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

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A R T I C L E   I N F O

Article history:
Received 25 January 2013
Accepted 28 May 2013

Keywords:
Diabetic retinopathy
Inflammation
Adenosine kinase

A B S T R A C T

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 A2A receptors (A2AAR) 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 A2AAR 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, A2AAR, 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 A2AAR 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 A2A adenosine receptor (A2AAR), a Gs-coupled

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http://dx.doi.org/10.1016/j.lfs.2013.05.024
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) (Löfler et al., 2007), decreases extracellular adenosine levels, and terminates the protective effect of A2AAR. 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 (Boisson, 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)pyrrolo[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 cyclooxgenases-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/6 (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 Celgro 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 μg/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 Na₂VO₄, 0.5 mM phenylmethylsulfonyl fluoride and 1:100 (v/v) of proteinase inhibitor cocktail (Sigma). Insoluble material was removed by centrifugation at 12,000 ×g at 4 °C for 30 min. Protein was determined by DC Protein
Immunolocalization studies

Immunofluorescence analysis was performed using fixed eye sections. Briefly, cryostat sections (7 μm) were fixed in 4% paraformaldehyde, blocked with Dako protein block serum-free and incubated with specific antibodies. Antibodies for β-actin, ICAM-1, ENT1, A2AAR 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.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>No</th>
<th>Body weight (g)</th>
<th>Blood glucose (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic</td>
<td>7</td>
<td>30.8 ± 0.59</td>
<td>198.6 ± 11.61</td>
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<tr>
<td>Non-diabetic + ABT 7002</td>
<td>7</td>
<td>30.0 ± 0.67</td>
<td>198.6 ± 7.93</td>
</tr>
<tr>
<td>Diabetic</td>
<td>7</td>
<td>24.1 ± 0.61</td>
<td>399.6 ± 11.22</td>
</tr>
<tr>
<td>Diabetic + ABT 702</td>
<td>7</td>
<td>23.4 ± 0.57</td>
<td>373.5 ± 6.82</td>
</tr>
</tbody>
</table>

Mean ± SD. ** P < 0.001 vs non-diabetic group. *** P < 0.0001 vs non-diabetic group.

Assay (Bio-Rad, Hercules, CA) and 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, ICAM-1, ENT1, A2AAR 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.

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 μM) (Sigma-Aldrich, Oakville, ON, Canada) was applied to 7 μm 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).

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′–3′)</th>
<th>Accession number</th>
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<tr>
<td>TNF-α</td>
<td>CCCTCACACTCGATCACTCTCT</td>
<td>NM_013693.2</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>CGCTCTCCCTTGAGAAGCTG</td>
<td>NM_010493</td>
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<tr>
<td>Iba-1</td>
<td>GTCTTGAAGCCATTTCTGGA</td>
<td>NM_019467</td>
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<tr>
<td>GAPDH</td>
<td>CAT GCC CTC CAA GGA GTAAGA</td>
<td>M32599</td>
</tr>
<tr>
<td>IRS</td>
<td>ACT GCC CTC CAT CCT TAGCT TCG</td>
<td>NR_003278</td>
</tr>
</tbody>
</table>

Mean ± SD. ** P < 0.001 vs non-diabetic group. *** P < 0.0001 vs non-diabetic group.

The results are expressed as mean ± 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 A2AR up-regulation in diabetic mice**

A2AR is the most likely candidate for mediating the anti-inflammatory effect of adenosine (Milne and Palmer, 2011). Diabetes
or inflammation is associated with up-regulation of A2AAR (Pang et al., 2010). The increased A2AAR expression may possibly represent an endogenous mechanism to combat the inflammation associated with diabetes induction. Consistent with this, diabetes induced up-regulation of A2AAR in the retina as compared with normal (Fig. 2). Treatment of ABT 702 reduced A2AAR expression in the diabetic mice as compared with vehicle-treated diabetic mice (Fig. 2).

Inhibition of adenosine kinase blocks A2AAR 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. Hyperglycemia 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. 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 ± 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).

Fig. 2. Inhibition of adenosine kinase blocks A2AAR up-regulation in diabetic mice. Representative Western blots and quantitative analysis of retinal A2AAR expression showing the effect of AK inhibition on A2AAR expression in the diabetic mouse retina (n = 4).

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.](image-url)
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 A2AAR**

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 μM potently inhibited AGA-induced TNF-α release. When the cells were pretreated with the A1AR antagonist 1,3-dipropyl-8-cyclopentylxanthine (CPX; 100 nM), the A2BAR antagonist 8-[4-[(4-cyanophenyl)arbamoylmethyl]oxy]phenyl]-1,3-di(n-propyl)xanthine hydrate (MRS1754; 1 μM), or the A3AR antagonist 3-propyl-6-ethyl-5[(ethylthio)carbonyl]-2-phenyl-3-pyridine-carboxylate (MRS1523; 10 μM), 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-a][1,3,5]triazin-5-ylamino]ethyl} phenol (ZM 241385) at concentrations (100 and 500 nM) capable of blocking A2AARs. These results suggest that ABT 702 inhibited AGA-induced TNF-α release from retinal microglia via the A2AAR.

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**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 μm; B) determined by RT-PCR analysis; GAPDH and 18S were used as reporter genes. The results represent the means ± SE of fold changes calculated using expression level, normalized to the level of the normal non-diabetic mice (n = 4–6).
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 A2AAR (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.
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, whereas 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. 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 ± SE of TUNEL-positive cells per retinal cross section (n = 4).](image-url)
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. 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 μg/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 ± SD of at least four different experiments.

Fig. 9. Inhibition of adenosine kinase blocks TNF-α release via A2AR. Cells were treated with vehicle or ABT 702 (20 μM) 30 min before AGA treatment in the presence of subtype-selective AR antagonists for A1AR (CPX, 100 nM), A3AR (ZM241385, 100 and 500 nM), A2AR (MRS 1754, 1 μM) and A3AR (MRS 1523, 10 μM). *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 extracellular 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 AK 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[3,2-α][1,3,5]triazin-5-ylamino]ethyl] phenol (ZM 241385), a selective A2AAR antagonist. These results suggest that ABT 702 inhibits AGA-induced TNF-α release in retinal microglia through A2AAR. A2AAR 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 A2AAR (Pang et al., 2010). High levels of A2AAR 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 a MAPK/ERK-dependent signaling pathway (Leung et al., 2005). ABT 702 inhibited the expression of both A2AAR 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 superoxides and nitrosyrlines 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.

Acknowledgments

This work was supported by Egyptian Cultural and Educational Bureau (NME and GIL), Department of Defense DM102155 (GIL) and Vision Discovery Institute (GIL).

References


