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# $A_{2A}$ Adenosine Receptor ($A_{2A}$AR) as a Therapeutic Target in Diabetic Retinopathy

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**In diabetic retinopathy (DR), abnormalities in vascular and neuronal function are closely related to the local production of inflammatory mediators whose potential source is microglia. $A_{2A}$ adenosine receptor ($A_{2A}$AR) has been shown to possess anti-inflammatory properties that have not been studied in DR. Here, we evaluate the role of $A_{2A}$AR and its underlying signaling in retinal complications associated with diabetes. Initial studies in wild-type mice revealed that the treatment with the $A_{2A}$AR agonist resulted in marked decreases in hyperglycemia-induced retinal cell death and tumor necrosis factor (TNF)-$\alpha$ release. To further assess the role of $A_{2A}$AR in DR, we studied the effects of $A_{2A}$AR ablation on diabetes-induced retinal abnormalities. Diabetic $A_{2A}AR^{-/-}$ mice had significantly more terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive cells, TNF-$\alpha$ release, and intercellular adhesion molecule-1 expression compared with diabetic wild-type mice. To explore a potential mechanism by which $A_{2A}$AR signaling regulates inflammation in DR, we performed additional studies using microglial cells treated with Amadori-glycated albumin, a risk factor in diabetic disorders. The results showed that activation of $A_{2A}$AR attenuated Amadori-glycated albumin-induced TNF-$\alpha$ release in a cAMP/exchange protein directly activated by cAMP-dependent mechanism and significantly repressed the inflammatory cascade, C-Raf/extracellular signal-regulated kinase (ERK), in activated microglia. Collectively, this work provides pharmacological and genetic evidence for $A_{2A}$AR signaling as a control point of cell death in DR and suggests that the retinal protective effect of $A_{2A}$AR is mediated by abrogating the inflammatory response that occurs in microglia via interaction with C-Raf/ERK pathway.** ***(Am J Pathol 2011, 178:2136–2145; DOI: 10.1016/j.ajpath.2011.01.018)***

Diabetic retinopathy (DR) is the leading cause of blindness in the Western world and the most common complication of diabetes.[1,2] DR has been categorized as a vascular-neuroinflammatory disease. Early features of DR include signs of retinal inflammatory reactions, breakdown of the blood-retinal barrier function and loss of retinal neurons.[3–5] Under these conditions, normally quiescent microglial cells become activated.[6,7] Activated microglia release glutamate, reactive oxygen species, and proinflammatory mediators, such as tumor necrosis factor (TNF)-$\alpha$.[8–10] The retinal expression of TNF-$\alpha$ has been reported to be associated with neuronal and endothelial cell death, hallmark features of the disease,[5,11] and inhibition of TNF-$\alpha$ has demonstrated beneficial effects in the prevention of early DR.[12] Thus, research on retinal microglia activation may provide insights into the pathogenesis of DR.[13]

Adenosine has been proposed to modulate a variety of physiological responses.[14] Under stress conditions, the local levels of extracellular adenosine are increased due to the increased need for energy supplied by ATP[15] and the increased degradation of released ATP.[16] The increased adenosine at inflamed sites can protect against cellular damage by activating $A_{2A}$ adenosine receptor ($A_{2A}$AR), a Gs-coupled receptor.[17] Treatment with an $A_{2A}$AR agonist blocked the inflammation and functional changes associated with diabetic nephropathy in wild-type diabetic mice, but not in the diabetic $A_{2A}AR^{-/-}$ mice.[18]

Recent efforts to understand how neurotoxic inflammatory cytokines are produced have shown that Ras/Raf/

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MEK/extracellular signal-regulated kinase (ERK) cascade is one of the attractive targets for intervention in human inflammatory-associated diseases, such as diabetes. However, this cascade 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 Ras/Raf/MEK/ERK kinase signaling to regulate cell functions.[19]

Previously we demonstrated the anti-inflammatory effect of $A_{2A}$AR in acute retinal inflammation.[20] Currently, we seek to determine the contribution of $A_{2A}$AR in retinal protection against diabetes-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 diabetes-induced retinal cell death by enhancing the anti-inflammatory signaling, including the interaction with C-Raf/ERK cascade. These findings suggest that $A_{2A}$AR agonists may be promising innovative agents in the treatment of diabetic retinopathy.

## Materials and Methods

### 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 Medical College of Georgia guidelines. The 8-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. 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. In pharmacological studies, age-, weight- and sex-matched C57BL/6 mice were rendered diabetic and then injected i.p. with vehicle dimethyl sulfoxide, or CGS21680 (25 $\mu$g/kg) every other day for the duration of the study ($n$ = 4/group to 6/group). The dose of CGS21680 was chosen according to what was previously reported.[21] Four weeks after the establishment of diabetes, pooled retina and vitreous samples were used for TNF-$\alpha$ analysis by enzyme-linked immunosorbent assay (ELISA) and frozen eye sections were prepared to examine retinal cell death by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) or microglial activation and intercellular adhesion molecule 1 (ICAM-1) expression by immunofluorescence.

### ELISA for TNF-$\alpha$

TNF-$\alpha$ levels in pooled retina and vitreous samples or 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.

Retinal and vitreous lysates from mouse eyes were prepared for TNF-$\alpha$ ELISA according to our previous method with minor modifications.[20] Briefly, pooled retina and vitreous from each eye were placed in 100 $\mu$L of lysis buffer [20 mmol/L imidazole HCl, 100 mmol/L KCl, 1 mmol/L MgCl2, 1% Triton X-100, 10 mmol/L NaF, 1 mmol/L Na3VO4, 1 mmol/L EGTA, and 1 mmol/L EDTA (pH 6.8)] supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) followed by homogenization with Ottawa sand using a Mini-Bead Beater (BioSpec Products, Inc., Bartlesville, OK). The lysate was cleared of debris by centrifugation at 10,000 $\times$ $g$ for 15 minutes (4°C), and 50 $\mu$L of the supernatant was used for ELISA directly without further dilution. The sample TNF-$\alpha$ levels were calculated from the standard curve and corrected for protein concentration.

### Immunofluorescence

Immunofluorescence analysis was performed using frozen retinal sections. Briefly, cryostat sections (15 $\mu$m) were fixed in 4% paraformaldehyde, blocked with 10% normal goat serum 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). Thereafter, sections were briefly washed with PBS and incubated with appropriate secondary antibodies. Slides were examined by confocal microscopy (LSM 510, Carl Zeiss Inc.). Specificity of the reaction was confirmed by omitting the primary antibody. Data (10 fields/retina; $n$ = 4 to 6 in each group) were analyzed using fluorescence microscopy and Ultra-View morphometric software to quantify the intensity of immunostaining.

### TUNEL

TUNEL was performed in frozen sections using the TACS-2 TdT Fluorescein *in situ* Apoptosis Detection Kit (Trevigen, Gaithersburg, MD) counterstained 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, the slides were incubated in a mixture of TdT, Mn + 2, and TdT dNTP for 1 hour at 37°C. The reaction was stopped with TdT Stop Buffer (Trevigen) for 5 minutes. After washing with deionized water, the slides were incubated with streptavidin-Fluorescein isothiocyanate (diluted 1:200) solution for 20 minutes at room temperature. Slides were counterstained, 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 the nuclei clearly labeled yellow were scored. After TUNEL assay, immunofluorescence was performed with the previously described method using the antibody against neuron-specific nuclear protein (NeuN; Chemicon, Temecula, CA).

### *Primary Retinal Microglia Culture*

Microglial cells were isolated from retinas of newborn Sprague Dawley rats according to a previous procedure[22] with minor modifications. Briefly, retinas were dissected from newborn Sprague Dawley 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 to 5 minutes and mixed with Dulbecco's modified Eagle's medium or Dulbecco's minimal essential medium/F12 (1:1) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Atlanta Biologicals, Atlanta, 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 $\mu$m), collected by centrifugation, re-suspended in the culture medium, and plated onto T150 cell culture flasks (Corning, Corning, NY) at a density of $2 \times 105$ cells/cm$^2$. All cultures were maintained in a humidified $CO_2$ incubator at 37°C and 5% $CO_2$, and were fed on the third day, and then once every 4 days. After 2 weeks, microglial cells were harvested by shaking the flasks at 100 rpm for 1 hour. The cell suspension was centrifuged and detached cells were replated in Dulbecco's modified Eagle's medium or Dulbecco's minimal essential medium/F12 (1:1) + 10% fetal bovine serum 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 for Iba1, a microglial marker. The morphology of microglia in culture was carefully examined by phase-contrast and fluorescent microscopy (data not shown).

### *Drug Treatment Effects on Cultured Microglial Cells*

Microglial cells were seeded at a density of $5 \times 10^5$ cells/well in a 24-well tissue culture plate, or $1 \times 10^5$ cells/well in a 96-well 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 adenosine receptor agonists (all from Sigma, St. Louis, MO), adenosine receptor antagonists [all from Sigma, except: 4-{2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl}fphenol (ZM 241385), which was from Tocris, Ellisville, MO], 8-pCPT-cAMP, PKI 14–22 amide, or Rp-Adenosine 3′,5′-cyclic monophosphorothioate (all from Sigma) at the indicated concentrations or vehicle dimethyl sulfoxide for 30 minutes at 37°C before activation of microglia. For microglia activation, Amadori-glycated albumin (AGA; Sigma) with undetectable endotoxin (<0.125 units/mL, 10 EU = 1 ng lipopolysaccharide; Lonza, Basel, Switzerland) was added to each well at a final concentration of 500 $\mu$g/mL. At indicated time points, cells were homogenized for Western blot analysis, and culture media were taken and analyzed for TNF-$\alpha$ by ELISA.

### *cAMP Determination*

Microglial cells in 96-well plates were pretreated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (500 $\mu$mol/L) for 10 minutes at 37°C followed by CGS21680 for 30 minutes. Cells were then treated with AGA for 4 hours. At the end of the incubation period, the cells were lysed (using 0.1 N HCl) and then scraped. cAMP content of the cell lysates was determined using a cAMP assay kit (Assay Design, Ann Arbor, MI) according to the manufacturer's instructions.

### *PKA Activity Assay*

The protein kinase A (PKA) activity assay is based on a solid-phase ELISA that uses a pre-coated synthetic peptide as a substrate for PKA, and a polyclonal antibody that recognizes the phosphorylated form of the substrate. PKA activity was estimated with a nonradioactive PKA activity assay kit (Assay Design) according to the manufacturer's instructions. Briefly, PKA control or lysates of treated microglial cells were added, followed by the addition of ATP to initiate the reaction. The kinase reaction was terminated and a phospho-specific substrate antibody was added to the wells that bind specifically to the phosphorylated peptide substrate. The phospho-specific antibody was subsequently bound by a peroxidase-conjugated secondary antibody. The assay was developed with tetramethylbenzidine substrate (TMB) and a color developed in proportion to PKA phosphotransferase activity. The color development was stopped with acid stop solution and the intensity of the color was measured in a microplate reader at 450 nm.

### *Protein Extraction and Western Blot Analysis*

Washed cultured cells were lysed in modified radioimmunoprecipitation assay buffer (Upstate, Lake Placid, NY) containing 50 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L EDTA, 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 minutes. Protein was determined by DC Protein Assay (Bio-Rad, Hercules, CA) and 50 $\mu$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 $\beta$-actin (Sigma), phospho-C-Raf (Ser338), phospho-B-Raf, C-Raf, B-Raf, phospho-ERK,

and ERK mitogen-activated protein kinase (Cell Signaling Technology, Beverly, MA) were detected with a horseradish peroxidase-conjugated antibody and enhanced chemiluminescence (ECL) (Amersham BioSciences, Buckinghamshire, UK). Intensity of immunoreactivity was measured by densitometry.

### *Data Analysis*

The results are expressed as mean ± SD differences among experimental groups that were evaluated by analysis of variance, and the significance of differences between groups was assessed by the post hoc test (Fisher's Protected Least Significant Difference) when indicated. Significance was defined as $P < 0.05$.

## *Results*

### *Role of $A_{2A}$AR in Diabetes-Induced Retinal Cell Death and Inflammation*

Significant death of retinal neural cells has been reported in experimental diabetes as early as 4 weeks.[23] Because activation of the $A_{2A}$AR has been implicated in the anti-inflammatory actions of adenosine in other tissues, we hypothesized that this receptor may also be effective in protecting retinal neurons from diabetes-induced inflammation and neurotoxicity. To test this hypothesis, we treated diabetic and nondiabetic mice with or without the $A_{2A}$AR-selective agonist, CGS21680, and determined the effects on diabetes-induced retinal cell death and TNF-$\alpha$ production. Quantitative analysis of TUNEL-Fluorescein

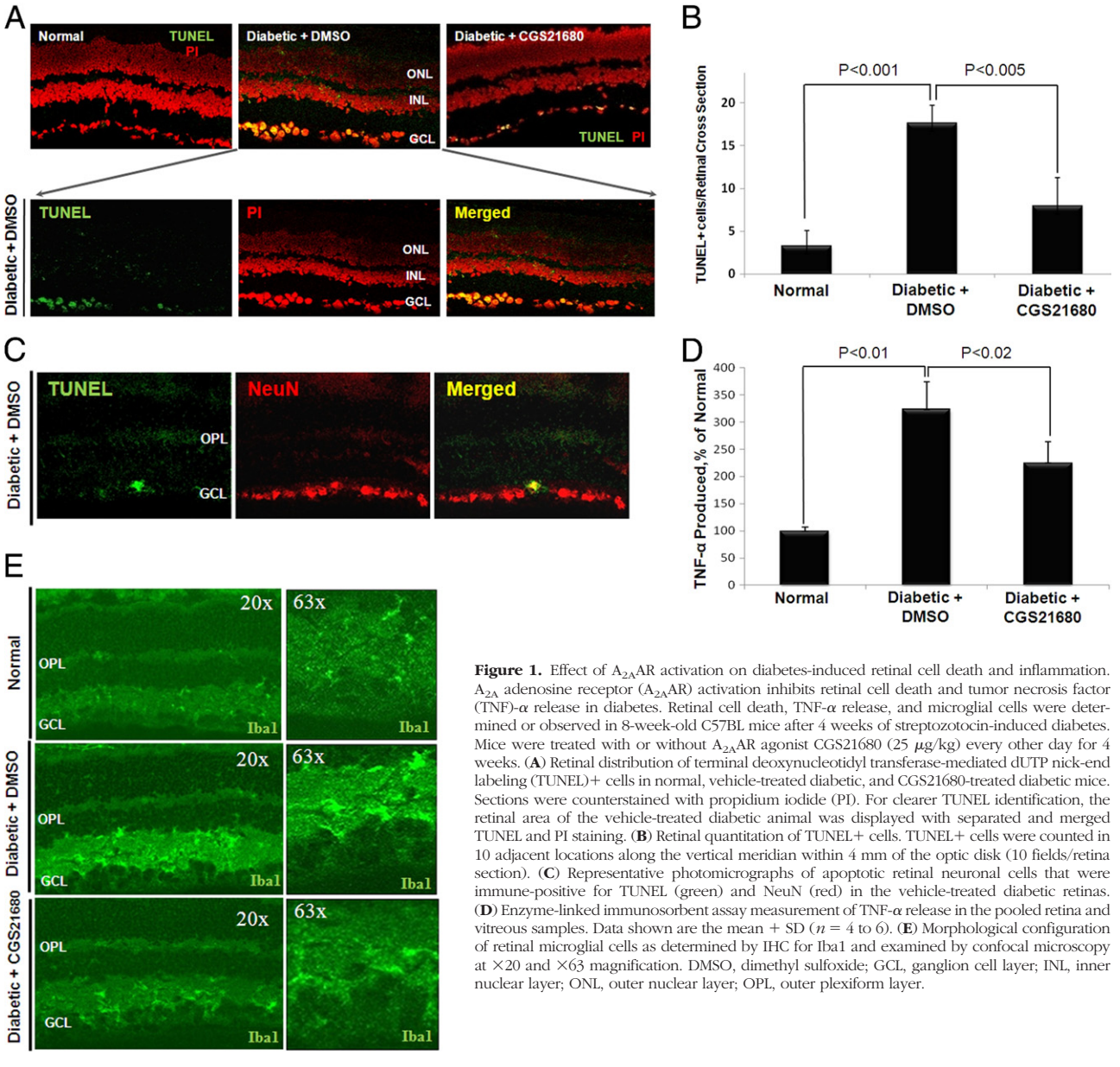


**Figure 1.** Effect of $A_{2A}$AR activation on diabetes-induced retinal cell death and inflammation. $A_{2A}$ adenosine receptor ($A_{2A}$AR) activation inhibits retinal cell death and tumor necrosis factor (TNF)-$\alpha$ release in diabetes. Retinal cell death, TNF-$\alpha$ release, and microglial cells were determined or observed in 8-week-old C57BL mice after 4 weeks of streptozotocin-induced diabetes. Mice were treated with or without $A_{2A}$AR agonist CGS21680 (25 $\mu$g/kg) every other day for 4 weeks. (**A**) Retinal distribution of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)+ cells in normal, vehicle-treated diabetic, and CGS21680-treated diabetic mice. Sections were counterstained with propidium iodide (PI). For clearer TUNEL identification, the retinal area of the vehicle-treated diabetic animal was displayed with separated and merged TUNEL and PI staining. (**B**) Retinal quantitation of TUNEL+ cells. TUNEL+ cells were counted in 10 adjacent locations along the vertical meridian within 4 mm of the optic disk (10 fields/retina section). (**C**) Representative photomicrographs of apoptotic retinal neuronal cells that were immune-positive for TUNEL (green) and NeuN (red) in the vehicle-treated diabetic retinas. (**D**) Enzyme-linked immunosorbent assay measurement of TNF-$\alpha$ release in the pooled retina and vitreous samples. Data shown are the mean + SD ($n = 4$ to 6). (**E**) Morphological configuration of retinal microglial cells as determined by IHC for Iba1 and examined by confocal microscopy at ×20 and ×63 magnification. DMSO, dimethyl sulfoxide; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; OPL, outer plexiform layer.

isothiocyanate 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 (in particular, neuronal cells) after 4 weeks of diabetes (Figure 1, A–C). The CGS21680 treatment significantly reduced the number of TUNEL-positive cells in the diabetic mice.

Next, we determined the treatment effect of CGS21680 on the levels of proinflammatory cytokine in the pooled retina and vitreous samples. Compared with age-matched nondiabetic animals, diabetic animals demonstrated a significant increase in TNF-$\alpha$ level (Figure 1D; $P < 0.01$) and the treatment with CGS21680 resulted in a marked reduction of diabetes-associated TNF-$\alpha$ release (Figure 1D; $P < 0.02$). Following this further, we have addressed an interesting feature (ie, acquisition of reactive microglial phenotype) that could be an important determinant for understanding the mechanisms by which CGS21680 affects TNF-$\alpha$ release. In this regard, we noted that when microglia encountered diabetic milieu, they transformed from their ramified resting state into an amoeboid shape, the activated and cytokines-releasing

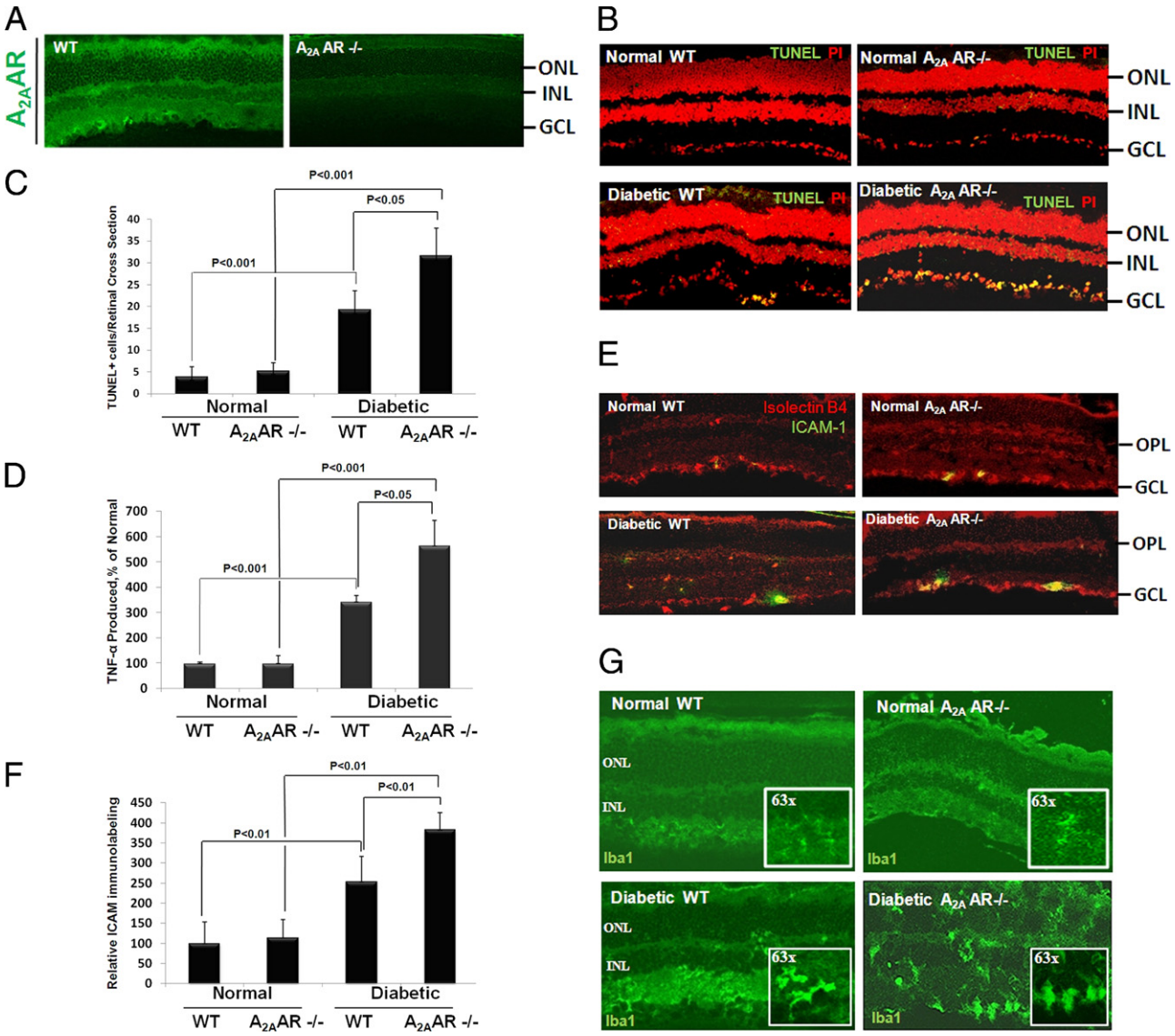


**Figure 2.** Effect of $A_{2A}$ adenosine receptor ($A_{2A}AR$) ablation on diabetes-induced retinal cell death and inflammation. $A_{2A}AR$ ablation leads to a significant retinal cell death and vascular inflammation in diabetes. Eight-week-old $A_{2A}AR^{-/-}$ mice in C57BL background and age-matched wild-type (WT) littermates were induced to diabetes with streptozotocin, and the retina was analyzed after 4 weeks of diabetes. (**A**) Retinal distribution of $A_{2A}AR$. Retinal sections from $A_{2A}AR^{-/-}$ and WT littermates were immunostained for $A_{2A}AR$. (**B**) Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)+ cell distribution in the retinal sections from $A_{2A}AR^{-/-}$ and WT littermates with and without diabetes. (**C**) Retinal quantitation of TUNEL+ cells. TUNEL+ cells were counted in 10 adjacent locations along the vertical meridian within 4 mm of the optic disk (10 fields/retina section). (**D**) Enzyme-linked immunosorbent assay measurement of tumor necrosis factor (TNF)-$\alpha$ release in the pooled retina and vitreous samples from $A_{2A}AR^{-/-}$ and WT littermates with and without diabetes. Data shown are the mean + SD ($n = 4$ to 6). (**E**) Intercellular adhesion molecule 1 (ICAM-1) distribution in the retinal sections from $A_{2A}AR^{-/-}$ and WT littermates with and without diabetes. Sections were stained for ICAM-1 and Iso-lectin B4. (**F**) ICAM-1/Iso-lectin B4 immunofluoresence were quantified in 10 adjacent locations along the vertical meridian within 4 mm of the optic disk (10 fields/retina section). (**G**) Configuration of Iba1-stained retinal microglia in sections from $A_{2A}AR^{-/-}$ and WT littermates with and without diabetes examined by confocal microscopy at ×20 and ×63 magnification. DMSO, dimethyl sulfoxide; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; OPL, outer plexiform layer.

state (Figure 1E). Although no gross difference in cell number was observed between diabetic and nondiabetic conditions, treatment of diabetic mice with CGS21680 attenuated the morphological transformation of ramified microglia into an activated amoeboid microglia (Figure 1E).

To further assess the role of $A_{2A}$AR in DR, we studied the effects of $A_{2A}$AR ablation on diabetes-induced retinal cell death and inflammation. Initially, immunofluorescence revealed that $A_{2A}$AR is localized in the ganglion cell layer and inner and outer plexiform layers (Figure 2A). Second, as shown in Figure 2, B and C, TUNEL+ cells in the retinas of diabetic $A_{2A}AR^{-/-\ mice}$ were greatly increased (~1.5-fold) compared with their age-matched diabetic WT littermates. Moreover, comparative analysis of TNF-$\alpha$ levels in the pooled retina and vitreous samples revealed that TNF-$\alpha$ release in the diabetic $A_{2A}AR^{-/-\ mice}$ was increased almost twofold compared with the diabetic WT littermates (Figure 2D; $P < 0.05$). To further characterize the role of $A_{2A}$AR in the retinal inflammation in diabetes, we determined the expression of ICAM-1 in the retinas of $A_{2A}AR^{-/-}$ and WT littermates with and without diabetes. As shown in Figure 2, E and F, ICAM-1 expression in diabetic $A_{2A}AR^{-/-\ mice}$ was increased significantly more than their age-matched diabetic WT mice. Finally, to determine whether the knockout of $A_{2A}$AR would alter microglia phenotype in unison with TUNEL and cytokine expression profiles during diabetes, we examined the morphological features of microglia in WT versus knockout animals. As shown in Figure 2G, an amoeboid phenotypic configuration indicating active form of microglia was observed in the diabetic WT mouse retina at 4 weeks, and this phenotypic became more obvious in $A_{2A}AR^{-/-}$ diabetic mice. Taken together, these results suggest that $A_{2A}$AR plays a crucial role in limiting retinal inflammation and neuronal cell injury associated with diabetes.

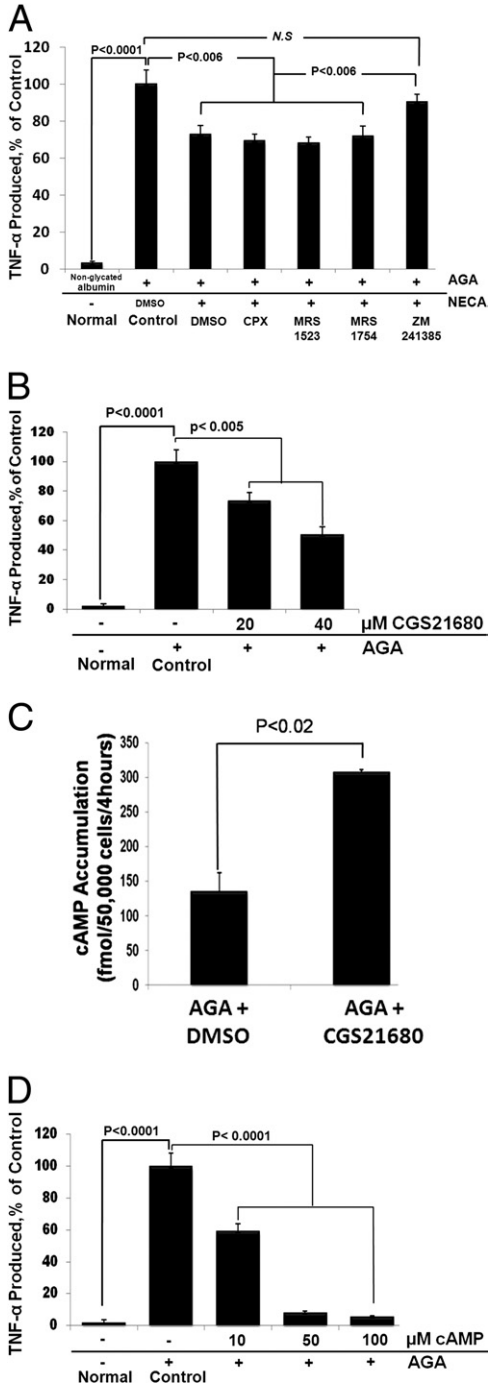


**Figure 3.** Identification of $A_{2A}$ adenosine receptor ($A_{2A}$AR) anti-inflammation signaling in retinal microglial cells. (**A**) Functional identification of AR subtypes in retinal microglial cells. Tumor necrosis factor (TNF)-$\alpha$ production was determined in retinal microglial cells treated with Amadori-glycated albumin (AGA) (500 $\mu$g/mL, 4 hours), in the presence or absence of the nonselective AR agonist adenosine-5′-*N*-ethylcarboxamide (1 $\mu$mol/L) and subtype-selective AR antagonists for $A_1$AR (8-cyclopentyl-1,3-dipropylxanthine, 0.1 $\mu$mol/L), $A_{2A}$AR (ZM 241385, 0.1 $\mu$mol/L), $A_{2B}$AR (MRS 1754, 1 $\mu$mol/L), and $A_3$AR (MRS 1523, 10 $\mu$mol/L). TNF-$\alpha$ levels were determined by enzyme-linked immunosorbent assay (ELISA). As a control for AGA, cells were also treated with nonglycated albumin (500 $\mu$g/mL, 4 hours). Data shown are the mean + SD of three experiments. (**B**) Effect of $A_{2A}$AR activation on AGA-induced TNF-$\alpha$ release. Retinal microglial cells were treated with AGA (500 $\mu$g/mL, 4 hours) in the presence of different doses of the $A_{2A}$-selective agonist, CGS21680. TNF-$\alpha$ levels were determined by ELISA. Data shown are the mean + SD of at least three different experiments. (**C**) Effect of CGS21680 on cAMP accumulation in 500 $\mu$g/mL AGA-treated microglia for 4 hours. Retinal microglial cells were pretreated with CGS21680, treated with AGA in the presence of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, and cAMP was determined with enzyme immunometric assay. Data shown are the mean + SD of three experiments. (**D**) Effect of cAMP on AGA-induced TNF-$\alpha$ release. Retinal microglial cells were treated with AGA (500 $\mu$g/mL, 4 hours) in the presence of different doses of cAMP. TNF-$\alpha$ levels were determined by ELISA. Data shown are the mean + SD of three experiments. CCPA, 2-chloro-N6-cyclopentyladenosine; CGS 21680, 2-p-[2-Carboxyethyl]phenethylamino-5′-N-ethylcarboxamidoadenosine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; ZM 241385, 4-{2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-a][1,3,5]triazin-5-ylamino]ethyl}fphenol; DMSO, dimethyl sulfoxide; MRS 1754, 8-[4-[((4-cyanophenyl) arbamoylmethyl) oxy]phenyl]-1,3-di(n-propyl) xanthine hydrate; MRS 1523, 3-propyl-6-ethyl-5[(ethylthio) carbonyl]-2-phenyl-4-propyl-3-pyridine-carboxylate; NECA, adenosine-5′-*N*-ethylcarboxamide; *N.S.*, not significant; ZM, ZM 241385, 4-{2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-a][1,3,5]triazin-5-ylamino]ethyl}phenol.

## *Activation of $A_{2A}$AR Attenuates AGA-Induced TNF-$\alpha$ Release in Retinal Microglial Cells*

After having shown that diabetic $A_{2A}AR^{-/-\ mice}$ exhibit profound retinal cell death and inflammation. Next we sought to explore a potential mechanism by which $A_{2A}$AR signaling regulates inflammation in DR with additional studies using microglial cells treated with AGA were performed. Treat-

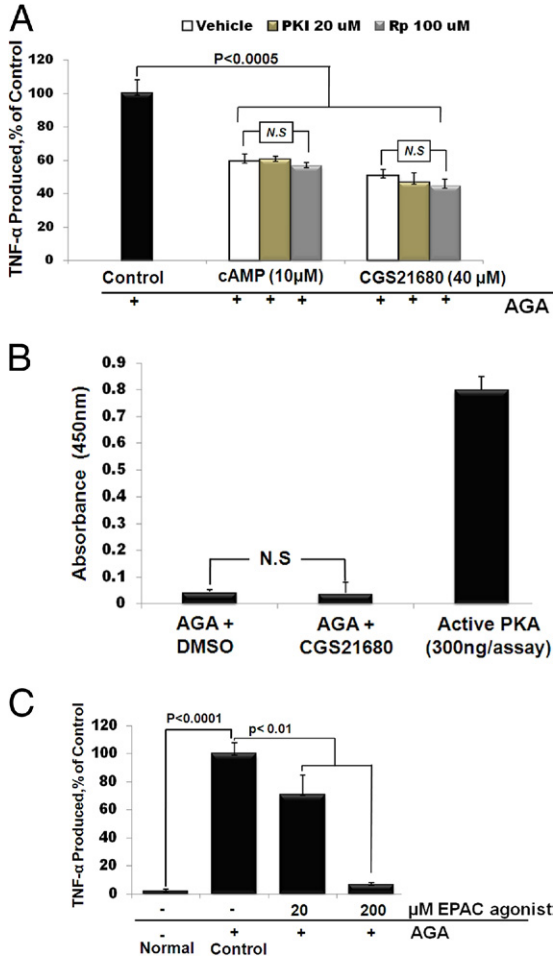


**Figure 4.** $A_{2A}$ adenosine receptor ($A_{2A}$AR)-cAMP mediated inhibition of tumor necrosis factor (TNF)-$\alpha$ release in activated microglia involves activity of exchange protein directly activated by cAMP (EPAC). (**A**) Effect of protein kinase A (PKA) inhibitors on Amadori-glycated albumin (AGA)-induced TNF-$\alpha$ release from microglial cells. Cells were pretreated with CGS21680 or 8-pCPT-cAMP in the presence of PKI 14–22 amide or Rp-adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMP), then treated with AGA (500 $\mu$g/mL, 4 hours), and TNF-$\alpha$ release was determined by enzyme-linked immunosorbent assay (ELISA). Data shown are the mean + SD of three experiments. (**B**) Effect of CGS21680 on PKA kinase activity in the AGA-treated microglial cells. Cells were pretreated with CGS21680, and then treated with AGA (500 $\mu$g/mL), and PKA kinase activity was determined by ELISA 4 hours after AGA treatment. (**C**) Effect of EPAC agonist on AGA-induced TNF-$\alpha$ release from microglial cells. Cells were pretreated with 8′-CPT-cAMP-O-Me (20 and 200 $\mu$mol/L), and then treated with AGA (500 $\mu$g/mL, 4 hours), and TNF-$\alpha$ release was determined by ELISA. Data shown are the mean + SD of three experiments. DMSO, dimethyl sulfoxide.

ment of microglia with AGA has been shown to simulate inflammation.[22,24,25] As shown in Figure 3A, treatment of retinal microglial cells with AGA, but not with nonglycated albumin, triggered a prominent increase in TNF-$\alpha$ release, which was potently inhibited (30% to 40%) by pre-incubation with 1 $\mu$mol/L nonselective AR agonist (adenosine-5′-*N*-ethylcarboxamide). Wdhen the cells were pretreated with the A1AR antagonist (8-cyclopentyl-1,3-dipropylxanthine; 0.1 $\mu$mol/L), the A2BAR antagonist [8-[4-[((4-cyanophenyl) arbamoylmethyl) oxy]phenyl]-1,3-di(n-propyl) xanthine hydrate (MRS 1754); 1 $\mu$mol/L], or the $A_3$AR antagonist [3-propyl-6-ethyl-5[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine-carboxylate; 2-chloro-N6-cyclopentyladenosine, 2-chloro-N6-cyclopentyladenosine (MRS 1523); 10 $\mu$mol/L], the inhibitory effect of adenosine-5′-*N*-ethylcarboxamide on TNF-$\alpha$ release was not affected. However, this effect was successfully blocked by the $A_{2A}$AR antagonist (ZM241385; 0.1 $\mu$mol/L). To confirm the role of the $A_{2A}$AR in regulating TNF-$\alpha$ release, we examined the effect of the selective $A_{2A}$AR agonist, CGS21680, in AGA-induced TNF-$\alpha$ release. As shown in Figure 3B, activation of $A_{2A}$AR with CGS21680 inhibited TNF-$\alpha$ release dose dependently. Together, these findings identify a signaling through $A_{2A}$AR as a critical control point for TNF-$\alpha$ release in AGA-treated retinal microglial cells.

Next, we sought to gain insights into the molecular mechanisms mediating the anti-inflammatory effects of $A_{2A}$AR signaling. $A_{2A}$AR is known as a G-protein coupled receptor that activates Adenylyl cyclase, thereby increasing intracellular cAMP. To verify that the inhibitory effect of $A_{2A}$AR activation on TNF-$\alpha$ release from activated microglia was mediated by elevating cAMP, we initially determined the effect of 2-p-[2-carboxyethyl]phenethylamino-5′-N-ethylcarboxamidoadenosine (CGS 21680) on cAMP production in AGA-stimulated microglia. In AGA-treated cells, pretreatment with CGS21680 resulted in approximately a twofold increase in cAMP level com-

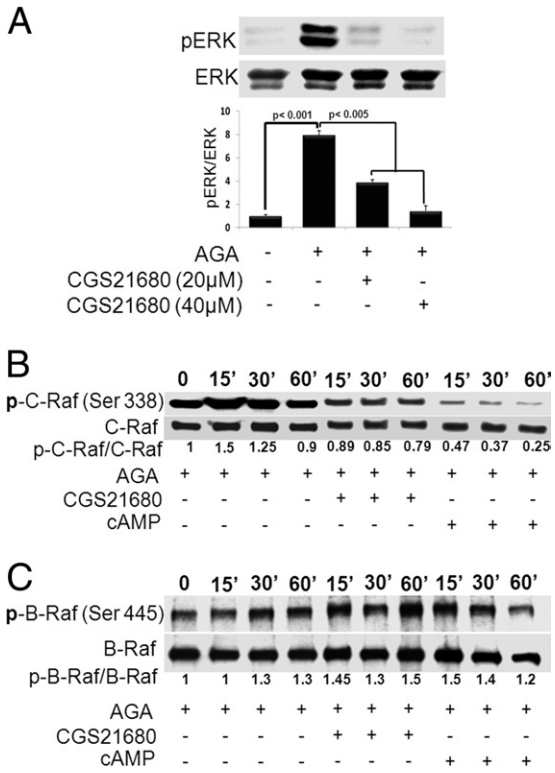


**Figure 5.** Role of $A_{2A}$ adenosine receptor ($A_{2A}$AR)-cAMP signaling in the mitogen-activated protein (MAP) kinase pathway. (**A**) Effect of $A_{2A}$AR activation on Amadori-glycated albumin (AGA)-induced extracellular signal-regulated kinase (ERK) MAP kinase activation. Retinal microglial cells were treated with AGA (500 $\mu$g/mL) in the presence or absence of CGS21680 (20 and 40 $\mu$mol/L) for 4 hours. Phospho-ERK (p-ERK) and total ERK MAP kinase in cell lysates were determined using Western blot analysis. Data shown are the mean + SD of three experiments. (**B**, **C**) Effect of $A_{2A}$AR activation on AGA-induced C-Raf (**B**) and B-Raf (**C**) activation. Retinal microglial cells were treated with AGA (500 $\mu$g/mL) in the presence or absence of CGS21680 (20 $\mu$mol/L) or 8-pCPT-cAMP (250 $\mu$mol/L) for the indicated times (0 to 60 minutes). Phospho-C-Raf (Ser338), phospho-B-Raf, total C-Raf, and B-Raf in the cell lysates were determined using Western blot analysis.

pared with a pretreated vehicle (Figure 3C). To assess the involvement of cAMP in TNF-$\alpha$ inhibition, retinal microglial cells were treated with AGA in the presence of a cell-permeable cAMP, 8-pCPT-cAMP (10 - 100 $\mu$mol/L), and TNF-$\alpha$ release was measured. As shown in Figure 3D, cAMP inhibited TNF-$\alpha$ release dose dependently. These results clearly demonstrate that inhibition of TNF-$\alpha$ release from activated microglial cells via the $A_{2A}$AR signaling pathway is cAMP-dependent.

### *$A_{2A}$AR-cAMP Mediated Inhibition of TNF-$\alpha$ Release in Activated Microglia Involves Activity of EPAC*

Recent studies have shown that protein kinase A (PKA) and the exchange protein directly activated by cAMP (EPAC) are downstream targets of cAMP. Therefore, we sought to determine whether both PKA and EPAC were involved in $A_{2A}$AR/cAMP-mediated anti-inflammatory signaling in AGA-treated microglia. As shown in Figure 4A, both CGS21680 and 8-pCPT-cAMP reduced TNF-$\alpha$ release significantly and this inhibition was not reversed by either Rp-cAMP or PKI 14–22, two different PKA inhibitors. To confirm this result, the effect of the cAMP-enhancing effect of CGS21680 on PKA kinase activity in the AGA-treated microglia was determined. Although PKA kinase activity was fully detectable in the positive control, this activity was almost undetectable in AGA-treated cells, and was also negligible following treatment with CGS21680 (Figure 4B). Next, we examine the role of EPAC in the cAMP-mediated anti-inflammatory signaling. As shown in Figure 4C, pretreating microglial cells with 20 and 200 $\mu$mol/L of the EPAC-selective agonist, 8′-CPT-cAMP-O-Me, dose-dependently inhibited AGA-induced TNF-$\alpha$ production. Taken together, these results demonstrate that the $A_{2A}$AR-cAMP signaling regulating AGA-induced TNF-$\alpha$ release from microglial cells involves the activity of EPAC, but not PKA.

### *$A_{2A}$AR Signaling Mediates the Anti-Inflammatory Effect via Interaction with AGA-Activated C-Raf/ERK Pathway in Retinal Microglial Cells*

Glycated albumin has been shown as a potent activator of Raf/ERK pathway in the course of TNF-$\alpha$ production (data not shown). Whether or not these kinases are modulated by the $A_{2A}$AR signaling was also examined here. As shown in Figure 5A, CGS21680 dose-dependently inhibited AGA-induced ERK activation. To further delineate the interaction between $A_{2A}$AR and mitogen-activated protein kinase signaling, we determined the effect of CGS21680 or 8-pCPT-cAMP on the activation of B-Raf or C-Raf, the upstream kinase of ERK, in AGA-treated retinal microglial cells. The results showed that phosphorylation of C-Raf occurred transiently at 15 minutes, whereas the phosphorylation of B-Raf was only slightly changed over the 60-minute experimental period. Both CGS21680 and 8-pCPT-cAMP inhibited AGA-induced activation of C-Raf, but not B-Raf (Figure 5, B and C). Collectively, these results suggest that the $A_{2A}$AR-signaling cross talks with C-Raf/ERK pathway at a site upstream of C-Raf and that this cross talk modulates AGA-induced TNF-$\alpha$ expression in retinal microglial cells.

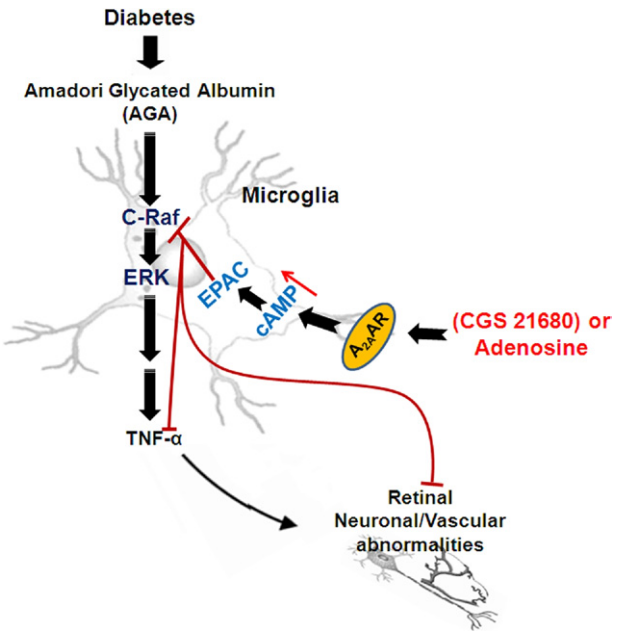


**Figure 6.** Proposed pathway of the Amadori-glycated albumin (AGA)-induced tumor necrosis factor (TNF)-$\alpha$ release, and $A_{2A}$ adenosine receptor ($A_{2A}$AR) agonists mediated anti-inflammation in the retinal microglial cells during diabetes. EPAC, exchange protein directly activated by cAMP; ERK, extracellular signal-regulated kinase.

## *Discussion*

The early signs of DR include retinal inflammatory reactions, breakdown of the blood-retinal barrier function, and loss of retinal neurons.[3–5] As the disease progresses, the retina may be damaged by oxidative stress induced by hyperglycemia, AGA, or advanced glycated proteins.[26–28] This stress damages vascular and neuronal tissues of the retina, and activates microglial cells. Activated microglia further exacerbate the damage by releasing proinflammatory cytokines and cytotoxic molecules in response to oxidative stress.[29] Thus, whereas the retina has traditionally been viewed as an immune privileged tissue, evidence is accumulating to support a role for local inflammation in the pathogenesis of DR.[3,11,30,31] This suggests that pharmacological interventions that reduce inflammation may be effective neuroprotectants for DR.[12,32]

Adenosine has many diverse functions, all of which are dependent on its interaction with specific receptor subtypes.[14] $A_{2A}$AR has been mostly studied for its potent anti-inflammatory effects.[33,34] High levels of $A_{2A}$ARs are found in macrophages and microglial cells that are poised, on activation, to abrogate the immune response.[35] We have recently demonstrated that the activation of $A_{2A}$AR, the dominant AR isoform in retinal microglial cells, blocks endotoxin-induced inflammation in these cells and in the retina.[20] Here, pharmacological experiments with AR agonists, as well as AR antagonists

have shown that $A_{2A}$AR is the most likely candidate for mediating the adenosine effect on TNF-$\alpha$ suppression in activated microglial cells. Treatment with the $A_{2A}$AR-selective agonist evokes the anti-inflammatory activity in AGA-activated retinal microglial cells via a cross talk with the Ras/C-Raf/MEK/ERK signaling. On activation of Gs-coupled receptor to regulate cell functions, cAMP increases to interact with the Ras/C-Raf/MEK/ERK signaling at a site upstream of C-Raf.[36] Activation of C-Raf involves phosphorylation at multiple activating sites, including Ser338, Tyr341, Thr491, Ser494, Ser497, and Ser499.[37] The cross talk between cAMP and the Ras/Raf/MEK/ERK signaling may lead to activation or inhibition of ERK activity, depending on the cell type. Evidence suggests that the expression of another Raf isoform, B-Raf, determines whether cAMP inhibits or activates ERK.[36] In cells that express B-Raf, cAMP activates B-Raf and ERK, whereas in cells that express C-Raf, cAMP inhibits C-Raf activity, thereby suppressing ERK phosphorylation. Based on our observation that the activation of $A_{2A}$AR-cAMP signaling inhibits ERK in retinal microglia, C-Raf is likely the major and functional Raf isoform in these cells. Moreover, the observed specificity in the cAMP signaling that is PKA-independent and EPAC-dependent suggests a novel mode in controlling the inflammatory events associated with microglia activation. A similar regulation of steroidogenesis has been observed for progesterone synthesis in ovarian granulosa cells.[38] Figure 6 illustrates the pathways of AGA, a diabetic inflammatory trigger, which lead to the TNF-$\alpha$ release in stimulated microglia, as well as the mechanism by which $A_{2A}$AR agonists block these processes.

The present study further showed that diabetic mice that lack $A_{2A}$AR exhibit significantly more retinal ICAM-1 expression, activated microglial cells, TNF-$\alpha$ release, and retinal cell death compared to diabetic mice that express $A_{2A}$AR. Increases in the expression of ICAM-1 have been causally related to the increased retinal vascular inflammation and permeability in diabetes.[3] Under stress conditions, the local levels of extracellular adenosine are elevated due to the increased need for energy supplied by ATP[15] and the degradation of released ATP.[16] This increased adenosine can protect against excessive cellular damage in a negative feedback manner.[17] However, a recent study that determined the ability of rat cardiac fibroblasts to release adenosine under conditions of ATP depletion suggested that protection by adenosine may be insufficient. Although decreases in cellular ATP level were accompanied by increased extracellular adenosine, extracellular adenosine released by cells cultured in the absence of insulin was significantly less compared to cells cultured in the presence of insulin.[39] Therefore, the apparently impaired protection in the WT diabetic mice with normal levels $A_{2A}$AR could reflect decreases in the release of adenosine due to the insulin deficiency.

Another important point of this work is the demonstration of the *in vivo* efficacy of CGS21680 in reducing retinal inflammation and cell death associated with diabetes. This evaluation of the *in vivo* effect of CGS21680 is essential with the aim to develop a receptor-based therapy for diabetic retinopathy devoid of the invasive side effects associated with the standard therapy, laser photocoagulation. 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 pharmacological strategies to safely use $A_{2A}$AR agonists in diabetic patients. Ongoing studies by our group are in progress to further elucidate the role of ATL370 and ATL313, two potent and selective $A_{2A}$R agonists, for their anti-inflammatory effects in diabetic retinopathy.

In summary, the current study provides preclinical evidence that the signaling through $A_{2A}$AR is a critical pathway for attenuating retinal cell death associated with diabetes. Furthermore, this study suggests that the function of $A_{2A}$AR agonists to inhibit C-Raf/ERK-mediated inflammatory cytokine release in activated microglia represents a novel therapeutic approach to treat ophthalmic complications associated with diabetes. Thus, with the discovery of new receptor-based therapies for diabetic retinopathy, $A_{2A}$AR holds significant potential as a therapeutic target.

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