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Potential roles of adenosine deaminase-2 in diabetic retinopathy

Nehal M. Elsherbiny [a,d,1], Mohammad Naime [a,1], Saif Ahmad [a], Ahmed M. Elsherbini [a], Shuaib Mohammad [a], Sadanand Fulzele [b], Azza B. El-Remessy [c], Mohammed M. Al-Gayyar [d], Laila A. Eissa [d], Mamdouh M. El-Shishtawy [d], Guichun Han [e], Richard White [f], Toque Flores Haroldo [g], Gregory I. Liou [a,*]

[a] Department of Ophthalmology, Georgia Regents University, Augusta, GA, United States
[b] Department of Orthopedics, Georgia Regents University, Augusta, GA, United States
[c] Program in Clinical and Experimental Therapeutics, University of Georgia, Augusta, GA, United States
[d] Department of Clinical Biochemistry, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt
[e] Department of Physiology and Pharmacology, Texas A&M University, United States
[f] Philadelphia College of Osteopathic Medicine, United States
[g] Department of Pharmacology and Toxicology, Georgia Regents University, Augusta, GA, United States

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ABSTRACT

The early activation of microglia that induces retinal inflammation in DR may serve as a target for therapeutic intervention of DR. Our demonstration that retinal inflammation is attenuated via adenosine receptor $A_{2A}AR$ supports the hypothesis that a mechanism to maintain extracellular concentrations of adenosine important in normal physiology is impaired in DR. Extracellular concentrations of adenosine are regulated by the interplay of equiliberative nucleoside transporter (ENT)s with enzymes of adenosine metabolism including adenosine deaminase-1 (ADA1), adenosine kinase (AK) and CD73. In the vertebrates but not rodents, a macrophage-associated ADA2 is identified. The role of ADA2 is, therefore, understudied as the sequencing probes or antibodies to mouse ADA2 are not available. We identified increased ADA2 expression and activity in human and porcine retinas with diabetes, and in Amadori glycated albumin (AGA)- or hyperglycemia-treated porcine and human microglia. In rodent as well as porcine cells, modulation of TNF-α release is mediated by $A_{2A}AR$. Quantitative analysis of normal and diabetic porcine retinas reveals that while the expression levels of ADA2, A2AAR, ENT1, TNF-α and MMP9 are increased, the levels of AK are reduced during inflammation as an endogenous protective mechanism. To determine the role of ADA2, we found that AGA induces ADA2 expression, ADA2 activity and TNF-α release, and that TNF-α release is blocked by ADA2-neutralizing antibody or ADA2 siRNA, but not by scrambled siRNA. These results suggest that retinal inflammation in DR is mediated by ADA2, and that the anti-inflammatory activity of $A_{2A}AR$ signaling is impaired in diabetes due to increased ADA2 activity.

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1. Introduction

DR is a leading cause of blindness among working-age adults [1]. Treatment options for DR remain limited and with adverse effects [2]. Discovery of new molecular entities with adequate clinical activity for DR remains one of the key research priorities in ophthalmology. Activation of retinal microglia in early diabetes is critical in causing the major complications in DR. Adenosine is elevated at sites of tissue damage resulting from inflammation [3] or hypoxia [4,5]. Adenosine can be formed intracellularly [6,7] and diffuse into the extracellular space via ENTs, or extracellularly from released ATP by ecto-nucleotidases, CD39 and CD73 [8]. Increased adenosine reuptake by ENT allows for adenosine conversion to AMP by adenosine kinase (AK), a predominant pathway for adenosine removal, which leads to inflammation [9]. The pathway for adenosine removal by ADA1 plays only a minor role in regulating adenosinergic function [10]; the major role of ADA1 is to maintain a low level of 2′deoxyadenosine for proper function in immune cells [11]. In addition to ADA1, ADA2 was found in mammals, lower vertebrates and insects as an extracellular enzyme. During inflammation, a disproportionate increase in ADA2 has been found in macrophage-rich tissues including blood [12,13]. The search for a rodent ADA2 gene (also known as cat eye syndrome critical region candidate 1, *CECR1*) by analysis at the critical region (at or near the

Abbreviations: CD73, ecto-5′-nucleotidase; ADA2, adenosine deaminase-2; AK, adenosine kinase; AGA, amadori glycated albumin; ENT1, equilibrative nucleoside transporter 1.

* Corresponding author.
E-mail address: giliou@gru.edu (G.I. Liou).

[1] These authors contributed equally to this work.

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human chromosome 22 pericentromere) in humans and the region of conserved synteny in mice has not been successful [14–16]. Extracellular adenosine can activate adenosine receptors, which are classified as different subtypes, based on their mechanism of signal transduction [17]. The A_2 receptors stimulate adenylate cyclase through Gs coupling [18]. The increased adenosine at inflamed sites exhibits anti-inflammatory effects to protect against cellular damage through A2AAR [19,20]. We have shown that diabetic A2AAR −/− mice had significantly more cell death, TNF-a release, and ICAM-1 expression compared with diabetic wild-type mice [21]. We have also shown that activation of A2AAR in the stressed retinal microglial cells was the most efficient in mediating TNF-α inhibition [22], and that treatment with the A2AAR agonist resulted in marked decreases in diabetes-induced retinal cell death and TNF-a release [21]. Our recent data demonstrating A2AAR agonist protects against diabetes-induced retinal inflammation suggests that abnormality in adenosine metabolism may contribute to retinal complications in diabetes. By determining the causal relationship between TNF-a release and the expression and activity of ADA2 in porcine models of DR, we present experimental results that suggest that impaired adenosine metabolism in diabetes is at least due to increased expression and activity of ADA2.

2. Materials and methods

2.1. Postmortem eye specimens

Human eyes, 9 non-diabetic and 8 diabetic, obtained from The Georgia Eye Bank (Atlanta, GA) followed the following selection criteria: >50 years old, either insulin requiring diabetes or no diabetes, and no life-support measures. The eyes were enucleated an average of 6.71 ± 0.84 h after death. Aliquots of the same eyes were used in our previous work [23].

2.2. Induction of diabetes

All animal procedures have been approved by the GRU Institutional Animal Care and Use Committee. The procedure of diabetes induction was slightly modified from a previously described procedure [24]. Twelve female Yorkshire pigs weighing 30 kg at arrival were used in the experiment. To six pigs, STZ (50 mg/kg in 0.1 mol/l Na-citrate buffer, pH 4.5) was administered in the ear vein over 1 min each day for 3 days. To the other six pigs, solvent alone was administered. Serum glucose concentrations were measured on a daily basis just prior to injection of STZ, every other day for the next two weeks, and every other week for the entire 12 weeks. The pigs were treated with a subcutaneous injection of short-acting insulin (Normulin, Novo Nordisk) to keep the blood glucose concentration between 350 and 550 mg/dL [25] (Table 1).

Table 1
Diabetic pig blood glucose levels (avg; mg/dl).

Blood glucose mg/dl	
D1	485
D2	412
D3	395
D4	277
D5	358
D6	236
N1	113
N2	75
N3	98
N4	124
N5	115
N6	73

2.3. Western blot analysis

Dissected individual pig retinas were homogenized in RIPA buffer (Upstate, Lake Placid, NY), supplemented with 40 mM NaF, 2 mM Na3VO4, 0.5 mM phenylmethylsulfonyl fluoride and 1:100 (v/v) of proteinase inhibitor cocktail (Sigma). Protein samples of 100 μg were loaded on a gradient gel (4–20%) (Pierce, Rockford, IL), transferred to nitrocellulose membrane and incubated with specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), which were detected with a horseradish peroxidase-conjugated antibody and enhanced chemiluminescence (ECL) (Amersham BioSciences, Buckinghamshire, UK). Intensity of immunoreactivity was measured by densitometry.

2.4. Quantitative real-time PCR

Total RNA was isolated from porcine retina using a Promega kit (Promega). Subsequently, cDNAs were generated from 1 μg of total RNA, using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and subjected to a 40-cycle PCR amplification. The ready-made primer and probe sets were ordered from Applied Biosystems Table 2. Three replicates were run for each gene for each sample in a 96-well plate. 18S RNA was used as the endogenous reference gene.

2.5. RNA interference

Microglial cells were transfected with porcine or control small interfering (si)RNAs Ambion) using HiPerFect (Qiagen) as described by our group [26] per manufacturer's instructions.

2.6. ADA2. activity assay

The ADA2 assay is based on the enzymatic deamination of adenosine at pH6 to inosine, which is converted to hypoxanthine by purine nucleoside phosphorylase (PNP). Hypoxanthine is then converted to uric acid and hydrogen peroxide (H2O2) by xanthine oxidase (XOD). H2O2 is further reacted with N-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline (EHSPT) and 4-aminoantipyrine (4-AA) in the presence of peroxidase (POD) to generatequinone dye, which is monitored in a kinetic manner. ADA1 activity is inhibited by ADA1-specific inhibitor EHNA (Diazyme Laboratories, Poway, CA).

Table 2
Primers used for qRT-PCR.

Gene	Primer sequence (5′-3′)	Accessionnumber
TNF-α	AGGAAGAGTTTCCAGCTGGCCC CAACGTGGGCGACGGGCTTA	NM_21402
ADA2	TCGGCACGAGCTCCGAGGAT TCTGGACGTGGCCGAGTGGA	AF384216
MMP-9	GACAGGCAGCTGGCAGAGGAAT GCCGGTTCCAGGGACTGCTT	NM_001038004
A2AAR	CACGCAGAGCTCCATCTTCA ACCAAGCCATTGTACCGGAG	XM_003483462.1
ENT1	CGGGAATTCGATTTCAGTGCCA GCAGGAAGGAGTTGAGGCAG	AJ606303
Cd73	AAGGCTCCACCCTGAAGAAGTA CGTCACGTGAATTCCGCC	XM_001927095.1
AK	CTGTGCATTACTGTACCCTCT CTTCCCGAGAACTCCCGTAT	XM_003359242
18S	TGCATGCTTGCAGGGCGGT GTCCTCGCTGCGGGTGTGGT	AY265350

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