Effect of agonists of adenosine receptors on inflammatory markers in human Muller cells

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We report the presence of adenosine receptors at molecular level and study their role in the inflammatory pathway under hyperglycemic condition. Human Muller cells were cultured in low (5 mM) and high (25 mM) glucose with 10% FBS and 1% P/S. Cells were starved in 0% FBS for 18 h and then treated with various agonists CCPA, CGS 21680, NECA and IB-MECA for 6, 12 and 24 h. The adenosine receptors were identified by immunocytochemistry. ELISA was used to measure the levels of TNF-α, IL-1β and ICAM 1. Four types of adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) were identified in human Muller cells. TNF-α content increased after agonist A_1 and A_3 treatment, but decreased after agonist A_{2A} and A_{2B} treatment. There was no significant effect on ICAM-1 and IL-1β. Stimulation of human Muller cells with adenosine A_{2A} agonist (CGS 21680) and adenosine A_{2B} agonist (NECA) reduces the level of TNF-α when exposed to high glucose, whereas A_1 adenosine agonist (CCPA) and A_3 adenosine agonist (IB-MECA) both positively and negatively regulate the TNF-α in hyperglycemia. However, none of these agonists has any significant role in affecting ICAM-1 and IL-1β.

Keywords: Adenosine receptors, agonists, diabetic retinopathy, inflammatory markers, MIO-M1 cells.

DIABETIC retinopathy (DR) is one of the most common complications of diabetes, causing vision impairment and blindness[1,2]. According to the International Diabetes Federation, diabetes currently affects 366 million people in the world and the number will rise to 552 million by 2030. Diabetic retinopathy has been classified as nonproliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR)[3,4]. Some characteristics of diabetic retinopathy include basement membrane thickening, alteration in blood flow, loss of retinal pericytes, increased proliferation of endothelial cells and formation of microaneurysms[4–6]. Progression through these stages leads to neovascularization and eventually loss of vision. The mechanism by which the diabetic risk factors initiate vascular disruption and disease progression in retinopathy remains unclear. However, *in vivo* and *in vitro* studies have shown diabetic retinopathy has features of inflammation which involve activation of multiple mediators such as adhesion molecules, chemokines and pro-inflammatory cytokines in migration of leukocytes towards infected or injured tissue[7]. During inflammation, the infected or injured tissue releases pro-inflammatory cytokines (tumour necrosis factor-alpha (TNF-α), interleukin-1-beta 1 (IL-1β) and chemokines (CCL 2 and CCL 5)), which induce the coordinate expression of numerous adhesion molecules such as E-selectin, intracellular adhesion molecules (ICAM)-1, vascular adhesion molecules (VCAM)-1 and chemokines[4–7].

Although pathogen does not play any role in diabetic retinopathy, research has been carried out on the vitreous fluid, serum, cells and diabetic animal models to demonstrate the upregulation of pro-inflammatory cytokines, chemokines and adhesion molecules in diabetic retinopathy. Increased level of IL-1β has been observed in the vitreous fluid of patients with diabetic retinopathy, retina from diabetic rats and retinal endothelial[8–11]. The expression of TNF-α markedly increased in vitreous, serum, ocular fibrovascular membranes from diabetic patients and in retina from animal models with diabetes mellitus[12–14]. It has been reported that retinal Muller cells play an important role in the initiation and progression of diabetic-retinopathy[15,16]. Muller cells are the principal glial cell of the retina which serve as structural support cells and spans its entire thickness[17]. According to some studies, Muller cells have a role in regulating blood flow in the retina and maintaining the blood retinal barrier[18,19]. Inflammatory markers present in glial cells of the retina are significantly induced in hyperglycemic conditions.

Adenosine is an endogenous purine nucleoside that is formed at sites of metabolic stress associated with injury or inflammation[20,21]. Its effects are being mediated through four types of receptors which are A_1, A_{2A}, A_{2B} and A_3 (ref. 22). These receptors are members of the G-protein-coupled receptors (GPCRs), which are actively involved in downstream signalling of various pathways. A_1 and A_3 receptors are coupled to inhibitory G protein ($G_{i/0}$), and their stimulation decreases the intracellular cyclic adenosine monophosphate (cAMP) concentration, whereas A_{2A} and A_{2B} are coupled to stimulatory G protein (G_s), and their stimulation increases cAMP concentration. It has been reported that A_1 and A_{2A} are activated by low concentration (0.01–1 μM), whereas A_{2B} and A_3 need higher volume (> 10 μm)[23–25]. In the present study, we mimic the hyperglycemic condition by growing the cells in high glucose and determine the presence of adenosine receptors in human Muller cells and the effect of their agonists on inflammatory markers under hyperglycemic condition.

Human Muller cells MIO-M1 were isolated from the neural retina of cadaveric donor eyes obtained from Moorfields Hospital Eye Bank[26]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% (vol/vol), fetal bovine serum (FBS) and 1% (vol/vol) penicillin/streptomycin in a humidified atmosphere

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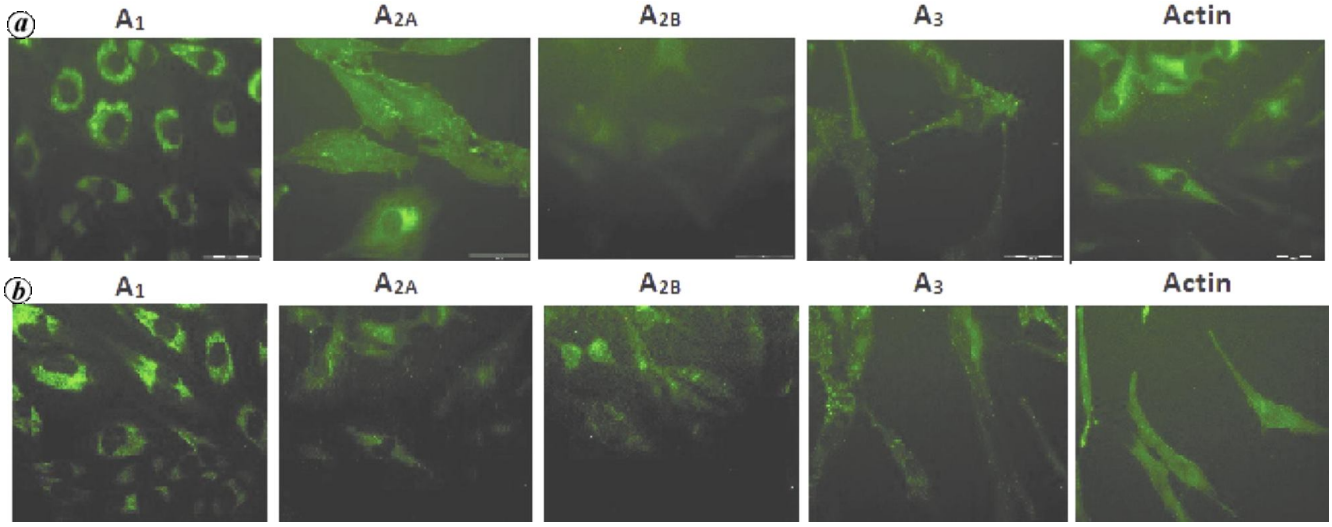

Figure 1. Detection of adenosine receptors in human Muller cells (MIO-M1). ***a***, Adenosine receptors in high glucose. ***b***, Adenosine receptors in low glucose. The cells were grown in DMEM containing low glucose (5 mM) and high glucose (25 mM) with 10% FBS for 48 h. Goat anti-actin antibodies were used as positive control. The images are representative of three independent experiments.

with 5% CO_2. For the experiment, the cells were grown in DMEM containing 5 mM glucose (low glucose) and 25 mM glucose (high glucose) with 10% FBS for 48 h, and then starved for 18 h in medium containing 0% FBS. Subsequently the cells were treated with 2-chloro-N6-cyclopentyladenosine (CCPA) (adenosine A_1 agonist), CGS 21680 (adenosine A_{2A} agonist), 5′-(*N*-ethylcarboxamido) adenosine (NECA) (adenosine A_{2B} agonist) and N^6-(3-iodobenzyl) adenosine-5′-*N*-methyluronamide (IB-MECA) (adenosine A_3 agonist) at three different concentrations (1, 10 and 100 μM) for 6, 12 and 24 h respectively.

The adenosine receptors were identified as described earlier[27], with modifications. Muller cells were seeded (50,000/well) onto six-well tissue culture plates containing sterile coverslips in a medium containing low glucose (5 mM) and high glucose (25 mM) and allowed to attach and proliferate in the respective medium for 2 days. The cells were fixed with cold methanol for 10 min at –20°C, rinsed twice with PBS, blocked at room temperature with 0.5% Tween (v/v) and 2% BSA (w/v) in PBS (blocking solution) for 1 h and rinsed twice with PBS. The cells were incubated overnight with primary antibodies (anti-A1 receptor, anti-A2A receptor, anti-A2B receptor and anti-A3 receptor) in blocking solution 1 : 100. Goat anti-actin antibodies were used as positive control. The following day, cells were washed twice with PBS and incubated for 1 h in a dark room with secondary antibodies conjugated with fluorescein isothiocyanate (FITC) in blocking solution at 1 : 200. After rinsing twice with PBS, the coverslips were mounted with antifading medium (ProLong Gold, Life Technologies, USA).

For preparation of cell lysates, the cells were washed with cold PBS and the monolayer was scrapped into 250 μl of lysis buffer (Mammalian Cell Lysis Kit, Sigma, USA)[28]. The total cell lysates were centrifuged at 12,000 *g* for 10 min at 4°C and the supernatant was frozen at –20°C. Protein concentration of the supernatant was quantified with the Bradford (Bio-Rad) protein assay.

ELISA was performed for TNF-α, IL-1β and ICAM-1 (R&D Systems, USA) according to the manufacturer's instruction. Drugs CCPA, CGS 21680, NECA and IB-MECA were procured from Sigma. All stock solutions were prepared in dimethylsulphoxide (DMSO) and stored at –20°C until used.

Results were expressed as mean ± SEM. Student's *t*-test was used to evaluate the results. $P < 0.05$ was considered statistically significant.

Before studying whether adenosine agonists alter inflammatory marker levels in human Muller cells (MIO-M1), we first determined the presence of adenosine receptors by immunofluorescence technique using specific anti-A1, anti-A2A, anti-A2B and anti-A3 antibodies (Figure 1). All subtypes of adenosine receptors are present in human Muller cells (MIO-M1).

TNF-α levels were increased in high glucose levels compared with low glucose ($P < 0.05$, Figure 2 *a*). Stimulation with CCPA (1 μM) significantly increased the TNF-α content after 6 h of treatment ($P < 0.05$, Figure 2 *b*), while 10 and 100 μM of CCPA decreased the TNF-α content. However, 12 h of treatment with 1 and 100 μM of CCPA significantly reduced the level of TNF-α, but 10 μM significantly increased the level of TNF-α ($P < 0.05$, Figure 2 *b*). After 24 h, 1 and 10 μM of CCPA increased the TNF-α content, but 100 μM significantly reduced the expression of TNF-α ($P < 0.05$, Figure 2 *b*). These results suggest that stimulation of human Muller cells in a hyperglycemic condition with CCPA can decrease/increase production of TNF-α. Stimulation with CGS 21680 (1, 10 and 100 μM), significantly decreased

TNF-α levels at 6 h after treatment in human Muller cells cultured in hyperglycemia ($P < 0.05$, Figure 2 *c*). However, the production of TNF-α increased after being treated with CGS 21680 (1, 10 and 100 μM) at 12 h. At 24 h, 1 μM of CGS 21680 significantly reduced the expression of TNF-α ($P < 0.05$, Figure 2 *c*). Similarly 10 μM of CGS 21680 decreased the TNF-α content, but 100 μM of CGS 21680 remained high. Based on these findings, stimulation of human Muller cells in a hyperglycemic condition with 1 μM of CGS 21680 can decrease production of TNF-α. Stimulation with NECA (1 μM) decreased the TNF-α content after 6 h of treatment but there was no significant reduction, while 10 μM of NECA significantly decreased the expression of TNF-α ($P < 0.05$, Figure 2 *d*) and 100 μM of NECA increased the level of TNF-α. After 12 h of treatment with 1 μM of NECA, the level of TNF-α decreased, while 10 and 100 μM of NECA significantly decreased the level of TNF-α ($P < 0.05$, Figure 2 *d*). After 24 h, 1 μM of NECA increased the TNF-α content, but 10 and 100 μM reduced the expression of TNF-α. Our results revealed that stimulation of human Muller cells in a hyperglycemic condition with 10 μM of NECA can decrease the production of TNF-α. Stimulation with IB-MECA (1, 10 and 100 μM), decreased TNF-α levels at 6 h after treatment in human Muller cells cultured in hyperglycemia, but significantly with 100 μM of IB-MECA ($P < 0.05$, Figure 2 *e*). After 12 h of treatment with 1 μM and 10 μM of IB-MECA, the level of TNF-α decreased significantly ($P < 0.05$, Figure 2 *e*) and 100 μM of IB-MECA increased the expression of TNF-α ($P < 0.05$, Figure 2 *e*). Also, 24 h of treatment with IB-MECA (1 μM) significantly increased the expression of TNF-α, while 10 and 100 μM decreased the TNF-α content. Our results demonstrated that stimulation of human Muller cells in a hyperglycemic condition with IB-MECA can decrease/increase production of TNF-α.

Human Muller cells grown in high glucose and low glucose did not alter IL-1β levels (Figure 3 *a*). The expression of IL-1β significantly increased after 24 h treatment with CCPA (1, 10 and 100 μM). Stimulation with NECA (10 μM) significantly increased IL-1β levels at 12 h after treatment in MIO-M1 cells cultured in high glucose medium ($P < 0.05$ versus non-treated; Figure 3 *d*).

Human Muller cells grown in medium containing 5 or 25 mM glucose and stimulated with four different adenosine agonists did not have altered levels of ICAM-1 (Figure 4).

In the present study, we provide evidence that cultured human Muller cells express A_1, A_{2A}, A_{2B} and A_3

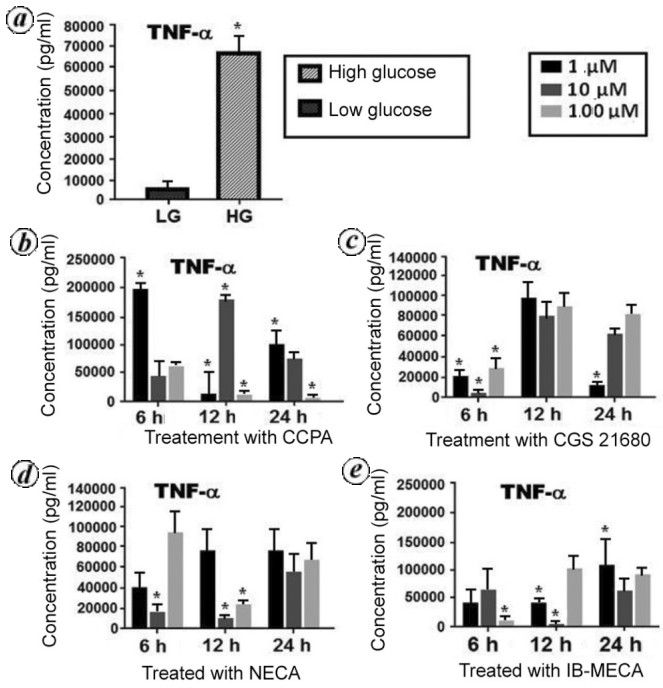

Figure 2. ELISA of TNF-α in human Muller cells (***a***) grown in low and high glucose without treatment as a control, (***b***) treated with 1, 10 and 100 μM of CCPA for 6, 12 and 24 h respectively, (***c***) treated with 1, 10 and 100 μM of CGS 21680 for 6, 12 and 24 h respectively, (***d***) treated with 1, 10 and 100 μM of NECA for 6, 12 and 24 h respectively and (***e***) treated with 1 M, 10 M and 100 μM of IB-MECA for 6, 12 and 24 h respectively. Data are represented as mean ± SEM. (***a***) *$P < 0.05$; high glucose compared to low glucose. (***b–e***) *$P < 0.05$; treated compared with control.

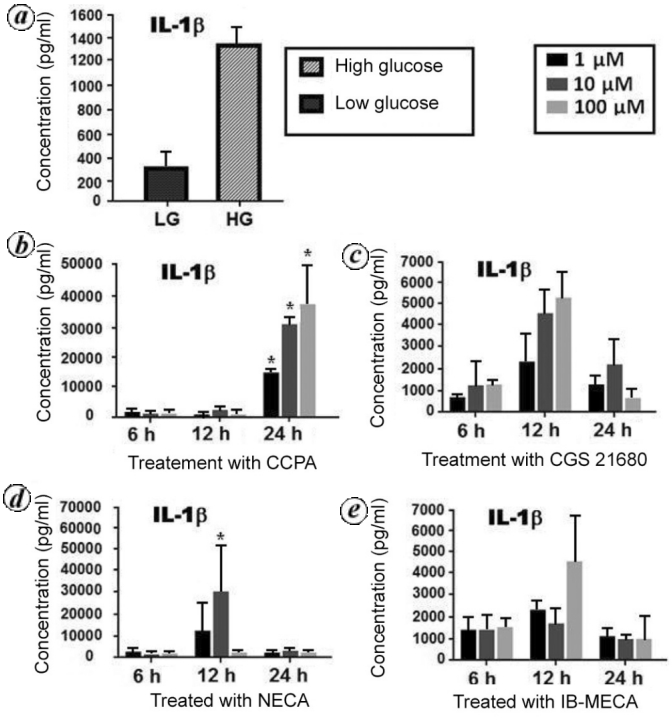

Figure 3. ELISA of IL-1β in human Muller cells (***a***) grown in low and high glucose without treatment as a control, (***b***) treated with 1, 10 and 100 μM of CCPA for 6 , 12 and 24 h respectively, (***c***) treated with 1, 10 and 100 μM of CGS 21680 for 6, 12 and 24 h respectively, (***d***) treated with 1, 10 and 100 μM of NECA for 6, 12 and 24 h respectively and (***e***) treated with 1, 10 and 100 μM of IB-MECA for 6, 12 and 24 h respectively. Data are represented as mean ± SEM. (***a***) *$P < 0.05$; high glucose compared to low glucose. (***b–e***) *$P < 0.05$; treated compared with control.

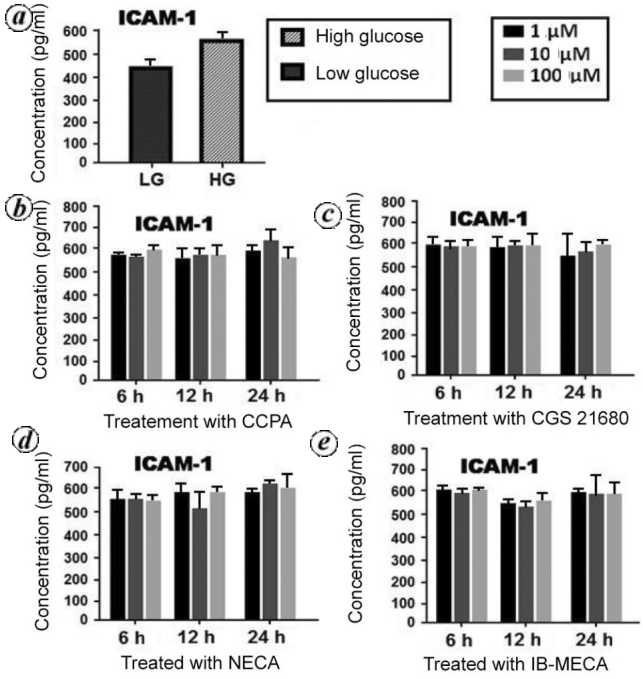

Figure 4. ELISA of ICAM-1 in human Muller cells (***a***) grown in low and high glucose without treatment as a control, (***b***) treated with 1, 10 and 100 μM of CCPA for 6, 12 and 24 h respectively, (***c***) treated with 1, 10 and 100 μM of CGS 21680 for 6, 12 and 24 h respectively, (***d***) treated with 1, 10 and 100 μM of NECA for 6, 12 and 24 h respectively and (***e***) treated with 1, 10 and 100 μM of IB-MECA for 6, 12 and 24 h respectively. Data are represented as mean ± SEM. (***a***) $*P < 0.05$; high glucose compared to low glucose. (***b–e***) $*P < 0.05$; treated compared with control.

adenosine receptors. Recent studies have shown that inflammation plays a major role in the pathogenesis of diabetic retinopathy[29,30]. Several inflammatory markers have been reported which might be involved in the pathogenesis of diabetic retinopathy, as mediators between leukocytes or as regulators of leukocyte adhesion and activation in retinal tissue[31]. Here we have studied the effect of adenosine agonist on the inflammatory markers such as TNF-α, IL-1β and ICAM-1 in hyperglycemic condition.

TNF-α is known as a pro-inflammatory cytokine which can be found in many types of cells, including astrocytes, macrophages, microglia and retinal glial cells[32]. In agreement with other studies, our results demonstrate that stimulation of human Muller cells (MIO-M1) with adenosine A_{2A} agonist and adenosine A_{2B} agonist can reduce the level of TNF-α in hyperglycemia[33–37]. These findings suggest that both adenosine agonists A_{2A} and A_{2B} serve pro-inflammatory role in the development of diabetic retinopathy. However, stimulation with both adenosine agonists A_1 and A_3 positively and negatively regulates TNF-α when exposed to high glucose concentration *in vitro*. These findings suggest that both A_1 and A_3 serve a dual role, pro-inflammatory and anti-inflammatory in the development of diabetic retinopathy.

The expression of IL-1β is known to be upregulated in the retina from diabetic rat, galactosemic mice and diabetic patients[37,38]. In addition, human monocytes, aortic, retinal vascular endothelial cells and Muller cells have been shown to upregulate IL-1β when exposed to high glucose concentration *in vitro*[39–41]. The present results showing no significant increases in IL-1β production, are in contrast to the recent work by Steinle *et al.*[6] A possible explanation for the discrepancy between our finding and the previous work is that we measured the IL-1β protein level after a short exposure (2 days) to high glucose, whereas the previous study measured the production of IL-1β after a prolonged exposure (5 days) to high glucose. In our results, similar to that of Liu *et al.*, glucose induces IL-1β expression in retinal vascular endothelial cells but not in Muller cells, astrocytes or microglia. Our study revealed that high glucose has no effect on ICAM-1, which is in agreement with the findings of Chen *et al.*[42,43].

In conclusion, our studies show that A_1, A_{2A}, A_{2B} and A_3 adenosine receptors are expressed in human Muller cells (MIO-M1). Our study also demonstrates the presence of adenosine receptors in human Muller cells (MIO-M1). Furthermore, our *in vitro* studies show that stimulation of human Muller cells with adenosine A_{2A} agonist (CGS 21680) and adenosine A_{2B} agonist (NECA) can reduce the level of pro-inflammatory cytokines such as TNF-α when exposed to high glucose, whereas A_1 adenosine agonist (CCPA) and A_3 adenosine agonist (IB-MECA) both positively and negatively (biphasic response) regulate TNF-α in hyperglycemia.

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