufacturer's instructions. Standards and samples were added and bound by the immobilized antibody. After washing an enzyme linked polyclonal antibody specific for the cytokine was added to the wells followed by a substrate solution yielding a colored product. The intensity of the color was measured at 450 nm. The sample levels were calculated from the standard curve and corrected for protein concentration.

Quantitative real time PCR

Total RNA was isolated from mouse retina using SV Total RNA Isolation kit (Promega, Madison, WI) following manufacturer's instructions, and the quality of the RNA preparations was monitored by absorbance at 260 and 280 nm (Helios Gamma, Thermo Spectronic, Rochester, NY). The RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA) using iScript reagents from Bio Rad on a programmable thermal cycler (PCR Sprint, Thermo Electron, Milford, MA). Fifty ng of cDNA was amplified in each qRT-PCR using a Bio Rad iCycler, ABgene reagents (Fisher Scientific) and appropriate primers (Table 2). Average of glyceraldehyde 3 phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA was used as the internal control for normalization.

Western blot analysis

Dissected individual mouse retinas were homogenized in modified RIPA buffer (Upstate, Lake Placid, NY), containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P 40, 0.25% deoxycholate, supplemented with 40 mM NaF, 2 mM Na_3VO_4 , 0.5 mM phenylmethylsulfonyl fluoride and 1:100 (v/v) of proteinase inhibitor cocktail (Sigma). Insoluble material was removed by centrifugation at 12,000 $\times g$ at 4°C for 30 min. Protein was determined by DC Protein

Table 1
Body weight and blood glucose levels in studied groups.

Group	No	Body weight (g)		Blood glucose (mg/dL)	
Non-diabetic	7	30.8	0.59	198.6	11.61
Non-diabetic + ABT 7002	7	30.0	0.67	198.6	7.93
Diabetic	7	24.1**	0.61	399.6***	11.22
Diabetic + ABT 702	7	23.4**	0.57	373.5***	6.82

Mean $\pm$ SD.

** P < 0.001 vs non-diabetic group.

*** P < 0.0001 vs non-diabetic group.

Assay (Bio Rad, Hercules, CA) and 100 μ g was boiled in Laemmli sample buffer, separated by SDS PAGE on a gradient gel (4 to 20%) (Pierce, Rockford, IL), transferred to nitrocellulose membrane and incubated with specific antibodies. Antibodies for β actin, ICAM 1, ENT1, A_{2A}AR and AK (Santa Cruz Biotechnology, Santa Cruz, CA) were detected with a horseradish peroxidase conjugated antibody and enhanced chemiluminescence (ECL) (Amersham BioSciences, Buckinghamshire, UK). The same filter was re probed with control antibodies, such as those for the actin. Intensity of immunoreactivity was measured by densitometry.

Immunolocalization studies

Immunofluorescence analysis was performed using frozen eye sections. Briefly, cryostat sections (7 μ m) were fixed in 4% paraformaldehyde, blocked with Dako protein block serum free and then incubated overnight at 4 °C with primary antibodies: rabbit anti Iba 1 (Proteintech Group), or goat anti ICAM 1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti AK (Santa Cruz Biotechnology, Santa Cruz, CA). Thereafter, sections were briefly washed with PBS and incubated with appropriate secondary antibodies. Slides were examined by fluorescent microscope. Specificity of the reaction was confirmed by omitting the primary antibody, or by using non immune IgG.

Immunohistochemistry of cleaved, activated caspase 3 was performed as follows. Retinas frozen sections were fixed in 4% paraformaldehyde, rinsed in PBS, blocked with 0.3% H₂O₂ then Mouse on Mouse (M.O.M.) Immunoglobulin Blocking Solution (Vector Laboratories, Burlingame, CA), and reacted with antibodies detecting cleaved, activated caspase 3 (Cell Signaling Technology) for 16 to 20 h at room temperature. Sections were washed, and reacted with M.O.M. biotinylated anti mouse Ig reagent (1:250), followed by M.O.M. ABC reagent. Color was developed with 3,3' diaminobenzidine (DAB) as substrate.

Terminal dUTP nick end labeling (TUNEL)

TUNEL was performed in frozen sections using the TACS 2 TdT Fluorescein In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg,

Table 2
The primer sets used for the detection of mouse genes by quantitative Real-Time PCR analysis.

Gene	Primer sequence (5'–3')	Accession number
TNF- α	CCCTCAGCTCAGATCATCTTCT GTCACGACGTGGGCTACAG	NM_013693.2
ICAM-1	CGCTGTGCTTTGAGAACTGTG ATACACGGTGATGGTAGCGGA	NM_010493
Iba-1	GTCCTTGAAGCGAATGCTGG CATTTCAAGATGCCAGATC	NM_019467
GAPDH	CAT GGC CTC CAA GGA GTAAGA GAG GGA GAT GCT CAG TGT TGG	M32599
18S	AGT GCG GGT CAT AAG CTT GC GGG CCT CAC TAA ACC ATC CA	NR_003278

MD) counter stained with propidium iodide, according to the manufacturer's suggestions. Briefly, sections were hydrated with alcohol 100%, 95%, and 70%, and then fixed in 3.7% paraformaldehyde. After washing, slides were incubated in mixture of TdT, Mn + 2, and TdT dNTP for 1 h at 37 °C. The reaction was stopped with TdT Stop Buffer for 5 min. After washing with deionized water, the slides were incubated with Streptavidin HRP (diluted 1:200) solution for 20 min at room temperature. Slides were counter stained, mounted, covered with coverslips and visualized by confocal microscopy (LSM 510, Carl Zeiss, Inc.). Apoptotic cells were identified as doubly labeled with TdT Fluorescein and propidium iodide and only nuclei that were clearly labeled yellow were scored.

Measurement of oxidative and nitrosative stress

The production of superoxide as oxidative stress, and peroxynitrite as nitrosative stress were measured in frozen eye sections using the oxidative fluorescent dye dihydroethidium (DHE) and nitrotyrosine immunofluorescent staining, respectively. DHE (2 μ M) (Sigma Aldrich, Oakville, ON, Canada) was applied to 7 μ m thick eye sections and the slides were then incubated in a light protected humidified chamber at 37 °C for 30 min. Cells are permeable to DHE. In the presence of superoxide, DHE is oxidized to fluorescent ethidium, which is trapped by intercalation with DNA. Ethidium is excited at 518 nm with an emission spectrum of 605 nm. The intensity of the fluorescence was quantified by Image J software (version 1.42; National Institutes of Health, Bethesda, MD). Nitrotyrosine levels in frozen eye sections were quantified by immunofluorescent histochemistry. Sections were stained with antibody for nitrotyrosine (Santa Cruz Biotechnology, Santa Cruz, CA). Images were observed using fluorescent microscope.

Data analysis

The results are expressed as mean $\pm$ SD. Differences among experimental groups were evaluated by analysis of variance, and the significance of differences between groups was assessed by the posthoc test (Fisher's PLSD). Significance was defined as P < 0.05.

Results

Body weight and blood glucose levels in studied group

The final body weight was lower after streptozotocin injection, and it was not affected by ABT 702 treatment. Blood glucose levels were higher in diabetic mice compared with non diabetic groups and they were not modified by ABT 702 treatment (Table 1).

Inhibition of adenosine kinase mitigates retinal inflammation in diabetic mice

Inflammation has been proposed to be important in the pathogenesis of DR. An early feature of inflammation is the release of cytokines leading to increased expression of endothelial activation markers such as Intercellular Adhesion Molecule 1 (ICAM 1) (Rangasamy et al., 2012). Consistently, ICAM 1 and TNF α expressions were markedly increased in the retinas of 8 week diabetic mice as compared with normal, non diabetics as revealed by qRT PCR (Fig. 1A, B) and Western analyses (Fig. 1C). Treatment with ABT 702 (1.5 mg/kg i.p., twice a week) reduced retinal ICAM 1 expression and retinal TNF α in the diabetic mice as compared with vehicle treated diabetic mice.

Inhibition of adenosine kinase blocks A_{2A}AR up regulation in diabetic mice

A_{2A}AR is the most likely candidate for mediating the anti inflammatory effect of adenosine (Milne and Palmer, 2011). Diabetes

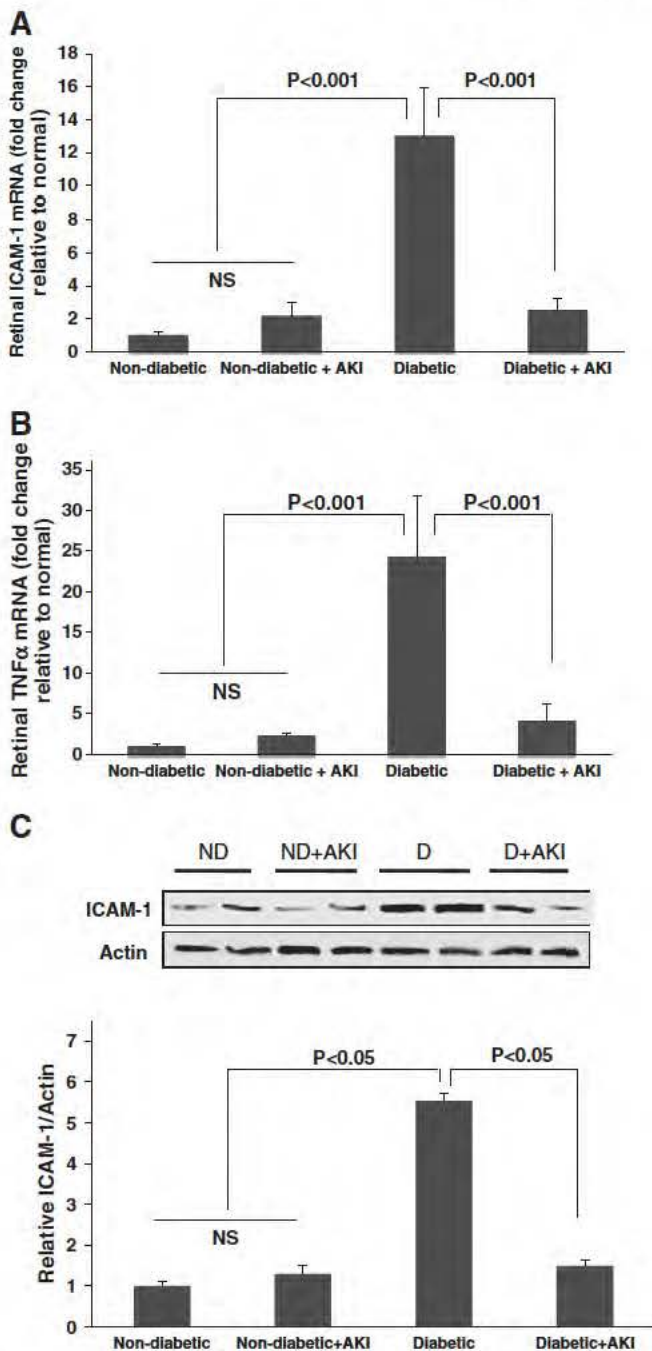


Fig. 1. Inhibition of adenosine kinase mitigates retinal inflammation in diabetic mice. A, B) Retinal expression of ICAM1 and TNF- α measured by R-T PCR. A) Effect of AK inhibition on ICAM1 expression in the diabetic mouse retina. B) Effect of AK inhibition on TNF- α expression in the diabetic mouse retina. GAPDH and 18S were used as reporter genes. The results represent the means $\pm$ SD of fold changes calculated using expression level, normalized to the level of the normal non-diabetic mice ($n = 4-6$). C) Representative Western blots and quantitative analysis of retinal ICAM1 expression showing the effect of AK inhibition on ICAM-1 expression in the diabetic mouse retina ($n = 4$).

or inflammation is associated with up regulation of $A_{2A}AR$ (Pang et al., 2010). The increased $A_{2A}AR$ expression may possibly represent an endogenous mechanism to combat the inflammation associated with diabetes induction. Consistent with this, diabetes induced up regulation of $A_{2A}AR$ in the retina as compared with normal (Fig. 2). Treatment of ABT 702 reduced $A_{2A}AR$ expression in the diabetic mice as compared with vehicle treated diabetic mice (Fig. 2).

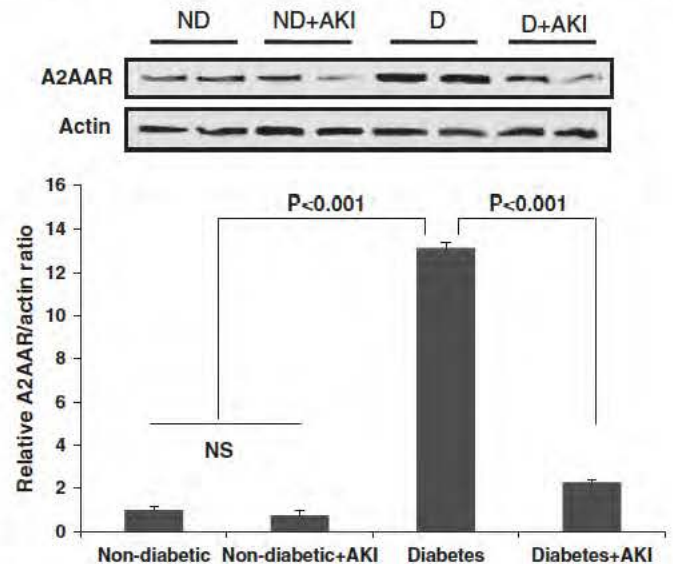


Fig. 2. Inhibition of adenosine kinase blocks $A_{2A}AR$ up-regulation in diabetic mice. Representative Western blots and quantitative analysis of retinal $A_{2A}AR$ expression showing the effect of AK inhibition on $A_{2A}AR$ expression in the diabetic mouse retina ($n = 4$).

Inhibition of adenosine kinase blocks ENT1 up regulation in diabetic mice

ENT1 plays an integral role in adenosine function in diabetes by regulating adenosine levels in the vicinity of adenosine receptors. Hypoglycemia up regulated ENT1 expression and adenosine transport in cultured human aortic smooth muscle cells (Leung et al., 2005). Consistent with this observation, diabetes induced up regulation of ENT1 in the retina as compared with normal (Fig. 3). The increase in ENT1 activity in diabetes may affect the availability of adenosine in the vicinity of adenosine receptors and, thus, alter vascular functions in diabetes. Treatment with ABT 702 reduced ENT1 expression in diabetic mice as compared with vehicle treated diabetic mice (Fig. 3).

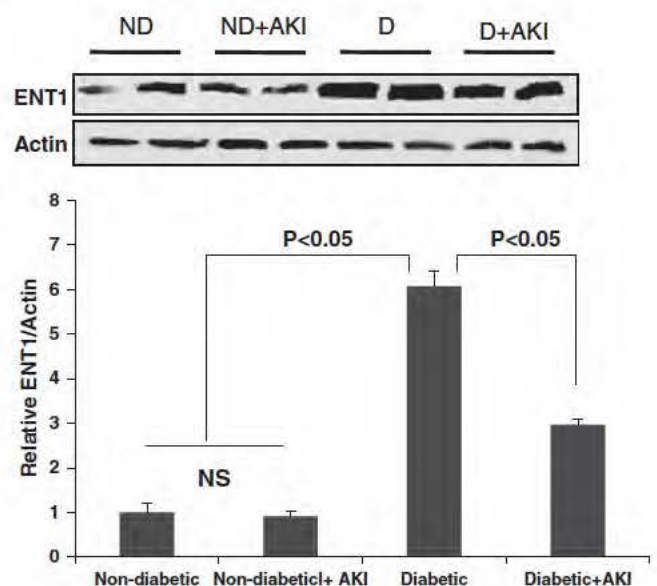


Fig. 3. Inhibition of adenosine kinase blocks ENT1 up-regulation in diabetic mice. Representative Western blots and quantitative Western analysis of retinal ENT1 expression showing the effect of AK inhibition on ENT1 expression in the diabetic mouse retina ($n = 4$).

Inhibition of adenosine kinase blocks adenosine kinase down regulation in diabetic mice

Sakowicz and Pawelczyk reported reduced AK activity in tissues of diabetic rat. They suggested that the expression of AK to some extent is controlled by insulin. Reduced AK expression is also reported in hypoxic tissues (Morote Garcia et al., 2008). The reduced AK expression may possibly represent an endogenous protective mechanism to raise extracellular adenosine levels. Consistent with these observations, AK expression was reduced in retinas of diabetic mice as compared with the normal (Fig. 4A, B). Treatment with ABT 702 blocked the diabetic effect on AK in diabetic mice as compared with vehicle treated diabetic mice (Fig. 4A, B).

Inhibition of adenosine kinase mitigates retinal microglial activation in diabetic mice

We next sought to explore a potential mechanism by which ABT702 regulates inflammation in DR. Through immunofluorescence, the effect of ABT702 treatment on microglial activation was determined by measuring Iba1 expression, which is up regulated in activated microglia in diabetic mice as compared with normal. Iba1 was found to be decreased in the AKI treated diabetic mice as compared

with vehicle treated diabetic mice (Fig. 5A). In addition, the level of Iba1 mRNA was markedly reduced in the retinas of AKI treated diabetic mice as compared with vehicle treated diabetic mice (Fig. 5B).

Inhibition of adenosine kinase mitigates oxidative and nitrosative stress in the retina of diabetic mice

Oxidative stress is a key pathogenic factor in DR (Madsen Bouterse and Kowluru, 2008). Diabetic mice showed a significant increase in DHE staining as compared with normal group and treatment with ABT 702 reduced DHE staining in diabetic mice retinas as compared with vehicle treated diabetic mice (Fig. 6A). In addition, immunofluorescent staining of nitrotyrosine, a stable product formed from the reaction of peroxynitrite with tyrosine residues and an index of nitrosative damage, was elevated in the retinas of diabetic mice as compared with that in normal retinas. ABT702 treated diabetic mice showed decreased nitrotyrosine staining as compared with vehicle treated diabetic mice (Fig. 6B).

Inhibition of adenosine kinase reduces retinal cell death in diabetic mice

Retinal cell death in diabetic and non diabetic animals treated and untreated with ABT702 was determined by immunostaining of cleaved,

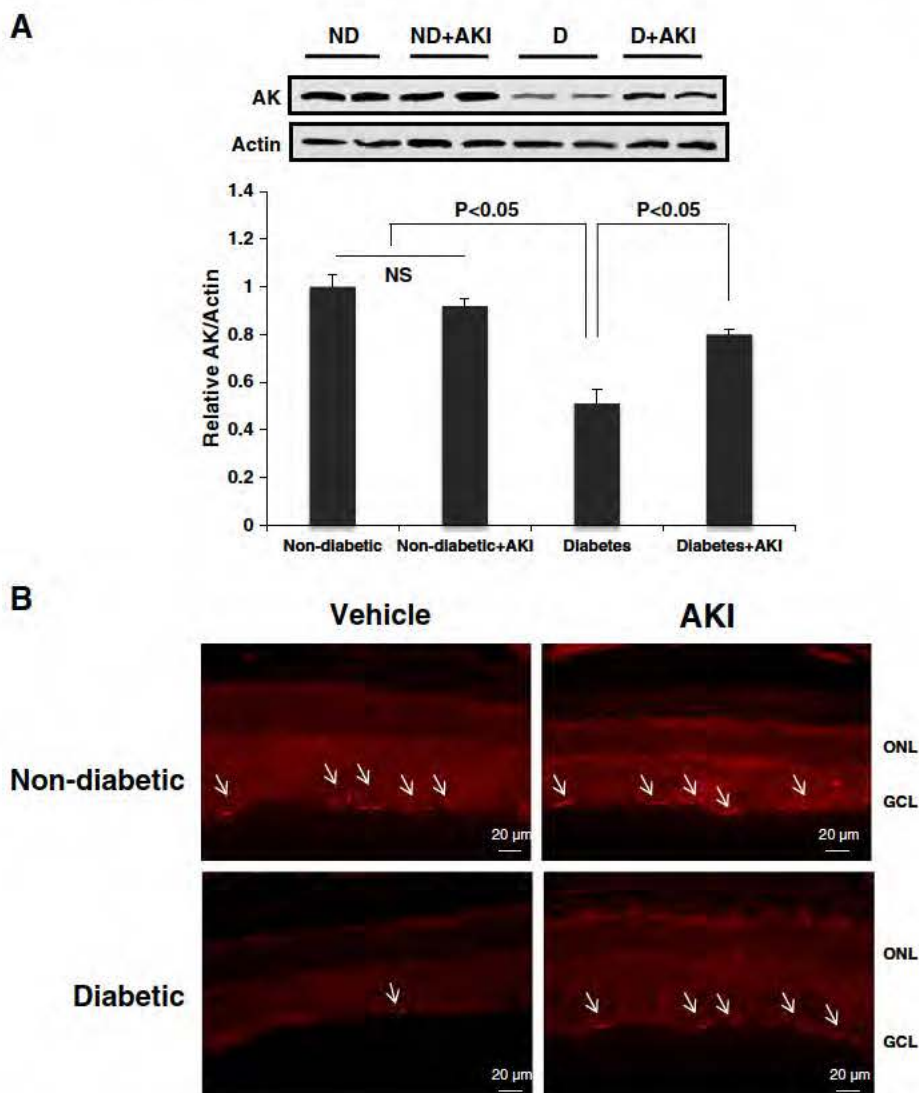


Fig. 4. Inhibition of adenosine kinase blocks adenosine kinase down-regulation in diabetic mice. A) Western blot analysis. B) Immunofluorescence staining. Arrows indicate AK distribution (n = 4–6).

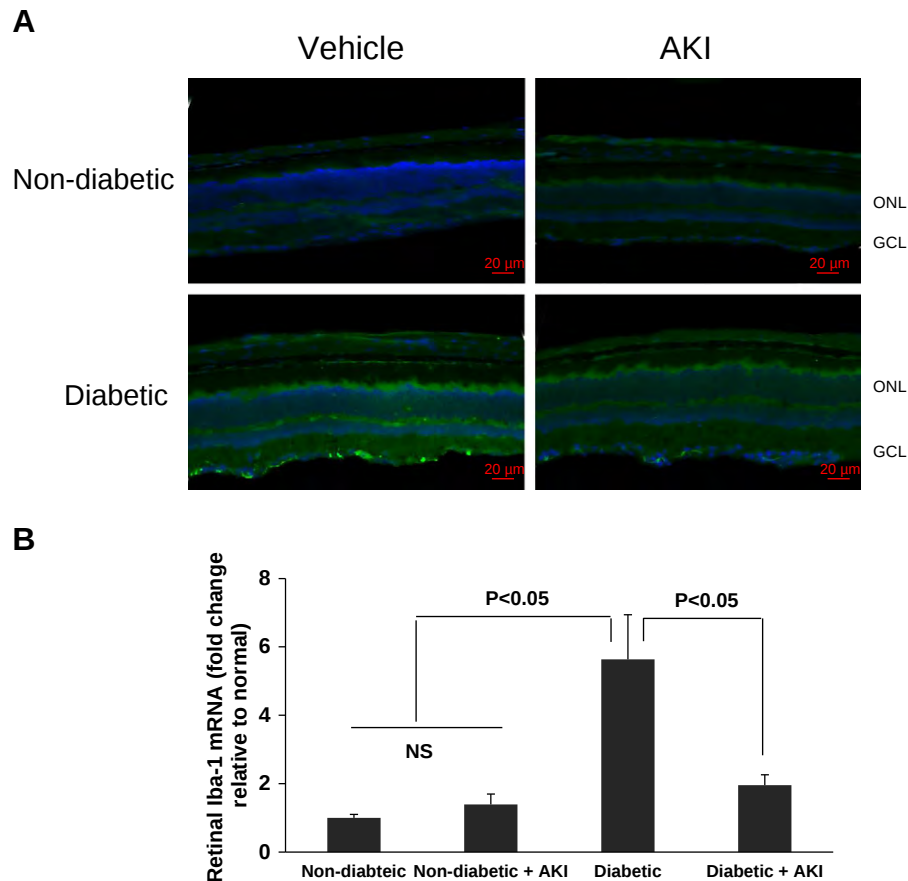


Fig. 5. Inhibition of adenosine kinase mitigates retinal microglial activation in diabetic mice. A) Effect of AK inhibition on Iba1 expression in the diabetic mouse retina determined by immunofluorescence staining. Scale bar: 20 μ m; B) determined by RT PCR analysis; GAPDH and 18S were used as reporter genes. The results represent the means $\pm$ SE of fold changes calculated using expression level, normalized to the level of the normal non-diabetic mice ($n = 4-6$).

activated caspase 3, a known marker for apoptosis, and by TUNEL. As shown by these methods, increased cell death appeared in the retinal ganglion cell layer of diabetic animals (Fig. 7A, B). Treatment with ABT702 blocked cell death in diabetic mice but did not affect treated normal controls (Fig. 7A, B). Taken together, the above findings suggest that AK inhibition plays a role in attenuating retinal oxidative stress, inflammation, and cell death by dampening microglial cell activation.

Inhibition of adenosine kinase is more effective than adenosine deaminase in blocking TNF α release in activated retinal microglial cells

The extracellular levels of adenosine are largely dependent on the intracellular activity of AK whereas the degradation of adenosine into inosine by adenosine deaminase (ADA) plays only a minor role in regulating adenosinergic function (Pak et al., 1994). This was further confirmed by others: endogenous adenosine levels in the brain are mainly dependent on the activity of AK (Gouder et al., 2004). To compare the anti inflammatory effect of the inhibitors of AK and ADA, we developed a cultured retinal microglia model. This model also helps elucidate the molecular mechanisms responsible for this effect. In this model, we determined the ability of ABT702 and EHNA, an ADA inhibitor, to affect TNF α release in retinal microglia in response to AGA treatment. EHNA at levels comparable to the present study was previously used to study the cardioprotective effect of adenosine metabolism inhibitors (Peart et al., 2001). Microglial cells were pretreated with the indicated concentrations of ABT702 and EHNA for 1/2 h then treated with AGA for 16 h. The supernatants were collected and assayed for TNF α by ELISA. As shown (Fig. 8), ABT 702 inhibited AGA induced

TNF α release in a dose dependent manner more significantly than EHNA.

Inhibition of adenosine kinase blocks TNF α release via A_{2A} AR

To identify the AR subtype(s) involved in ABT 702 inhibitory effect on TNF α release in the retinal microglia in response to AGA, we examined the effect of the ABT 702 in the presence of AR subtype selective antagonists. The concentrations of each antagonist chosen for this study were based on the affinity and selectivity for the recombinant mouse AR subtypes determined by radioligand binding studies, and was applied to rat retinal microglial cells previously (Liou et al., 2008; Ibrahim et al., 2011b). As shown in Fig. 9, cells pretreated with vehicle showed a significant increase in AGA induced TNF α release compared with vehicle treated control cells. Treatment with ABT 702 at a concentration of 20 μ M potently inhibited AGA induced TNF α release. When the cells were pretreated with the A_{1A} AR antagonist 1,3 dipropyl 8 cyclopentylxanthine (CPX; 100 nM), the A_{2B} AR antagonist 8 [4 [(4 cyanophenyl) arbamoylmethyl] oxy] phenyl] 1,3 di(n propyl) xanthine hydrate (MRS 1754; 1 μ M), or the A_{3A} AR antagonist 3 propyl 6 ethyl 5 [(ethylthio)carbonyl] 2 phenyl 4 propyl 3 pyridine carboxylate (MRS 1523; 10 μ M), the inhibitory effect of ABT 702 on TNF α release was not affected. However, this effect was successfully blocked by 4 {2 [7 amino 2 (2 furyl)]1,2,4}triazolo [2,3 α][1,3,5]triazin 5 ylamino]ethyl} phenol (ZM 241385) at concentrations (100 and 500 nM) capable of blocking A_{2A} ARs. These results suggest that ABT 702 inhibited AGA induced TNF α release from retinal microglia via the A_{2A} AR.

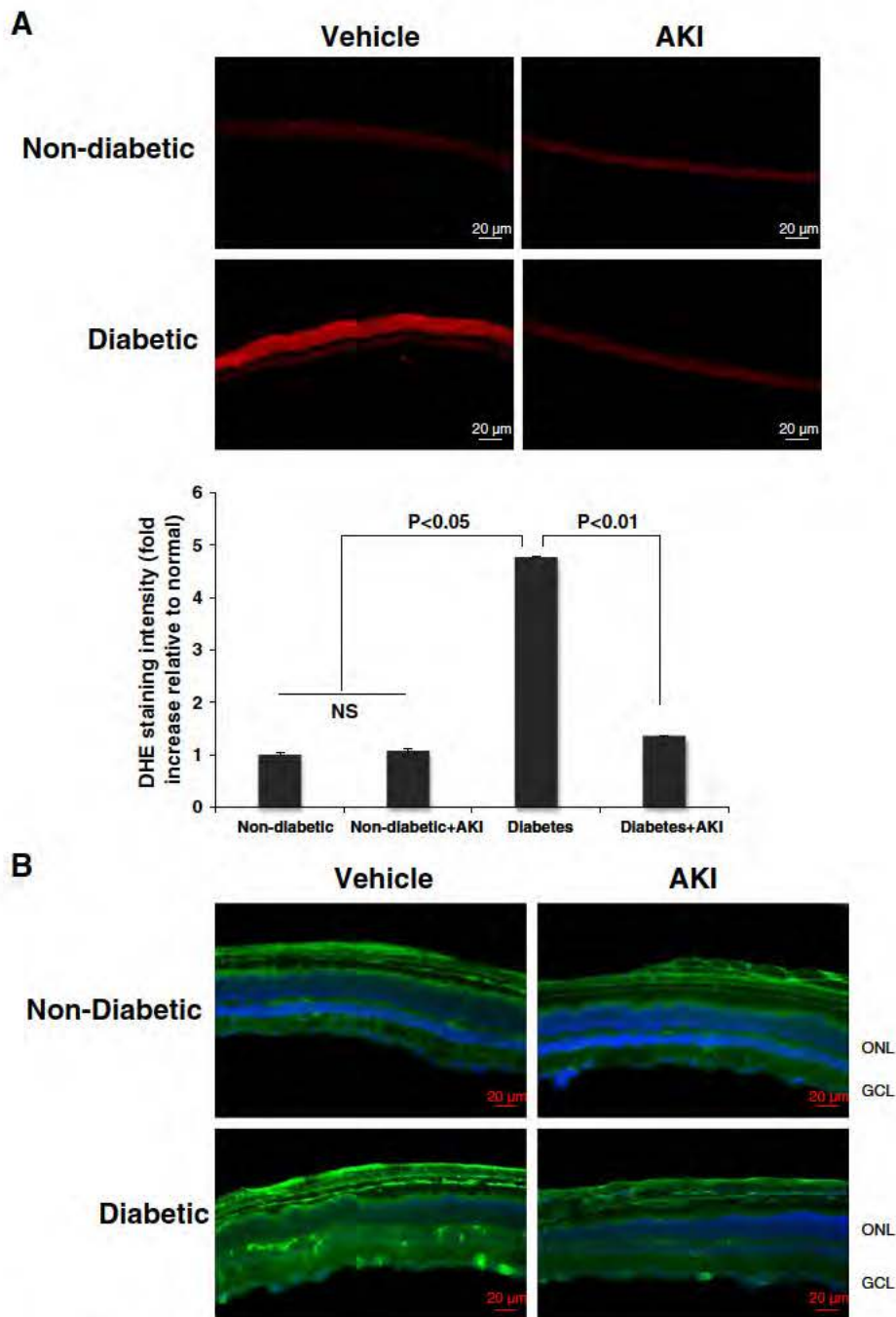


Fig. 6. Inhibition of adenosine kinase mitigates oxidative and nitrosative stress in the retina of diabetic mice. Effect of AK inhibition on diabetes-induced oxidative and nitrosative stress in the retina. A) Oxidative stress measured by DHE staining. The fluorescence intensity indicates the level of superoxides production. The results represent the means $\pm$ SE of fold changes calculated using intensity, normalized to the level of the normal non-diabetic mice ($n = 4-6$). B) Nitrosative stress determined by immunofluorescence for nitrotyrosine. Scale bar: 20 μ m.

Discussion

Biochemical studies have shown that inflammatory reactions (Joussen et al., 2004), including TNF α release, are relatively early events that occur in response to diabetes before vascular dysfunction involving acellular capillary formation and neovascularization (Kern and Barber, 2008). Moreover, TNF α has been shown to recruit leukocytes, cause vascular breakdown and promote neuronal injury at high levels (Joussen et al., 2009). Thus, treatments targeting early features of DR would provide long term vascular benefits. Adenosine released

at inflamed sites exhibits anti inflammatory effects through A_2AAR (Bong et al., 1996). Although adenosine and its agonists are protective in animal models of inflammation, their therapeutic application has been limited by systemic side effects such as hypotension, bradycardia, and sedation (Williams, 1996). Moreover, adenosine usually disappears very rapidly in physiological or inflammatory conditions due to rapid re uptake and subsequent intracellular metabolism (Möser et al., 1989). The use of AK inhibitors represents one possible way to amplify the endogenous therapeutic effects of site and event specific accumulation of extracellular adenosine while minimizing hemodynamic toxicity.

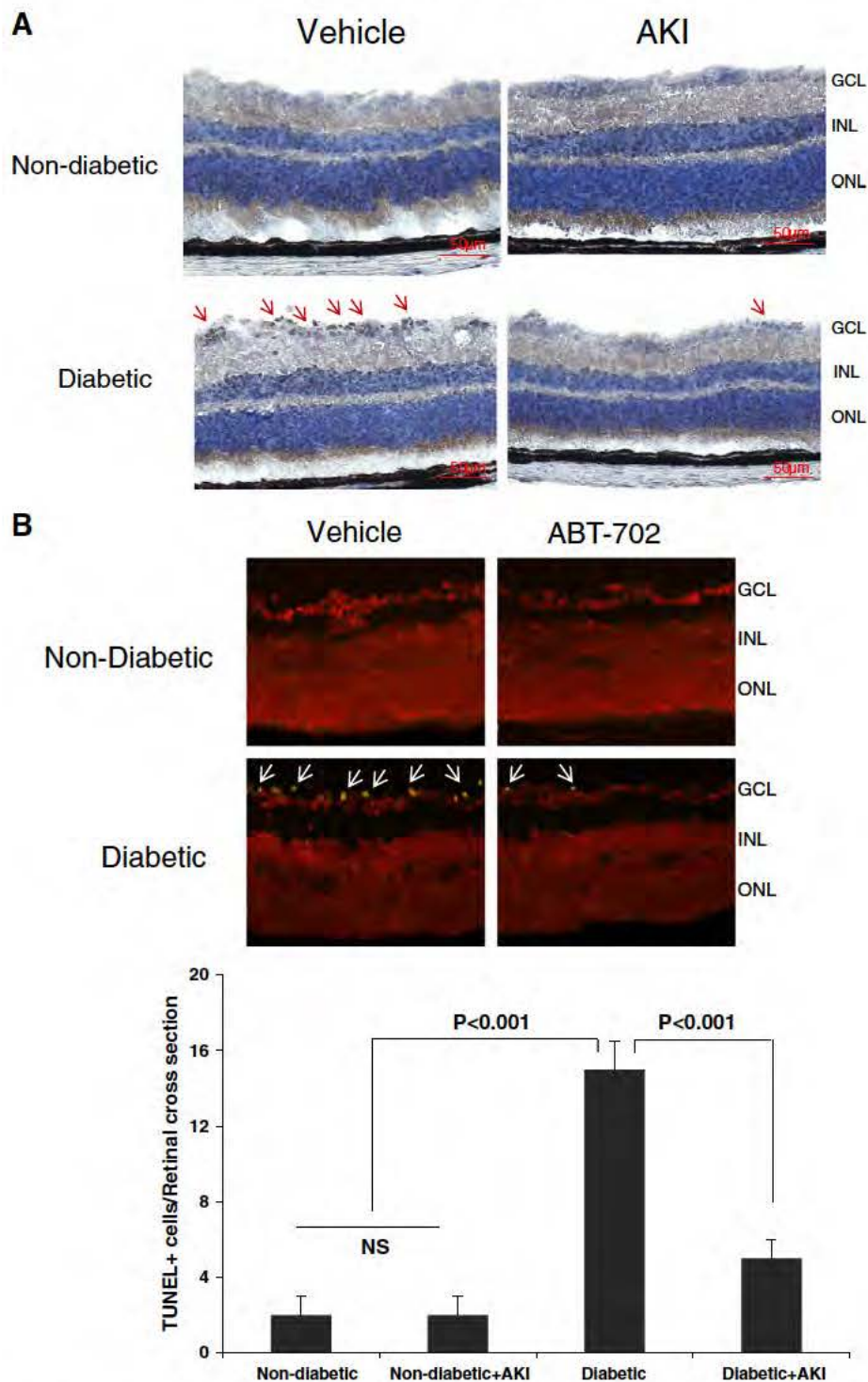


Fig. 7. Inhibition of adenosine kinase reduces retinal cell death in diabetic mice. A) Representative images with arrows show the localization of the apoptotic marker cleaved, activated caspase-3 in the ganglion cell layer in diabetic retina sections. ABT702 attenuated diabetes-induced cell death in the retina. B) Representative images with arrows showing the localization and quantitative analysis of TUNEL-positive cells in the ABT702-treated diabetic mice. The results represent the means $\pm$ SE of TUNEL-positive cells per retinal cross section ($n = 4$).

Endogenous adenosine levels in the brain are mainly dependent on the activity of AK, the key enzyme of adenosine metabolism (Gouder et al., 2004). This notion is based on several lines of evidence: 1) transgenic mice overexpressing AK are highly susceptible to stroke induced brain injury (Pignataro et al., 2007); 2) pharmacological inhibition of AK provides seizure suppression in various models of epilepsy (Ugarkar et al., 2000); 3) inhibition of AK in hippocampal slices

increases endogenous adenosine and depresses neuronal firing, where as inhibition of adenosine deaminase has little or no influence (Huber et al., 2001); 4) AK activity is regulated in response to brain injury and is subject to developmental regulation (Studer et al., 2006; Pignataro et al., 2008). We demonstrated that AK has the same importance in the retina. In the present work, intraperitoneal injection of ABT 702 was found to cause a significant inhibition of ICAM 1 and TNF α

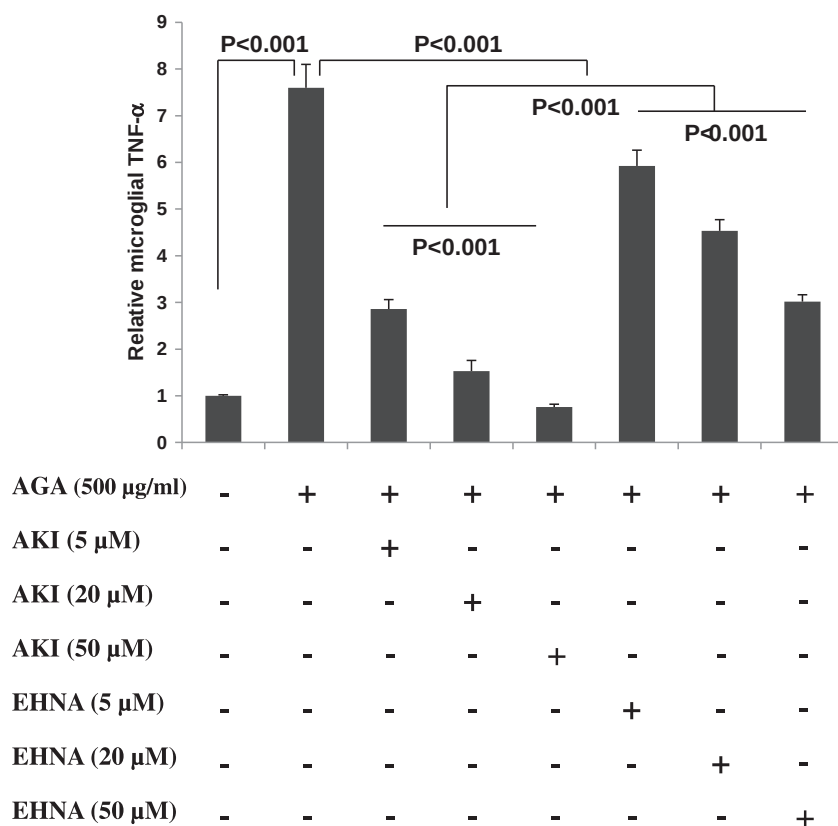


Fig. 8. Inhibition of adenosine kinase is more effective than adenosine deaminase in blocking TNF- α release in activated retinal microglial cells. Retinal microglial cells were treated with AGA (500 μ g/mL, 16 h) in the presence of different doses of EHNA (ADA inhibitor), and ABT 702. TNF- α levels were determined by ELISA. Data shown are the mean $\pm$ SD of at least four different experiments.

mRNA as well as protein levels in the retina of diabetic mice, suggesting the curative effect of ABT 702 on inflammation associated with STZ diabetic model. ABT 702 also prevented up regulation of Iba1;

supporting the hypothesis that ABT 702 reduces retinal inflammation through attenuation of microglia activation. Following this, we used primary culture of rat retinal microglial cells to gain insights into the

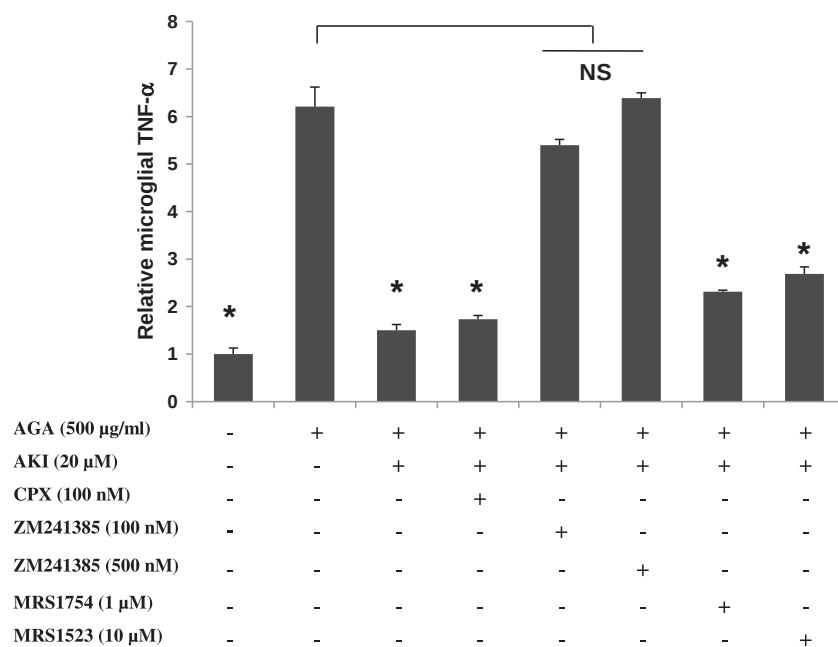


Fig. 9. Inhibition of adenosine kinase blocks TNF- α release via A_{2A} AR. Cells were treated with vehicle or ABT 702 (20 μ M) 30 min before AGA treatment in the presence of subtype-selective AR antagonists for A_1 AR (CPX, 100 nM), A_{2A} AR (ZM241385, 100 and 500 nM), A_{2B} AR (MRS 1754, 1 μ M) and A_3 AR (MRS 1523, 10 μ M). *Significant compared to AGA treated microglial cells ($P < 0.05$).

mechanism of ABT 702's anti inflammatory effect. The results indicate that treatment of ABT 702 inhibited AGA induced TNF α release. Furthermore, ABT 702 was more effective than ADA inhibitor in inhibiting TNF α release, suggesting a major role for AK in the regulation of extra cellular adenosine.

The ability of ABT 702 to mitigate AGA induced TNF α release suggests the importance of inhibiting AK activity in ameliorating this inflammatory response through increasing adenosine levels. To test this hypothesis, the inhibitory effect of ABT 702 on AGA induced TNF α release was examined in the presence of AR subtype selective antagonists in the retinal microglial cells. This inhibitory effect was successfully blocked only by 4-[2-[7-amino-2-(2-furyl)-1,2,4-triazolo[2,3- α]-1,3,5-triazin-5-ylamino]ethyl]phenol (ZM 241385), a selective A_{2A}AR antagonist. These results suggest that ABT 702 inhibits AGA induced TNF α release in retinal microglia through A_{2A}AR. A_{2A}AR mediates the suppressive effects of adenosine in macrophages as well as microglial cells (Kreckler et al., 2006).

Diabetes or inflammation is associated with up regulation of A_{2A}AR (Pang et al., 2010). High levels of A_{2A}ARs are found in macrophages and microglial cells that are poised, on activation, to abrogate the immune response (Trincavelli et al., 2008). In addition, hyperglycemia is associated with increased ENT1, possibly via an MAPK/ERK dependent signaling pathway (Leung et al., 2005). ABT 702 inhibited the expression of both A_{2A}AR and ENT1 in the diabetic retina suggesting its ability to attenuate diabetic conditions. Further, we demonstrated that ABT 702 injection attenuated diabetes induced reduction in AK expression. In the brain, AK expression is decreased following onset of injury thus potentiating the adenosine surge as a potential neuroprotective mechanism. Indeed, expression levels of AK might have a crucial role in determining the degree of brain injury (Li et al., 2008).

Next, we studied the effect of ABT 702 on oxidative and nitrosative stress in the retina in diabetes. In diabetes the retina experiences increased oxidative stress (Kowluru and Kanwar, 2007), and reactive oxygen species (ROS) are considered as a causal link between elevated glucose and the metabolic abnormalities important in the development of diabetic complications (Brownlee, 2001). ABT 702 decreased super oxides and nitrotyrosine levels in diabetic retina. The ability of ABT 702 to reduce inflammatory stress in the retina may rely on its inhibitory effect on oxidative and nitrosative stress.

Further, we studied the effect of ABT 702 on retinal cell death. Diabetes induced retinal oxidative and nitrosative stress have been positively correlated with neuronal cell death (Asnaghi et al., 2003). Treating diabetic mice with ABT 702 blocked the increases in oxidative and nitrosative stress and significantly reduced cell death as revealed by decreased cleaved caspase 3 immunostaining and TUNEL assay in treated diabetic retinas. Neurons are highly susceptible to oxidative stress, which can induce apoptosis; therefore, it is likely that diabetes induced oxidative stress leads to neuronal injury.

Finally, despite all the advantages for ABT 702 as a potential effective therapy for DR, given that the administration of ABT 702 by i.p. injection is invasive and stressful, oral administration of ABT 702 may be necessary but should be carefully developed (Kowaluk et al., 2000).

Conclusions

The data presented here provide experimental evidence that targeting AK can inhibit diabetes induced retinal abnormalities that are postulated in the development of DR by potentially amplifying the endogenous therapeutic effects of site and event specific accumulation of extracellular adenosine. Thus, ABT 702 appears to be a useful therapy to possibly inhibit the development/progression of retinopathy, the sight threatening complication faced by diabetic patients.

Conflict of interest

We have no conflict of interests.

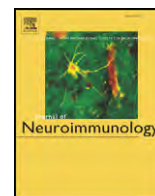
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Inhibition of adenosine kinase attenuates inflammation and neurotoxicity in traumatic optic neuropathy

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ABSTRACT

Traumatic optic neuropathy (TON) is associated with apoptosis of retinal ganglion cells. Local productions of reactive oxygen species and inflammatory mediators from activated microglial cells have been hypothesized to underlie apoptotic processes. We previously demonstrated that the anti-inflammatory effect of adenosine, through A_{2A} receptor activation had profound protective influence against retinal injury in traumatic optic neuropathy. This protective effect is limited due to rapid cellular re uptake of adenosine by equilibrative nucleoside transporter 1 (ENT1) or break down by adenosine kinase (AK), the key enzyme in adenosine clearance pathway. Further, the use of adenosine receptors agonists are limited by systemic side effects. Therefore, we seek to investigate the potential role of amplifying the endogenous ambient level of adenosine by pharmacological inhibition of AK. We tested our hypothesis by comparing TON induced retinal injury in mice with and without ABT 702 treatment, a selective AK inhibitor (AKI). The retinal protective effect of ABT 702 was demonstrated by significant reduction of Iba 1, ENT1, TNF α , IL 6, and iNOS/nNOS protein or mRNA expression in TON as revealed by western blot and real time PCR. TON induced superoxide anion generation and nitrotyrosine expression were reduced in ABT 702 treated mice retinal sections as determined by immunofluorescence. In addition, ABT 702 attenuated p ERK1/2 and p P38 activation in LPS induced activated mouse microglia cells. The results of the present investigation suggested that ABT 702 had a protective role against marked TON induced retinal inflammation and damage by augmenting the endogenous therapeutic effects of site and event specific accumulation of extracellular adenosine.

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1. Introduction

Traumatic optic neuropathy (TON) is partial or complete loss of function of optic nerve (ON) due to either a direct injury or indirectly after head trauma sequelae, such as edema, hemorrhage, and concussion (Steinsapir and Goldberg, 2011). Unfortunately, there are currently no proven treatments that can prevent the damage associated with an acute TON. Optic nerve injury mediated loss of retinal ganglion cells (RGCs) through apoptosis has been hypothesized due to several

underlying common mechanisms, including lack of neurotrophin support, increased extracellular glutamate levels, damage from free radicals, and disruption of cellular homeostasis (Pang et al., 2010). All these mechanisms cause activation of microglial cells and inflammatory responses such as release of free radicals, cytokines, and prostaglandins and complement molecules (Lucas et al., 2006). Therefore, counteracting inflammation may possess neuroprotective effect in TON.

Adenosine is a ubiquitous homeostatic purine nucleoside that accumulates extracellularly in response to metabolic stresses such as hypoxia and inflammation. Activation of either G protein coupled adenosine receptors (ARs; A1R, A2AR, A2BR, and A3R) by extracellular adenosine can modulate cell signaling. However, A2A receptor activation significantly modulates neuronal integrity and neuroprotection by adenosine receptor modulation has been demonstrated in several model systems (Lusardi, 2009). In accordance, we have demonstrated that A_{2A}AR signaling had a protective effect in traumatic optic neuropathy by attenuating microglia induced inflammatory response (Ahmad et al., 2013).

ARs agonists have limited therapeutic use due to systemic side effects (Fredholm et al., 2005). However, a promising alternative might

Abbreviations: TON, traumatic optic neuropathy; TNF- α , tumor necrosis factor- α ; ELISA, enzyme-linked immunosorbent assay; ROS, reactive oxygen species; MAP kinase, mitogen-activated protein kinase; ERK, P38, extracellular signal-regulated kinase; AR, adenosine receptor; AKI, adenosine kinase inhibitor; ABT-702, 2-p-[2-Carboxyethyl]phenethylamino-5'-N-ethylcarboxamidoadenosine; LPS, lipopolysaccharides.

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be the augmentation of the adenosine levels by targeting enzymes or nucleoside transporters that regulate the extracellular levels of adenosine (Shen et al., 2012). Metabolic clearance of adenosine occurs through key enzyme adenosine kinase (ADK) and evidence shows that the inhibition of this enzyme increases extracellular adenosine levels in cell and tissues (Boison and Shen, 2010). Indeed, the inhibition of ADK has been proven to possess potential therapeutic usefulness in a wide range of neurological disorders (Boison, 2008). In this context, we previously reported that pharmacologic inhibition of ADK augments adenosine and exerts activity in retina of diabetic mice (Elsherbiny et al., 2013). Here, we seek to investigate the retinal protective role of ABT 702, a selective adenosine kinase inhibitor against marked TON induced retinal inflammation and damage.

2. Materials and methods

2.1. Experimental design

All procedures with animals were performed in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare publication, NIH 80-23) and Georgia Regents University, Augusta, GA, USA guidelines. Eight to ten week old male wild type (WT) mice (16 mice in each group) in C57BL/6 background were used for experiments. Mice were anesthetized according to standard protocol and limbal conjunctival peritomy was performed on one eye of each mouse. Forceps dissection under the conjunctiva posteriorly allowed access to the optic nerve, upon which pressure was placed 1 mm posterior to the globe until pupillary dilation was noted (approximately 10 s). Blood vessel close to optic nerve was carefully avoided in TON surgery. Mock operated contralateral eye served as the control. After one week, all mice were sacrificed. Eyes were enucleated and sectioned for histological analysis. Retinas were harvested for Western or Real Time PCR analysis. In pharmacologic studies, age, weight and sex matched C57BL/6 mice were rendered optic nerve crush and then injected i.p. with vehicle (DMSO), or ABT 702 (AKI, Adenosine Kinase Inhibitor) (1.5 mg/kg bwt, i.p.) every other day for the duration of the study ($n = 4-6$ /group).

2.2. Western blot analysis

Protein expression was measured by western blotting. In brief, washed cultured cells or retinal tissues were lysed in modified RIPA buffer (Upstate, Lake Placid, NY), containing 50 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 1% Nonidet P 40, 0.25% deoxycholate, supplemented with 40 mmol/L NaF, 2 mmol/L Na_3VO_4 , 0.5 mmol/L phenylmethylsulfonyl fluoride and 1:100 (v/v) of proteinase inhibitor cocktail (Sigma). Insoluble material was removed by centrifugation at $12,000 \times g$ at 4°C for 30 min. Protein was determined by Bradford method (Bio Rad, Hercules, CA). 50–100 μg was boiled in Laemmli sample buffer, separated by SDS PAGE on a gradient gel (4 to 20%) (Pierce, Rockford, IL), transferred to nitrocellulose membrane and incubated with specific antibodies. Antibodies for β -actin (Sigma), Iba1 (Wako, Japan), ADK, iNOS, nNOS, nitrotyrosine and ENT1 from Santa Cruz Biotechnology Inc., CA, and phospho ERK and ERK (Cell Signaling Technology, Beverly, MA) were detected with a horseradish peroxidase conjugated antibody and ECL chemiluminescence (Amersham BioSciences, Buckinghamshire, UK). Intensity of immunoreactivity was measured by densitometry.

2.3. Immunohistochemical analysis

Immunofluorescence analysis was performed using frozen retinal sections. Briefly, cryostat sections (10 μm) were fixed in 4% paraformaldehyde, blocked with 10% normal goat serum (NGS) and then incubated overnight at 4°C with primary antibodies: Rabbit anti nitrotyrosine, rabbit anti Iba 1 (Wako Pure Chemical, Wako, TX), or mouse anti

pERK1/2 antibody (Cell signaling technology, USA). Thereafter, sections were briefly washed with 1X PBS T (0.1%) or 0.3% Triton X 100 and incubated with appropriate secondary antibodies (Invitrogen). Slides were examined under the fluorescence microscope (Carl Zeiss). Specificity of the reaction was confirmed by omitting the primary antibody. Data (10 fields/retina, $n = 4-6$ in each group) were analyzed using fluorescence microscopy and Ultra View morphometric software or Image J software (NIH) to quantify the intensity of immunostaining.

2.4. Real time PCR (isolation of RNA, synthesis of cDNA)

Total RNA was isolated from mouse retina using SV Total RNA Isolation kit (Promega, Madison, WI) following manufacturer's instructions, and the quality of the RNA preparations was monitored by absorbance at 260 and 280 nm (Helios Gamma, Thermo Spectronic, Rochester, NY). The RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA) using iScript reagents from Bio Rad in a programable thermal cycler (PCR Sprint, Thermo Electron, Milford, MA). 50 ng of cDNA was amplified in each real time PCR using Bio Rad iCycler, ABgene reagents (Fisher scientific) and appropriate primers (Table 1). An average of glyceraldehyde 3 phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA was used as the internal control for normalization.

2.5. Analysis of dihydroethidium (DHE) fluorescence for the detection of superoxide

The detection of superoxide anion in the mouse eye sections was performed as described previously (Ahmad et al., 2013). In brief, mouse eyes were frozen in OCT and stored at -80°C until use. Enzymatically intact eye sections were thawed in room temperature, rehydrated with PBS, incubated with dihydroethidium (DHE; 10 μM /L in PBS) for 30 min at 37°C in a humidified chamber protected from light. After incubation, sections were washed with PBS. DHE is oxidized on reaction with superoxide to ethidium, which binds to DNA in the nucleus and emits red fluorescence. For the detection of ethidium, samples were examined with a fluorescence microscope (Axioskop 2 plus with AxioCam; Carl Zeiss, Germany; Excitation/Emission wavelengths: 518/605 nm). DHE fluorescence was quantified using Image J software (NIH).

2.6. Mouse microglia cell culture, drugs treatment

The mouse microglial cell line EOC 20 was obtained from the American Type Culture Collection (ATCC CRL 2469, Manassas, VA, USA). Cells were maintained at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, 0.5% penicillin/streptomycin, 4 mM L-glutamine, and 20% conditioned medium from bone marrow derived L-mac cells (ATCC CRL 2420) as a source of colony stimulating

Table 1

The primer sets used for the detection of mouse genes by quantitative real-time PCR analysis.

Gene	Primer sequence (5'-3')	Accession number
TNF- α	CCCTCACACTCAGATCATCTTCT GTCACGACGTGGGCTACAG	NM_013693.2
ENT1	CAAGTATTTTCAAAACCGCTGGAC GAAACGAGTTGAGGCAGGTGAAGAC	Am J Physiol Heart Circ Physiol 299:H847–H856, 2010
Iba-1	GTCCTTGAAGCGAATGCTGG CATTCTCAAGATGGCAGATC	NM_019467
iNOS	ACA TCG ACC CGT CCA CAG TAT CAG AGG GGT AGG CTT GTC TC	Primer Bank ID 6754872
IL-6	TAGTCCTTCTACCCCAATTTC TTGGTCTTAGCCACTCCTTC	NM_031168.1
GAPDH	CAT GGC CTC CAA GGA GTAAGAGAG GGA GAT GCT CAG TGT TGG	M32599
18S	AGT GCG GGT CAT AAG CTT GC GGG CCT CAC TAA ACC ATC CA	NR_003278

factor 1. EOC 20 cells were grown to 60% confluence at which point their media were removed and replaced with fresh medium. Microglial cells were seeded in a 6 well tissue culture plate. One day after seeding, the wells were washed with DMEM and incubated in the same media with various treatments. Cells were pretreated with ABT 702 (AKI 20 μ M, Tocris, Ellisville, MO), SB203580 (P38 inhibitor, 20 μ M) and U0126 (MEK inhibitor, 20 μ M) at the indicated concentrations reported previously or vehicle dimethylsulfoxide (DMSO) for 30 min at 37 °C before LPS (50 ng/ml for 24 h) treatment. At indicated time points, cells were harvested and homogenized for Western blot analysis and culture media were taken and analyzed for TNF α by ELISA. For immunocytochemistry, mouse microglia cells were grown in 4 well chambers and after the drug treatment and indicated time point cells were fixed in 4% paraformaldehyde for 20 min and then washed with PBS and the slides were stored at 4 °C for immunofluorescence study.

2.7. Enzyme linked immunosorbent assay (ELISA) for TNF α

TNF α levels in the supernatants of culture media were estimated with ELISA kits (R&D, Minneapolis, MN) as per the manufacturer's instructions. Standards and samples were added and bound by the immobilized antibody. After washing, an enzyme linked polyclonal antibody specific for the cytokine was added to the wells followed by a substrate solution yielding a colored product. The intensity of the color was measured at 450 nm. The sample levels were calculated from the standard curve and corrected for protein concentration.

2.8. Statistics

The results are expressed as mean $\pm$ SD. Data were analyzed by Graph Pad PRISM software. Differences among experimental groups

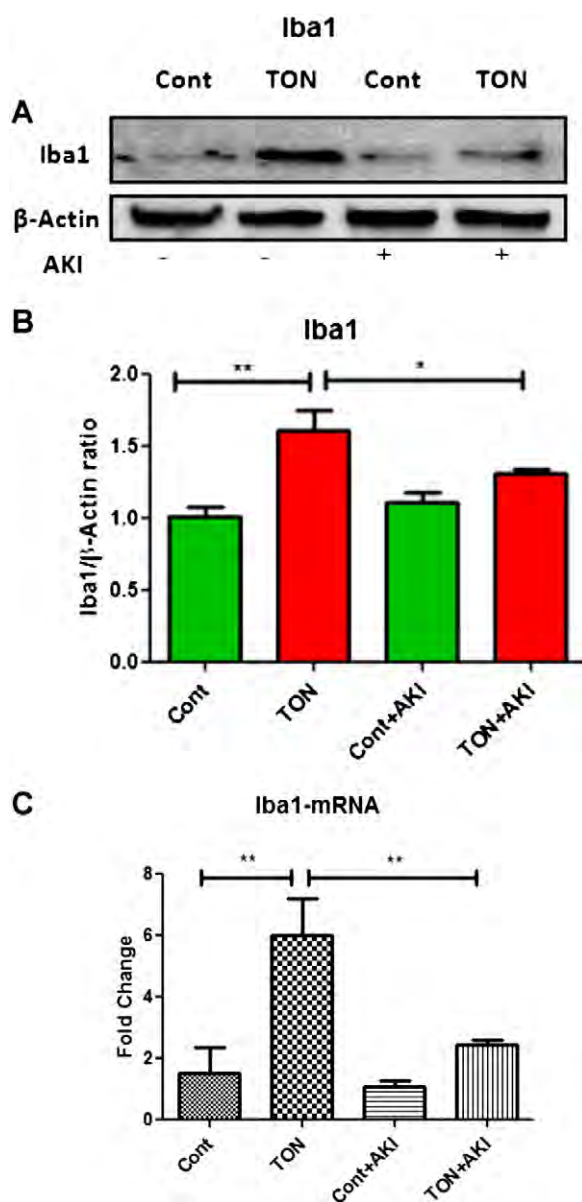


Fig. 1. Effect of adenosine kinase inhibitor (AKI, ABT-702) on retinal microglia activity (Iba-1 expression level) in TON. A) Immunoblotting analysis of microglial activation marker Iba-1 expression in TON vs. control in the retina, with and without AKI. B) Densitometry analysis of Iba-1 and actin ratio by Image J software, NIH. C) RT-PCR analysis for Iba-1 mRNA level in retinal tissue. Data shown are the mean $\pm$ SD (n = 4–6). *P < 0.05, **P < 0.01, and ***P < 0.001.

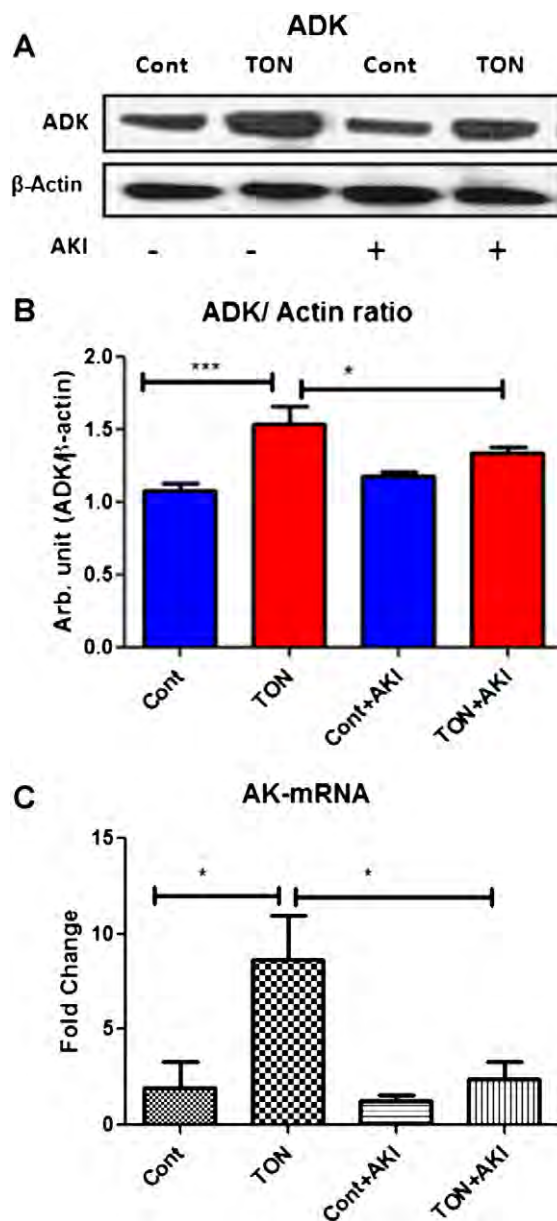


Fig. 2. Effect of adenosine kinase inhibitor (AKI, ABT-702) on ADK expression in the TON retinal tissue. A) Western blot analysis of ADK protein expression in retinal tissue in TON vs. control, with and without AKI treatment. B) Densitometry analysis was done for ADK and β-actin ratio by Image J software. C) Retinal ADK mRNA was determined by Real-Time PCR in TON vs. control, with and without AKI effect. Data shown are the mean $\pm$ SD (n = 4). *P < 0.05, **P < 0.01, and ***P < 0.001.

were evaluated by analysis of variance (one way ANOVA), and the significance of differences between groups was assessed by the posthoc test (Newman Keuls multiple comparison). Significance was defined as $P < 0.05$.

3. Results

3.1. TON induced microglial activation and its attenuation by adenosine kinase inhibitor

We have previously reported that TON activates microglia which leads to increased neurotoxicity and inflammation in retina and endogenous adenosine plays anti inflammatory role by activating its receptor A_2AAR which further activates cAMP and inhibits MAPKinase pathway

(Ahmad et al., 2013). Here we showed that inhibiting adenosine kinase significantly reduced the hyper activation of microglia. Western blot data showed that treatment with AKI (ABT 702) significantly reduced the Iba1 protein and mRNA expression in TON (Fig. 1A C) ($p < 0.05$ and $p < 0.01$). In our earlier study, we observed that when microglia encountered TON milieu, they became activated as indicated by increased Iba 1 expression and lead to the Retinal Ganglion Cell death (Ahmad et al., 2013).

3.2. Role of AKI in retinal Adenosine kinase expression level in TON

We determined the effect of optic nerve crush on the expression of adenosine kinase in the retina. Our group has reported recently that adenosine kinase upregulated in retinal tissue of diabetic retinopathy (Elsherbiny et al., 2013). Adenosine kinase (ADK) converts endogenous adenosine into AMP. During stress condition exogenous adenosine is required in more quantity to work with its receptor but it seems that adenosine kinase plays negative role in traumatic condition by converting adenosine into AMP. Here, compared with the contralateral eyes, the eyes with crushed optic nerves demonstrated a significant increase in the levels of ADK protein and mRNA expression. The treatment with AKI resulted in a marked reduction of TON associated ADK protein and mRNA up regulation (Fig. 2A C). These results demonstrated that under TON associated stress, ADK expression increased which means

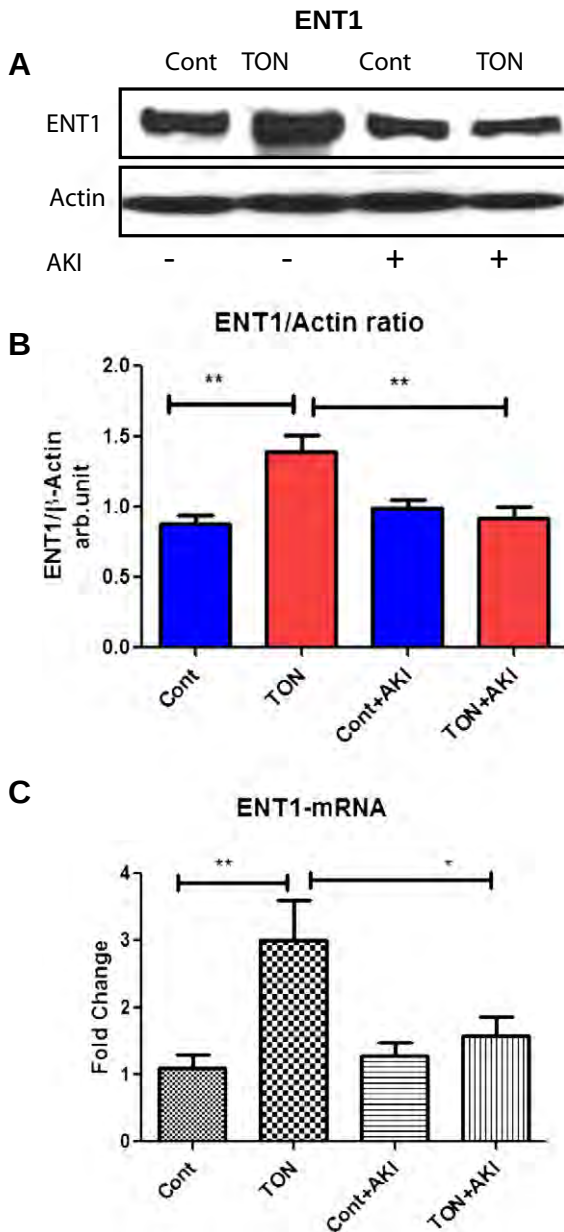


Fig. 3. Role of AKI treatment on the retinal ENT1 expression level in the mouse model of TON. A) Analyses of ENT1 protein expression in TON model with and without AKI treatment by western blot. B) Densitometry analysis was done for ENT1 and β -actin ratio by Image J software (NIH). C) Retinal ENT1 mRNA was determined by Real-Time PCR in TON vs. control, with and without AKI effect. Data shown are the mean $\pm$ SD ($n = 4$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

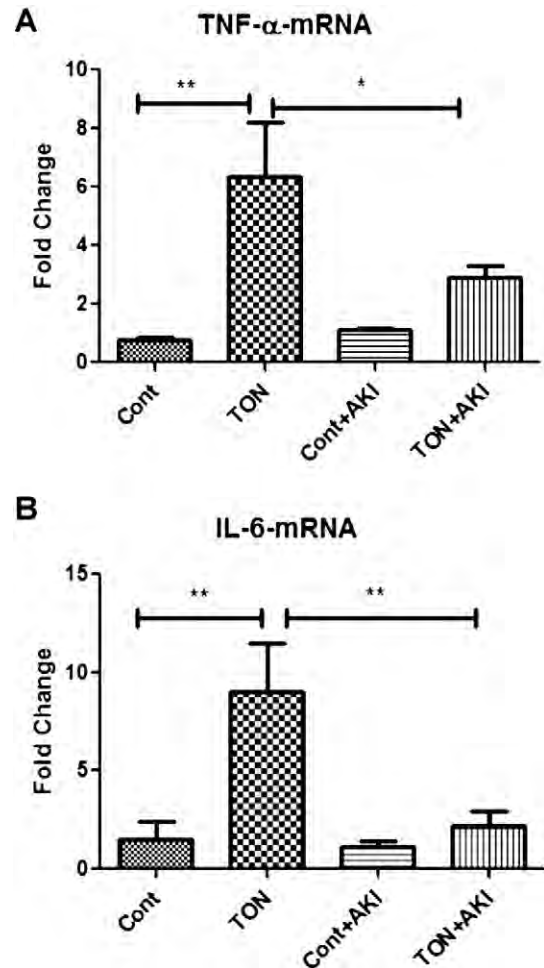


Fig. 4. Effect of AKI treatment on pro-inflammatory cytokines expression in TON retinal tissue. A) and B) Real-Time PCR analysis of TNF- α and IL-6 mRNA expression in the retina of TON, with and without ABT-702 treatment. Data shown are the mean $\pm$ SD ($n = 4$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

less adenosine availability outside but ADK inhibition may reverse this process.

3.3. Role of AKI in retinal Equilibrative nucleoside transporter 1 (ENT1) expression in TON

We next determined the effect of optic nerve crush on the levels of ENT1 expression in the retina. ENT1 regulates adenosine transportation from inside to outside and vice versa. Compared with the contralateral eyes, the eyes with crushed optic nerves demonstrated a significant increase in the levels of ENT1 protein and mRNA. ABT 702 treatment in the eyes with crushed optic nerves significantly reduced TON associated ENT1 protein and mRNA expression ($p < 0.01$ & $p < 0.05$) (Fig. 3A C) ($n = 4$ 6).

3.4. Role of inflammation and its attenuation by AKI in TON mice

As shown in Fig. 7A and B, RT PCR analysis of mRNA expression of TNF α and IL 6 in the retinas of TON were notably increased as compared with TON contralateral eye. These cytokines were over produced by the hyper activation of microglia during traumatic condition (Ahmad et al., 2013). AKI treatment significantly reduced mRNA expression of TNF α and IL 6 in the retinas of TON mice (Fig. 4A, B) ($p < 0.05$ and $p < 0.01$) ($n = 4$ 6).

3.5. Effect of AKI in retinal iNOS/nNOS expression

Furthermore, we examined the effect of AKI on retinal inducible and neuronal nitric oxide (iNOS/nNOS) expression level. iNOS is inducible only in pathological condition by inflammation or cytokines. After iNOS is induced, it is expected to produce large amount of nitric oxide (NO), which leads to RGS death during retinal traumatic condition. Similarly, overproduction of NO by nNOS has been reported in acute and chronic neurodegeneration. Here we found increased iNOS protein and mRNA expression in TON eye as compared with control. nNOS protein expression was also elevated in TON. Treatment with AKI significantly attenuated iNOS and nNOS protein and mRNA expression level when compared with TON ($p < 0.001$, $p < 0.05$ and $p < 0.01$) (Fig. 5A E).

3.6. Effect of AKI in retinal oxidative stress

Since we found that iNOS/nNOS expression are upregulated in TON, we determined the effect of optic nerve crush on the levels of oxidative stress in the retina by two methods (DHE and nitrotyrosine staining) that measure superoxide generation and Nitrogen Reactive Species (NRS). Compared with the contralateral eyes, the eyes with crushed optic nerves demonstrated a significant increase in the levels of superoxide determined by DHE and nitrotyrosine expression in eye section.

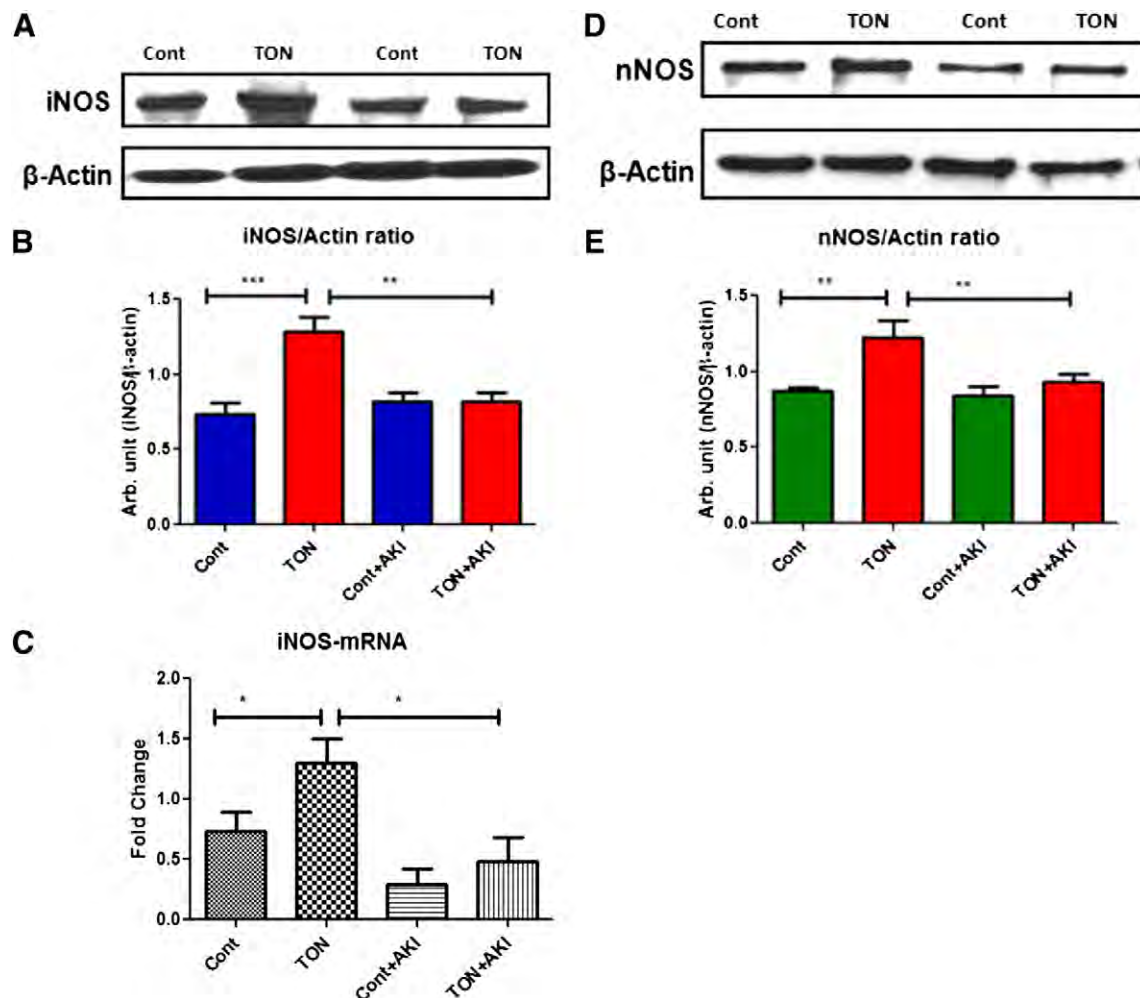


Fig. 5. Effect of AKI treatment on the retinal levels of iNOS/nNOS in the mouse model of TON. A) and B) Retinal iNOS protein expression was determined by western blotting in TON vs. control with and without AKI. Densitometry analysis of iNOS and β -actin band ratio was done by Image J software, NIH. C) RT-PCR analysis was done for iNOS mRNA expression in TON vs. TON + AKI group. D) and E) Retinal nNOS protein expression was determined by western blotting in TON vs. control with and without AKI. Densitometry analysis of nNOS and β -actin band ratio was done by Image J software, NIH. Data shown are the mean $\pm$ SD ($n = 4$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

The AKI treatment resulted in a marked reduction of TON associated superoxide production and nitrotyrosine expression compared with TON (Fig. 6A–D) ($p < 0.01$ & $p < 0.05$) ($n = 4–6$).

3.7. AKI treatment attenuates LPS induced TNF α release in mouse retinal microglia cells

We found that TON mice exhibit retinal inflammation. We next sought to explore a potential mechanism by which ADK signaling regulates inflammation in TON. To explore this, additional studies using mouse microglia cells treated with LPS were performed. Microglia with LPS or AGS treatment has been shown to simulate inflammation (Ibrahim et al., 2011; Elsherbiny et al., 2013). As shown in Fig. 7C, the treatment of retinal microglia cells with LPS triggered a prominent increase in TNF α release. AKI treatment significantly reversed this process as compared with LPS induced cells ($p < 0.01$). p P38 and p ERK1/2 inhibitor were used to compare AKI effect and there were no significant changes.

3.8. Adenosine kinase signaling mediates the anti inflammatory effect via interaction with LPS activated MAPK pathway in mouse microglia cells

Here we studied the role of AKI on MAPKinase pathway and we checked the p P38 and p ERK1/2 activation in the LPS induced microglia cells. Western blot analysis showed that LPS significantly activated MAPKinase signaling and inhibition of ADK by ABT 702 reduced its effect. These results demonstrated that adenosine kinase inhibition control the adenosine accumulation outside, and during traumatic

and stress condition adenosine activates one of its receptor A_{2A} ARs to block the MAPKinase activation which further inhibits activation of microglia. To prove this, we performed the immunofluorescence experiment that shows LPS treatment stimulated microglia (Iba 1) and p ERK1/2 activation. Fig. 7D shows the co localization of microglia marker Iba 1 (red) and p ERK1/2 (green) with nucleus staining DAPI. Treatment with AKI inhibited the MAPKinase and microglia activation as compared with LPS treated cell.

4. Discussion

Inflammation plays a key role in many CNS diseases, including neural injury, infections and other diseases (Zheng et al., 2012). In case of optic nerve injury, inflammatory responses are immediately activated followed by activation of glial cells along with release of inflammatory molecules. In TON, influx of activated microglia play key role in retinal damage by secreting pro inflammatory cytokines and cytotoxic molecules in response to oxidative stress. We previously demonstrated that extracellular adenosine has an anti inflammatory effect in the retinal microglial cells near RGC mediated by adenosine receptor A_{2A} (A_{2A}AR) signaling (Ibrahim et al., 2011). Therapeutically, adenosine and its agonists have protective effect in various animal models of inflammation, hypoxia and ischemia but are limited there by systemic side effects such as hypotension, bradycardia, and sedation (Williams, 1996). In addition, physiological or inflammatory conditions limits adenosine availability because of its rapid reuptake via nucleoside transporters (NTs) and subsequently metabolized intracellularly (Moser et al., 1989).

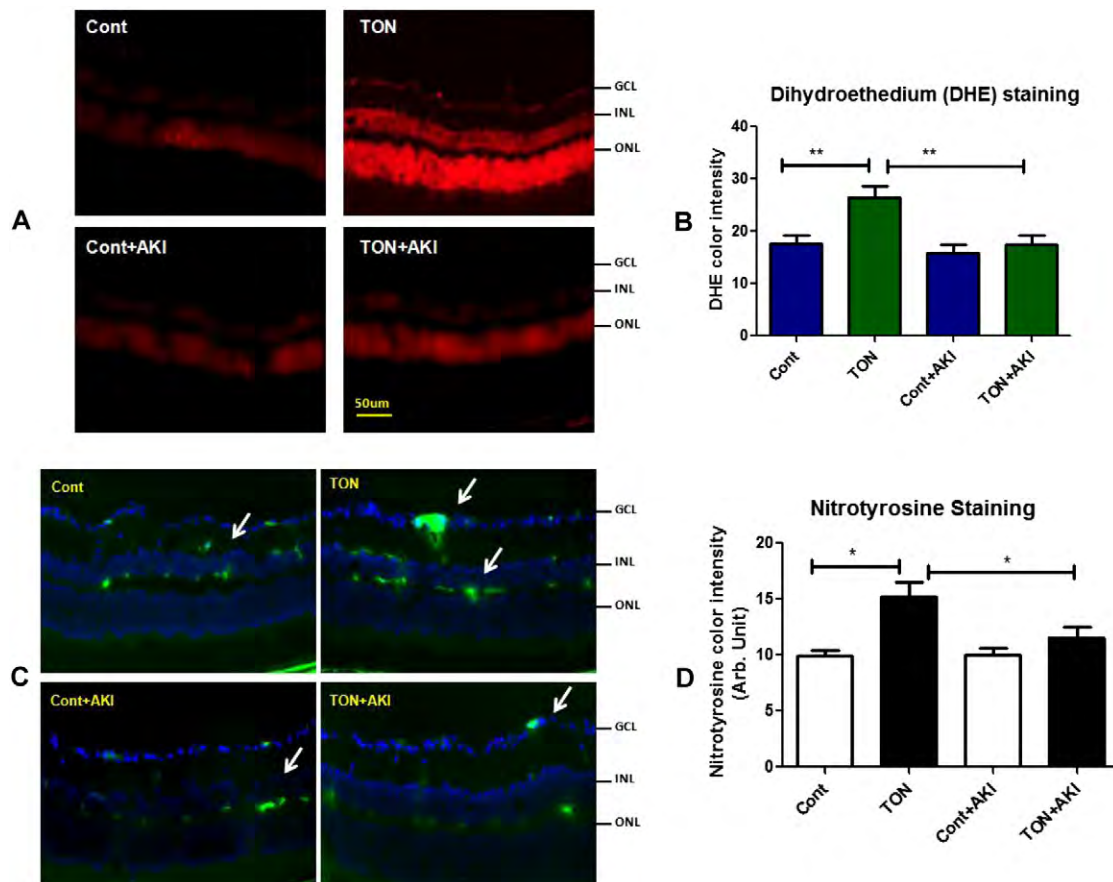


Fig. 6. Effect of adenosine kinase inhibition by AKI on superoxide production and nitrotyrosine expression. A) and B) Dihydroethidium (DHE) staining was performed in frozen retinal section in TON vs. TON + AKI group. Color intensity was calculated by Image J Software. C, D) Immunofluorescence analysis of nitrotyrosine was done in the frozen retinal sections, TON vs. TON + AKI. Sections were stained with nitrotyrosine antibody with Alexa flour 488 (green) and DAPI (blue). Color intensity was measured by Image J Software. Data shown are the mean $\pm$ SD ($n = 4–6$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Adenosine kinase (ADK) is thought to be the principal enzyme responsible for regulating the level of adenosine under physiological conditions. Thus, use of ADK inhibitors represents an effective alternative for greater therapeutic effects of extracellular adenosine at particular site and event along with lower hemodynamic toxicity. Pharmacologic inhibition of ADK has been reported to exert beneficial effects in different disease models (Ugarkar et al., 2000; Vljakovic et al., 2011; Annes et al., 2012). In our earlier study, we also

demonstrated that ABT 702, a selective ADK inhibitor had a protective role in diabetic retina due to its potential to amplify therapeutic effects at site of injury (Elsherbiny et al., 2013).

The activation of microglia plays an important role in inflammatory response in TON (Zheng et al., 2012). We previously reported that TON milieu caused microglia activation as indicated by increased Iba 1 expression (Ahmad et al., 2013). In the present study, ABT 702 treatment inhibited TON induced increase of retinal Iba 1 levels. Further,

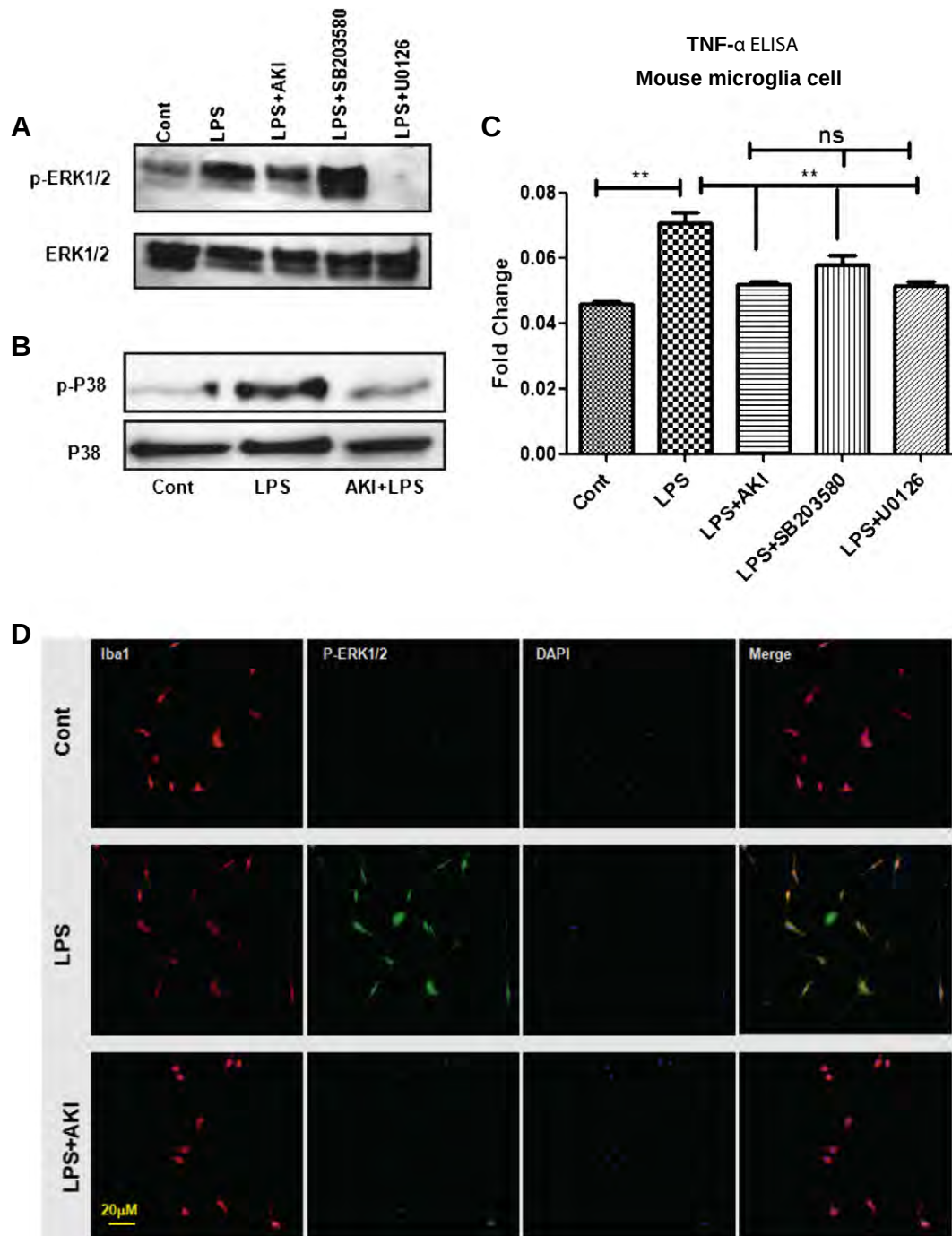


Fig. 7. Role of MAPKinase in the ADK-mediated anti-inflammation: LPS-induced TNF-α release in the mouse microglia cells and its attenuation with AKI. A) LPS induced activation of ERK in the mouse microglia cells. Phosphorylated (p) ERK and its total protein were determined by western blot analysis in control, LPS and LPS+AKI cell lysates. B) Phosphorylated P38 and its total protein were determined by western blot analysis in control, LPS and LPS+AKI cell lysates. C) TNF-α release was measured by ELISA in LPS induced mouse microglia cells in control, LPS and LPS+AKI cell lysates supernatants. D) Immunofluorescence analysis of Iba-1 and pERK1/2 expression level in microglia cells. LPS activates Iba-1 and pERK1/2 as compared with control, and AKI treatment inhibited their activation in LPS treatment. Iba-1 and pERK1/2 were stained with Alexa fluor 594 (red) and Alexa fluor 488 (green) respectively and DAPI (blue) for nucleus. U0126 (MEK inhibitor) and SB203580 (P38 inhibitor) were used to compare AKI. Data shown are the mean ± SD (n = 4). *P < 0.05, **P < 0.01, ***P < 0.001.

activated microglia released inflammatory molecules such as IL 6, and TNF α , which may be toxic to neurons and other glial cells (Smith et al., 2012). Here, we found that ABT 702 reduced retinal increase of mRNA levels of TNF α and IL 6 in TON. These findings suggest that ABT 702 exerted its protective effect by augmenting the anti-inflammatory mechanism of adenosine mediated by attenuation of microglia activation.

Furthermore, we found up-regulated protein and mRNA levels of adenosine kinase in mouse retina with TON. The up-regulation of ADK was previously demonstrated in diabetic retinopathy (Pang et al., 2010). Greater levels of ADK in activated macrophages and microglial cells abrogate the immune response. Earlier research showed that adenosine level dramatically increases in extracellular space in ischemic condition (Hagberg et al., 1987), and also in the rat model of transient ischemia the concentration of adenosine in the cerebrospinal fluid was increased four fold (Meno et al., 1991). Previous reports have shown that a beneficial role of elevated adenosine in ischemic condition, and the increased adenosine accumulation appears to be protective in brain cell injury (Phillis et al., 1991; Tatlisumak et al., 1998). However, adenosine kinase influences adenosine reuptake by converting into AMP. During ischemic or traumatic condition cells need more adenosine but on the other side adenosine kinase inhibits adenosine production. In current study ABT 702 significantly reduced the adenosine kinase protein and mRNA expression. Thus, the inhibition of adenosine kinase seems beneficial for the adenosine signaling. Our results are in agreement with others where adenosine kinase inhibition with selective ADK inhibitors showed increased adenosine level in brain cells and retinal inflammation (White, 1996; Tatlisumak et al., 1998; Elsherbiny et al., 2013).

In addition, increased ENT1 was previously demonstrated in human aortic smooth muscle cells by hyperglycemia (Leung et al., 2005). Similar to this study, we found increased ENT1 protein and mRNA levels in mouse retina with TON (Fig. 3). AKI treatment attenuated its expression level in retinal tissue. ENT1 transports adenosine from intercellular to extracellular and vice versa. Previously it was reported that ENT1 expression was increased in high glucose (Liou, 2010), which may affect the availability of adenosine for its receptor to work as anti-inflammatory in diabetes. Thus, we may say that in TON ENT1 up-regulation is pathologically implicated and causes low concentration of adenosine by reuptake intercellular, but its low expression may be beneficial in TON. This result is supported by our recent work where we have shown that ABT 702 markedly decreased ENT1 expression in diabetic retinopathy (Elsherbiny et al., 2013).

Further, we investigated the effect of ABT 702 treatment on TON induced oxidative stress. We previously demonstrated increased oxidative stress in mice retina with TON (Ahmad et al., 2013). Here, we studied the effect of ABT 702 treatment on superoxide anion, iNOS/nNOS and nitrotyrosine levels in mice retina with TON. Nitration of tyrosine residues is evident in several retinal inflammatory and neurodegenerative diseases (Gouder et al., 2004), which occurs due to reaction of tyrosine with reactive nitrogen species such as peroxynitrite (Pacher et al., 2007). These reactive nitrogen species are formed by reaction of superoxide anion and Nitric oxide (NO), thus, serving as a likely indicator simultaneous generation of NO and superoxide (El Remessy et al., 2003). However, it is reported that retinal ganglion cell loss during retinal hypoxia regulated by NO (Kaur et al., 2006). Under pathological conditions, NO is synthesized by the inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS). Studies reported the expression of nNOS and iNOS in glial cells, infiltrating leukocytes and in RGCs in hypoxic retina (Kashiwagi et al., 2003; Kaur et al., 2006). The produced NO from nNOS and iNOS contributes to neurotoxicity resulting in cell death and axonal damage (Kaur et al., 2008). Report suggests that NO triggered several pathways including N-methyl-D-aspartate (NMDA) mediated intracellular Ca^{2+} influx and CREB mediated apoptotic proteins which results to neuronal death (Mishra et al., 2002). Increased NO production is shown to mediate MAPK activation during hypoxia in cerebral cortical nuclei of

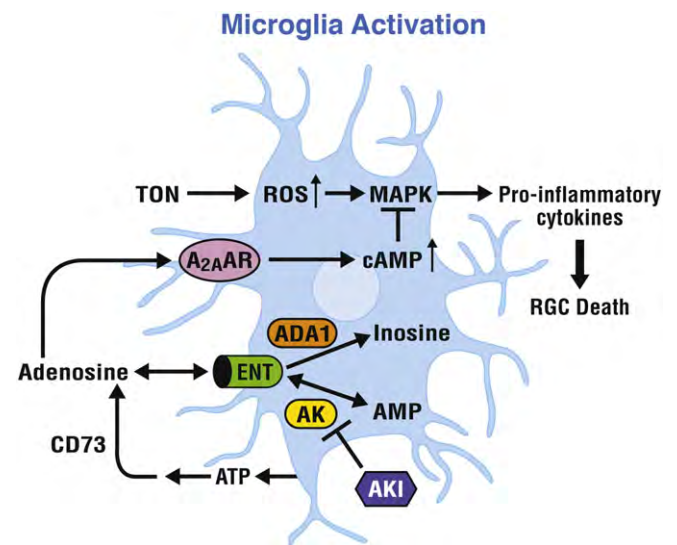


Fig. 8. Proposed possible molecular mechanism of anti-inflammation in Traumatic Optic Neuropathy.

newborn piglets (Mishra et al., 2004). In the current study, we found increased levels of iNOS/nNOS expression in TON retinal tissue, and increased superoxide anion and nitrotyrosine levels in mouse retinal sections with TON. Treatment with ABT702 attenuated TON induced oxidative and nitritative stress in mouse retina with TON. These findings suggested that ABT 702 attenuated TON induced activated microglia production of NO and reactive oxygen species by augmenting adenosine signaling.

We investigated the role of ABT 702 treatment in MAPK activation in mouse microglia in vitro. In Fig. 7D immunocytochemistry results revealed that Iba1 and p ERK1/2 co-localized in LPS induced mouse microglia. In our previous study we have reported that LPS activates ERK1/2 phosphorylation (Ahmad et al., 2013). Using mouse microglia cells we have shown that LPS treatment induces pERK1/2 and p P38 MAPK activation, which leads to increased TNF α release. Activation of MAPK has been demonstrated as a major signaling cascade for TNF α production in microglia (Ajizian et al., 1999). In this study, data shows that AKI treatment reduced the phosphorylation of ERK and P38 in microglia cells. This result was an agreement with our previous finding where A_{2A}AR agonist attenuated increased TNF α release in activated microglial cells through MAPK pathway (Ahmad et al., 2013).

In conclusion we may demonstrate that inhibition of adenosine kinase attenuates TON induced inflammation and neurotoxicity by stimulating adenosine signaling and inhibiting MAPK pathway in activated retinal microglia cells (Fig. 8).

Disclosure

This report is according to journal guidelines and ethical issues. All authors have no conflicts of interest.

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